DEVELOPMENT OF FUNCTIONAL BIOENGINEERED MUSCLE MODELS AND A NOVEL MICRO-PERFUSION SYSTEM

by

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Chapter 1

Introduction

PROJECT OVERVIEW

Tissue engineering combines the principles of medical, life sciences, and engineering fields toward the development of biological substitutes to restore, maintain, or improve tissue function. Our laboratory has previously developed methods to bioengineer various 3-dimenisonal contractile tissues, including cell-based cardiac and skeletal constructs. These methods involve seeding cells on a fibrin gel, where cells then proliferate and/or exhibit spontaneous contractility, resulting in compaction of the fibrin gel. During culture the cell front moves radially inwards, towards the center of the culture surface and results in the formation of bioengineered 3-dimensional tissues.

Fibrin-based biomaterials are well-suited for bioengineering contractile tissues, as it promotes cell growth, wound healing, and tissue regeneration (1-6). Fibrin closely replicates extracellular matrix (ECM) components of native muscle tissues and its degradation kinetics are compatible with the rate of new tissue formation (7). Therefore all of the bioengineered tissue models described in this dissertation were developed using fibrin gel casting strategies (1; 5; 8-10). However, the construct design was altered for each model (Figure 1.1).

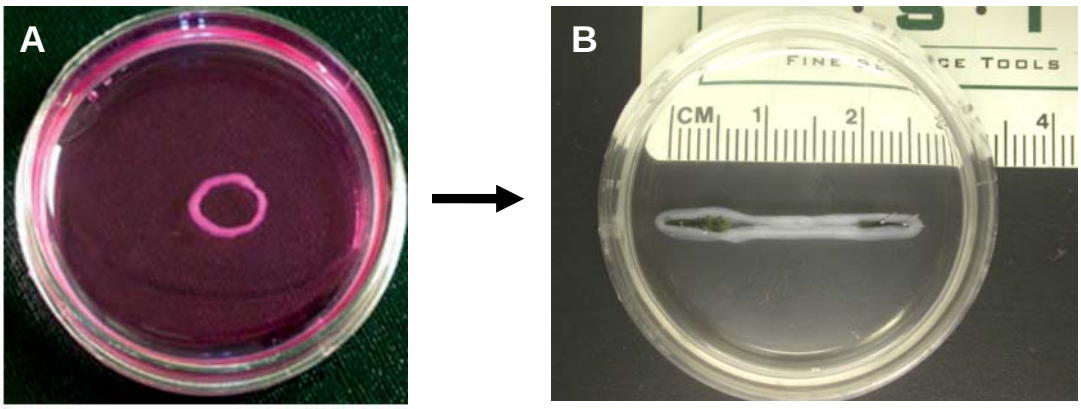

Figure 1.1 Bioengineered smooth muscle tissues.
A) Ring-shaped bioengineered internal anal sphincter, developed from primary rabbit smooth muscle cells. **B)** Strip-design of a bioengineered vascular smooth muscle strip, developed from primary human aortic vascular

It is imperative that bioengineered tissues have a high degree of functional testing in order to evaluate tissue-specific function as well as suitability for future clinical applications (11). Functional testing for all bioengineered muscle tissues described was evaluated using a custom built force transducer and data acquisition software (12; 13). In addition, cellular characterization of bioengineered muscle tissues was also performed.

Finally, as bioengineered tissue models were developed, there became a need for improved methods for culture of bioengineered tissues, to accommodate theincreased metabolic demand of the 3-dimensional tissue constructs as compared to traditional monolayer culture systems. The final section of this research describes a micro-perfusion system which promotes continuous fluid flow to support the increased metabolic requirements of bioengineered tissues

during culture. In addition, exposure of bioengineered tissues to micro-perfusion resulted in some functional improvement of tissues.

Bioengineered *in vitro* muscle tissues may provide alternative treatment modalities for muscle dysfunction/disease, providing transplantable tissue substitutes which can have direct applicability. In addition to potential clinical applications, bioengineered tissues can be used as a 3-dimensional model system for basic research and in new drug development as a tool to evaluate efficacy and safety.

HYPOTHESIS AND SPECIFIC AIMS

Congestive heart failure (CHF) is a major medical challenge in developed countries. There are currently 4.8 million patients in the U.S. living with CHF with an estimated cost to society of $23.2 billion annually(14). Heart transplantation has been the most successful treatment option to treat severe CHF(15). Although the short-term survival rate stands at 81%, widespread applicability of heart transplantation is often limited by a chronic shortage of donor organs(16). Cardiac tissue engineering may provide a potential solution to the donor heart crisis in the future. Research in the area of cardiovascular tissue engineering is focused on the development of functional 3-dimensional cardiac and vascular tissue *in vitro (6)*. Tissue engineered heart muscle could be used clinically to patch areas of myocardial infarction while vascular constructs may be utilized as bypass grafts, particularly in small diameter applications. Tissue engineering models also allow the detailed study of critical events in organogenesis, such as

the establishment of cell-cell communication, construction and modification of the ECM and angiogenesis (17). There is an unprecedented need to develop models of tissue engineered cardiovascular constructs.

Our overall working hypothesis is that primary mammalian smooth muscle cells derived from gastrointestinal and vascular sources can be cultured in the presence of a rapidly degrading fibrin gel to form functional 3-dimensional muscle tissue constructs in vitro.

Specific Aim 1 – Determine the optimal cell culture conditions (seeding density, viability in culture, and tissue-specific function, with force generation as the end variable measured) for the development of 3-dimensional "ring" models of anal sphincter and colon tissue, bioengineered in a fibrin matrix utilizing primary rabbit smooth muscle cells.

Specific Aim 2 – Determine the optimal cell culture conditions (seeding density, viability in culture, and tissue-specific gene expression and functionality) for the development of a 3-dimensional *in vitro* muscle "strip" model, bioengineered in a fibrin matrix with primary human aortic VSM cells.

Specific Aim 3 – Design, fabricate, and test a micro-perfusion system to support the culture of bioengineered 3-dimensional heart muscle. The micro-perfusion system should provide a continuous supply of oxygenated and supplemented media to bioengineered tissues, and also to replicate sheer

stress conditions observed in vivo. The goals of the micro-perfusion system are to: 1) increase functionality (force generation, gene expression, viability, total protein) of bioengineered tissues, and 2) replace the use of a standard cell culture incubator.

COMMONALITY THROUGH THESIS RESEARCH

There are several re-occurring themes in this body of work. Firstly, and most important, the research described in this thesis describes novel bioengineered models for smooth muscle cells and technology to support the culture of 3-dimensional construct utilizing custom-build perfusion apparatus. Collectively, this body of work adds knowledge base to two critical areas of tissue engineering research; development of functional bioengineered models and ancillary technology to support these models. The field of tissue engineering is fairly new with incremental additions to its knowledge base. Typically, exploratory research is the norm to establish emerging trends; dominant designs have not been developed within the field. Therefore, the current body of work is aligned to the general trends within the field of functional cardio-vascular tissue engineering.

Another common theme throughout this body of work is the utilization of fibrin to support the formation of bioengineered models for gastrointestinal, vascular, and cardiac muscle constructs. Several studies have shown that fibrin has specific characteristics which make it suitable as a biomaterial for tissue engineering applications. Firstly, the formation fibrin gel by the polymerization of fibrinogen molecules utilizing thrombin has been fairly well studied with simple

reaction kinetics (18). The properties of the fibrin gel have shown to support the formation of 3-dimensional models of tissue constructs, due to the resemblance to ECM components found in normal mammalian muscle tissue (3; 19). In addition, the degradation kinetics of fibrin can easily be controlled by changing the properties of the gel and/or cross-linking the fibrin molecule to stabilize the gel (20). This provides an opportunity to modulate the degradation rate of fibrin to match the properties of new tissue formation.

RELATIONSHIP OF THESIS RESEARCH TO WORK AT THE AHL

The overall research objective within the Artificial Heart Laboratory is to develop functional models of cardiovascular structures in vitro and to develop supporting technology to modulate the phenotype of the resulting tissue constructs. The current body of work is aligned with both of these objectives. The first part of this thesis is focused on the development of novel models for vascular structures. The second part of this work is focused on the development of a novel bioreactor system to support the culture of bioengineered tissue constructs. Collectively, the work described in this thesis has been designed and executed with the overall global objectives of the AHL in mind.

RELATIONSHIP OF THESIS WORK TO THE FIELD OF FUNCTIONAL TISSUE ENGINEERING

The research described in this thesis has been designed to address critical scientific and technological gaps in the current knowledge base in the field of functional tissue engineering. Specific Aim 1 has been designed to introduce researchers in gastrointestinal research to the numerous advantages functional 3-dimensional tissue constructs have to offer. The sphincter and colon muscle models described in this thesis are the only published bioengineered models of gastrointestinal structures developed in vitro. This gives researchers in the field a tremendous advantage over existing systems for basic research. Specific Aim 2 has been designed to extend the work descried in the area of strip models for vascular tissue. Published work has shown the utility of supporting the culture of vascular smooth muscle cells within a fibrin matrix. However, this body of work is not well aligned with the field of functional tissue engineering, completely ignoring the need for testing contractile properties. In this thesis, the contractile properties of bioengineered vascular tissue fabricated utilizing human smooth muscle cells have been evaluated. In Specific Aim 3, a novel micro-perfusion system is described to support the culture of bioengineered heart muscle. In this part of the thesis, an often ignored need to develop supporting technology for bioengineered constructs has been addressed.

Collectively, this body of work described in this thesis has been designed and executed with the overall field of functional tissue engineering in mind. Every study is aimed to address a critical knowledge gap, either scientific or technological, thereby providing an incremental addition to the field.

1.6 OUTLINE OF THESIS

Chapter 2 and 3 provide a broad overview of the field of cardiac and vascular tissue engineering, respectively. Seminal work is highlighted and critical technological challenges which need to be addressed in the field are highlighted. Chapter 4 describes ring models of anal sphincter and colon tissue. Chapter 5 provides a detailed description of the formation of a strip model for vascular tissue. Chapter 4 and 5 provide detailed methods for the formation of bioengineered constructs as well as thorough characterization of the models. Chapter 6 describes a novel micro-perfusion system to support the culture of bioengineered heart muscle. Detailed description of the perfusion system is provided and data to demonstrate functional improvement in heart muscle constructs resulting from micro-perfusion are presented. Chapter 7 provides a summary of the research presented in this thesis.

Chapter 2

Engineering the Heart Piece by Piece:

State of the Art in Cardiac Tissue Engineering

SUMMARY

According to the National Transplant Society (2006), more than 7,000 Americans in need of organs die every year only for lack of lifesaving organs. Bioengineering 3-dimensional organs *in vitro* for subsequent implantation may provide a solution to this problem. The field of tissue engineering in its most rudimentary form is focused on the developed of transplantable organ substitutes in the laboratory.

The objective of this chapter is to introduce important technological hurdles in the field of cardiac tissue engineering. This chapter starts with an overview of tissue engineering, followed by an introduction to the field of cardio-vascular tissue engineering, and finally summarizes some of the key advances in cardiac tissue engineering; specific topics discussed in this chapter include cell sourcing and biomaterials, *in vitro* models of cardiac muscle, and bioreactors. The chapter concludes with thoughts on the utility of tissue engineering models in basic research as well as critical technological hurdles that need to be addressed in the future.

OVERVIEW OF TISSUE ENGINEERING

Tissue engineering is a rapidly evolving field involving collaborative expertise from diverse disciplines including engineering, medical, and life sciences (21-25). In its simplest form, the aim of tissue engineering is to promote functional regeneration of damaged tissue utilizing cells cultured *in vitro* within 3-dimensional scaffolds to repair and/or replace damaged tissue in patients. Tissue engineering strategies are focused on four main areas of research: cell sourcing, scaffold design, functional tissue development and finally, development of viable commercialization models (Figure 2.1). The identification of a suitable cell source remains a formidable challenge, especially for cardiac applications as adult derived cardiomyocytes are difficult to obtain and non-proliferative *in vitro,* thereby limiting their applicability. The main areas of opportunities for cell sourcing include human embryonic stem cells, adult derived stem cells and autologous cells derived from patients. The choice of cell source would vary significantly depending on the application; autologous derived skeletal muscle cells can be utilized for cardiac regeneration, while autologous derived cardiac cells may not be the most feasible choice. Selection of suitable scaffolding material depends on the ability of the material simulate properties of the extracellular matrix (ECM), promote cell viability and proliferation, possess easily controllable degradation kinetics and have a high degree of immune tolerance when implanted *in vivo*. There are several matrices currently available which

meet many of these requirements, while new and improved biomaterials with improved functionality are continuously being developed. The next stage typically requires successful colonization of the scaffold by the cells; the viability of the cells during culture within the scaffold, the ability of the cells to maintain differentiated phenotype and the ability of the cells to functional interaction with the biomaterial become important considerations. Once cellularization of the scaffold has occurred, guided phenotypic maturation of the cells becomes important to promote tissue formation which closely resembles the physiological make-up of normal mammalian tissue. It becomes necessary to provide mechanical, electrical, and chemical/hormones cues to support the functional development of the tissue. Bioreactors need to be implemented to induce electro-mechanical stimulation of the bioengineered tissue, leading to gene expression that closely resembles the gene expression of *in vivo* tissue. The development of micro-perfusion systems becomes increasing important to replicate the physiological flow conditions observed *in vivo*. As tissue growth and maturation occurs, vascularization and/or functional innervation of the bioengineered tissue construct become important.

The task of engineering functional 3-dimensional constructs is often confounded by the degree of characterization required to evaluate the physiological performance of the construct. Functional tests often require *in vitro* assessment of mechanical (force, pressure, compression) and histological/biological (gene/protein expression and distribution) properties. The *in vitro* testing phase is followed by *in vivo* evaluation of biocompatibility and

immune acceptance/tolerance. During the next stage of testing, it becomes important to demonstrate the ability of the bioengineered construct to functionally integrate with the host tissue to promote the regeneration of damaged tissue using suitable animal models. This is followed by clinical evaluation of the bioengineered constructs to demonstrate the ability to translate the research to the clinical setting. The bioengineered constructs are then evaluated by guidelines established by regulatory process, such as the Food and Drug Administration (FDA), which serve to evaluate the safety and efficacy of the bioengineered constructs. For definitive success of bioengineered functional constructs, they must meet the challenges of commercialization, including development of business models backed by strong financial resources.

INTEGRATION OF CORE TECHNOLOGIES

Development of tissue engineering technologies requires collaborative efforts from diverse scientific disciplines. This model of scientific collaboration is fairly well established in many scientific endeavors and the novelty of tissue engineering places additional challenges in implementing successful collaborations. Development of core technologies for tissue engineering research requires expertise from engineering, medical and life sciences disciplines. Evaluating the phases of research for one particular example, development of a functional 3-dimensional cardiac patch, will illustrate the flow of information and technology between the three key players (Figure 2.2).

Identification of the need for an alternative therapy to treat cases of myocardial injury would need to begin within the medical profession. Although there have been several therapeutics options available to treat acute myocardial infarction, one can see the advantage of utilizing a functional tissue engineered 3-dimensional patch. The first and foremost problem would be identification, isolation, purification, characterization of a suitable cell source, typically carried out by the cell biologists. The next step would be the development of bioactive biomaterials by the engineering team and would require expertise in biomaterial synthesis, characterization and induction of bio-activity thereby allowing functional interaction with cells. The ability of the cells to functional interact with the biomaterial and promote the formation of 3-dimensional cardiac muscle would depend on many factors; attachment of the cells to the fibers of the biomaterials via integrin mediated mechanisms and ability of the cells to maintain differentiated phenotype during colonization of the scaffold. Understanding and manipulating cell-material interaction necessitates scientific input from engineering as well as the life sciences experts.

During the early stages of research, there needs to be an effort directed towards the development of small animal models, bioreactor technology to simulate physiological parameters and modulate the fluid environment of the constructs by developing continuous perfusion systems as well as the utilization of an array of biochemical markers. Every group of experts has to contribute to their full potential to head these initiatives. Later stages of research involve development of large animal models as well as the ability to develop feedback

control for bioreactors and real time monitoring of material properties and biochemical markers. This again demonstrates the need for true genius by scientists from each discipline.

A true collaborative effort between various disciplines is imperative to the success of each phase and it is crucial to promote the exchange of technology between each phase, revisiting the problem definition at during every stage of the process. This simple example serves to demonstrate the degree of complex interactions and exchange of information required at the very early stages of scientific development between scientists from medical, engineering and life sciences. Development of a successful model to accomplish this degree of scientific and technological collaboration will be a significant challenge for the field of tissue engineering.

Tissue engineering has traditionally lacked a model for cohesive research. The general tendency has been for the establishment of laboratories by single investigators in an academic setting. This has led to the development of excellent technological development in a single area of tissue engineering, without emphasis on the functional interaction across disciplines. Although single laboratories claim to collaborate with disciplines outside their area of expertise, or even recruit research personnel across these boundaries, we do not believe this is adequate for the specific research challenge at hand, nor is it done in such a way that it changes and improves the overall culture of the tissue engineering research community in terms of promoting increased multidisciplinarity. Rather, multi-disciplinary centers need to be established,

often through the collaborative efforts of professional from academic, government and industrial background. Leadership needs to be jointly established by all participating members. Funding will need to be secured by diverse sources like federal agencies, philanthropic donations and support from industry. Intellectual property will need to be managed in a manner that promotes a significant degree of openness to allow technology development will protecting ownership of the inventors. In our opinion, this will be the only through successful way to develop tissue engineering models.

POTENTIAL CLINICAL APPLICATIONS OF BIOENGINEERED CARDIO-VASCULAR CONSTRUCTS

The field of cardiac tissue engineering includes not only heart muscle, but also tri-leaflet valves, cell based cardiac pumps/ventricles, and vascular grafts. Although the focus of this article is on cardiac muscle, other areas of cardiac tissue engineering will be briefly discussed for completion. Most of these fields are at early stages of technological development. There is an opportunity to have a significant impact on the future of treatment modalities for cardio-vascular disorders (Figure 2.3).

The most successful strategy for treatment of end stage congestive heart failure has been surgical transplantation. However, widespread applicability of heart transplantation is often limited by a chronic shortage of donor organs(15; 26; 27). Cardiac tissue engineering may provide a potential solution to the donor heart crisis in the future. The clinical utility of cardiac patches could be

particularly advantageous in cases of acute myocardial infarction, where integration of bioengineered patches with host myocardium could theoretically improve the contractile function of the failing left ventricle (Figure 2.3A) (28-34).

The current treatment options for vascular substitution require the use of the mammary artery or saphenous vein for by-pass grafting. The major disadvantages of this strategy is the need for invasive patient surgery and the limited quantity of autologous grafting material available. Synthetic vascular substitutes like Dacron and ePTFE lack the cellular components thereby limiting functional performance and have limited utility in small diameter applications. Patients often suffer from thrombus formation and intimal hyperplasia. Tissue engineering alternatives could potentially eliminate some of these complications and may be particularly suitable for small diameter applications (Figure 2.3B) (35-39). The ability to fabricate hollow tubular structures surrounded by smooth muscle cells with a lining of endothelia cells on the luminal surface would constitute a viable tissue engineered alternative.

Heart valve substitution is the only effective treatment for end-stage heart valve diseases when repair is not feasible (40). Current options are limited to mechanical valves (41) and biological (42) substitutes. Mechanical heart valves often require long term anticoagulation while biological substitutes lack the mechanical durability for long term function. Valve replacement for pediatric patients is further limited due to the inability of the prosthetic valve to grow with the pediatric patient, necessitating several re-operations during the childhood of a patient. Tissue engineering offers the potential of generating heart valves of

virtually any size, which would grow with pediatric patients(43). In addition to the many challenges of tissue engineering already discussed, the ability to engineer tri-leaflet valves is confounded by a complex 3-dimensional geometry necessitating complex mold design (Figure 2.3C) (44-48).

The ability to engineer cell based cardiac pumps may provide an alternative to mechanical Left Ventricular Assist Devices (LVADs)(49). LVADs are used as a bridge to transplantation, transporting oxygenated blood from the apex of the heart directly to the aorta, completely bypassing the failing left ventricle. A cell based alternative would be composed of a hollow chamber surrounded by contracting cardiac cells with one-way valves for unidirectional flow and embedded electronics for feedback control (Figure 2.3D). Sensors would receive electrical input from the SA node, permitting contractions that are synchronized with the heart. The cell based alternatives to mechanical LVADs would offer a higher degree of immune tolerance reducing the need for long term anti-coagulation.

CELL SOURCING

A critical issue in cardiac tissue engineering is the choice of cells for construct development. An ideal cell source for cardiac tissue engineering should possess the following characteristics: 1) proliferative, 2) easy to harvest, 3) nonimmunogenic, 4) ability to differentiate. Depending on the specific application, potential cell types for cardiac tissue engineering may include

(although not limited to): vascular smooth muscle, skeletal myoblasts, fetal cardiomyocytes, mesenchymal stem cells, endothelial progenitors, bone marrow cells, umbilical cord cells, fibroblasts, embryonic stem cells. The composition of cells is also an important consideration in cardiac tissue engineering. Cardiac tissue *in vivo* is complex, being composed of multiple cell types. Cardiac myocytes are the main component of the heart, however a combination of endothelial cells, fibroblasts, smooth muscle cells, neural cells, and leukocytes make up approximately 70% of the totally cells in the working myocardium (50). Non-cardiomyocytes play an important role in normal cardiac development and function. Using a triple-cell-based culture of cardiomyocytes, endothelial cells, and embryonic fibroblasts, a 3-dimensional model of vascularized cardiac tissue has recently been developed (51). The presence of endothelial cells led to increased cardiomyocyte proliferation, while the presence of embryonic fibroblasts decreased endothelial cell death and increased their proliferation; thereby demonstrating the importance and complexity of functional interactions between the various cell types. Future development of *in vitro* cardiac models which incorporate multiple cell types should lead to improved graft/transplant survival with increased functionality.

Utilization of adult derived stem cells would provide a cell source that can be derived from patients and are therefore autologous. Potential sources of adult stem cells for cardiac tissue engineering include bone marrow, peripheral blood, and skeletal muscle. There have been promising results using adult stem cells, demonstrating viability of transplanted cells, increased angiogenesis in the infarct

zone and improved left ventricular function. Studies evaluating the feasibility of cell transplantation provide a good model to understand the utility of various cell types for myocardial regeneration (52-54). Cell transplantation therapies have utilized adult derived stem cells skeletal myoblasts (55-60), bone marrow derived cells(61-65), and endothelial progenitor cells (66; 67) or embryonic stem cells(68-72).

Autologous skeletal myoblasts show expression of contractile proteins and form well-differentiated myotubes *in vitro* and after transplantation in the heart; however the nascent myotubes do not form electrical junctions with host myocardium and thus are unlikely to participate in a functional syncytium and deliver respective work load. Also, myoblasts do not differentiate towards a more cardiomyocyte-like phenotype upon *in vitro* or *in vivo* physiological stimulation and have not demonstrated fatigue resistance required for cardiac application. In addition, the time needed to process and expand autologous myoblast cultures would make it difficult to deliver them in a timely fashion to a patient in need. Although adult stem cells are a promising option for tissue engineering, at the present time, their true potential remains unclear. An important question that remains is whether it is possible to induce transdifferentiation of adult stem cells at the necessary efficiency. Recent research effort is focused on growth factors and culture conditions that promote cardiac lineage commitment and genes that may be introduced to induce cardiac differentiation (73).

Although the utilization of human embryonic stem (ES) cells is compounded with ethical, political, and scientific differences of opinion, there is

great potential for the use of ES cells in cardiac regeneration. The motivation for the utilization of embryonic stem cells are the unlimited proliferative capacity of these cells and ability to differentiate into multiple cells types including all cells found in the myocardium. Various studies have demonstrated that mouse and human ES cells can differentiate into functional cardiomyocytes (74-76). Genetically engineered ES cells can be cultured to yield large-scale generation of viable cardiomyocytes for tissue engineering and/or implantation (77-79), thereby demonstrating the feasibility of utilizing ES cells for cardiac tissue engineering applications. Recent studies demonstrating the presence of stem/progenitor cells in the heart may provide an alternative cell source for cardiac regeneration and repair (80).

A significant amount of research is directed towards evaluating the feasibility of utilizing different cell types for cardiac regeneration. Although a clear direction has not evolved as yet, several promising options are being evaluated. In concluding this section, it may be valuable to compare cell therapy approaches with tissue engineering methods for cardiac regeneration. The main advantage that tissue engineering has to offer is the fabrication of a functional 3-dimensional tissue construct which may be layered onto the damaged myocardium. This presents the opportunity for functional interaction between the transplanted construct and the host thereby permitting functional augmentation due to the contractions of the construct.

BIOMATERIALS

Biomaterials guide the phenotypic maturation of the cardiac cells during tissue formation by promoting functional cell attachment and subsequent remodeling to form functional 3-dimensional tissue constructs(81-84). There are several requirements for an ideal biomaterial for cardiac tissue engineering applications. The material should support the attachment, viability and proliferation of cardiac cells in culture. The porosity (pore size and distribution) should accommodate the colonization of different cell types found in cardiac tissue. The size, orientation and distribution of biomaterial fibers should mimic the properties of the cardiac extracellular matrix. The degradation kinetics of the biomaterial should match the rate of tissue formation by the cardiac cells while producing non-toxic degradation products. The mechanical properties of the material (tensile strength, elasticity) need to be comparable to the extracellular matrix of the heart. In addition, for *in vivo* applications the biomaterial should be immune tolerant, functionally integrate with host myocardium and support the formation of electro-mechanical coupling as well as construct neovascularization.

Several biomaterials have been utilized to engineer functional 3-dimensional cardiac tissue *in vitro(85)*. Two classes of biomaterials currently utilized to engineer functional 3-dimensional cardiac tissues are polymeric scaffolds (polyglycolic acid(86-89)) and hydrogels (collagen(90-93), fibrin (unpublished data), and alginate(94)). The main advantage of using polymeric scaffolds is the ability to provide stable mechanical support to promote cardiac tissue formation. Polymeric scaffolds can sustain higher mechanical loads and

therefore have been preferred for many cardio-vascular tissue engineering applications. Hydrogels, on the other hand provide, have been shown to provide an environment that more closely stimulates the extracellular matrix of cardiac muscle. Several medications, including the utilization of cross-linking agents have been implemented to improve the mechanical strength of the gels.

The currently utilized biomaterials have shown to adequately support cardiac cell remodeling (95-97). Bioengineered constructs generated by both polymeric scaffolds and hydrogels have shown to exhibit contractile, histological and biochemical characteristics similar to normal mammalian cardiac muscle (19; 98; 99). However, as new models of cardiac muscle are engineered in the laboratory with the desire to functionally match the performance of normal heart muscle, a new generation of novel biomaterials would also need to be developed.

There has been an emphasis towards the development of “smart” biomaterials that are receptive to changes in the physiological environment and are adaptive to changes in the degree of tissue maturation(100-103). The overall goal is to program a feedback loop that changes the properties of the materials as changes in the physiology of the cells take place. Consider a hypothetical case in which cardiac cells are seeded into the 3-dimensional architecture of a “smart” material. As tissue remodeling takes place, the cardiac cells generate extracellular matrix components, mainly collagen. The rate of synthesis of collagen would depend on several variables; viability of the cardiac fibroblasts, mechanical conditioning and stimulation with soluble factors and the rate of

synthesis of collagen would change over time depending on the balance of these factors. One goal of a “smart” material would be to “sense” the change in collagen production and “program” material degradation to match the rate of collagen synthesis. Conceptually, one unit of collagen production will result in one unit of material degradation.

Although conceptual at this stage, there have been several interesting examples of the development of “smart” materials (Figure 2.4). The ability of matrix metalloproteinases (MMPs) to recognize and cleave specific amino acid sequences makes it a suitable mechanism for the functionality of smart biomaterials(104). A similar concept has been developed by utilization of changes in pH upon cellular endocytosis of biomaterials (105). The pH within the endosome is acidic, which can be utilized to cleave acid sensitive carriers of biomaterials thereby promoting the controlled release of bioactive factors. Changes in the oxidative state of tissue have also been proposed as a potential mechanism for the development of smart materials (106; 107).

The goal in the development of “smart” materials is to engineer biomaterials with specifically targeted cell attachment sites, targets for cleavage by changes in the physiological environment all linked to a polymer backbone containing internalized growth factors (Figure 2.4). The objective would be to utilize the cell attachment site to deliver the biomaterial to specific targets while changes in the physiological state of the cells would promote the release of growth factors in the local environment. For example, matrix metalloproteins

cleavage at specific sites results in controlled release of the bone morphogenetic proteins into the local environment.

IN VITRO MODELS OF HEART MUSCLE

When considering the functional requirements that tissue engineered heart muscle needs to satisfy, one must aim to recapitulate the anatomy of mammalian cardiac muscle. Although it may not be feasible to develop a perfect replica of mammalian cardiac tissue *in vitro*, certain key characteristics define conditions that would be pre-requisite for the development of tissue models of heart muscle. Identification of a suitable cell source, preferably adult derived autologous stem cells with physiological compositions of cardiac myocytes, fibroblasts and endothelial cells would be necessary. One would need novel biomaterials with properties that replicate the extracellular matrix of the heart. The functional integration of cardiac cells with biomaterials would need to result in contractile, histological, biochemical and electro-physical properties comparable to mammalian heart muscle. Immune tolerance and neovascularization would also be additional constraints placed on tissue engineered cardiac constructs.

Our discussion on *in vitro* models of heart muscle is divided into 2 sections. In the first section we discuss models published by investigators across the globe and in the second part we discuss 3 models that have been developed in our laboratory.

Carrier and colleagues utilized fibrous meshes made from polyglycolic acid (PGA) as a scaffolding material for culturing neonatal cardiac myocytes(108). PGA was processed into fibrous meshes to form disk-shaped constructs with a very high degree of porosity and seeded with primary cardiac myocytes(109; 110). These studies were the first to demonstrate the formation of functional cardiac muscle utilizing scaffolding based strategies. Eshcenhagen developed a model of cardiac muscle by casting a mixture of neonatal cardiac myocytes and collagen into plastic molds(111; 112). The resulting tissue constructs, termed Engineered Heart Tissue (EHT), were capable of generating fairly high twitch forces(113). Eschenhagens model was the first 3-D model of cardiac muscle *in vitro*. Okano utilized temperature sensitive polymer surfaces (TSS) to engineer 2-dimensional sheets of cardiac cells, which were stacked to form 3-dimensional tissue constructs (114-117). The most attractive feature of this model is the utilization of the TSS which eliminates the need for synthetic scaffolding materials in the contractile region of the tissue engineering cardiac muscle. Akins utilized a rotating bioreactor system to promote the organization of isolated cardiac cells on the surface of microcarrier beads (118). Li utilized a gelatin mesh(119-123) while Leor utilized alginate sponges(124; 125) to support cardiac tissue formation.

Our laboratory has spent the past several years evaluating the feasibility of utilizing different platforms to engineer functional 3-dimensional cardiac muscle *in vitro*(1; 126-131). We have currently developed 3 models of contractile heart muscle (Figure 2.5): 1) Cardioids are formed by the self organization of

primary cardiac cells in the absence of synthetic scaffolding materials (Figure 2.5A), 2) Bioengineered heart muscle (BEHMs) are formed in the presence of a rapidly degrading fibrin gel (Figure 2.5B), and 3) Smart material integrated heart muscle (SMIHM) utilizes polymeric scaffolding to support cardiac tissue formation (Figure 2.5C).

Cardioids are formed by the spontaneous delamination of a cohesive monolayer of primary cardiac cells (132). The tissue culture surface utilized for cardioid formation is specifically engineered to control surface protein concentration. The surface protein promotes cell attachment during initial stages of cultures while initiating delamination during the later stages of culture. In addition, soluble factors introduced via media changes, promotes the cardiac cells to generate ECM components during the entire culture period. The cardiac cells and ECM self-organize by controlled guidance of the protein adhesion molecules. The main advantage of this model is the ability to promote self organization of a 3-dimenisonal tissue construct without the use of synthetic scaffolding material.

BEHMs are formed by the remodeling of primary cardiac cells in the presence of a biodegradable fibrin gel (133). During BEHM formation, the cardiac cells are layered on the surface of the fibrin gel and allowed to remodel, promoting the formation of functional 3-dimensional heart muscle. Cardiac cells degrade the fibrin gel and produce ECM components during the initial stages of construct formation. As this process continues, the fibrin gel is gradually degraded and eventually replaced by endogenous ECM within 2 weeks of

culture. The scaffolding material promotes ECM production while providing temporary support for the cells. The most attractive feature of this model is the formation of heart muscle in a relatively short time period, within 4-5 days.

SMIHMs utilize a “smart” material to control the formation of heart muscle (134; 135). The material is considered to be “smart” because it closely resembles cardiac ECM *in vivo* and the material can undergo controlled degradation upon formation of viable heart muscle. Utilizing this technology, we have engineered SMIHMs by cellularization of these smart materials using primary cardiac cells and shown close resemble to normal heart muscle tissue. Our current efforts are focused on controlling the rate of material degradation to match the rate of tissue formation. We are also in the process of programming a feedback loop in the material to respond to changes in the rate of collagen synthesis.

BIOREACTORS AND MICROPERFUSION

Bioreactors are an integral aspect of all tissue engineering research and are particularly important in the cardio-vascular field due to the hemodynamic requirements of bioengineered constructs. Several classes of bioreactors have been utilized in functional tissue engineering(136). Bioreactors have been utilized during scaffold cellularization to obtain a uniform cell distribution within the 3-dimensional architecture of biomaterial. Bioreactors have also been utilized to provide physical stimulation to pre-formed 3-dimensional constructs during long term *in vitro* culture; for example, electro-mechanical stimulation to

promote maturation of cardiac constructs. In addition to bioreactors, micro-perfusion systems play an important role in functional tissue engineering. The role of micro-perfusion systems has been primarily to deliver a continuous supply of enriched media to the bioengineered construct during *in vitro* culture. The goal is to closely replicate the *in vivo* condition whereby a continuous blood supply promotes nutrient exchange as well as removal of waste products. Bioreactor/micro-perfusion systems have been described for several cardio-vascular systems including tissue engineered blood vessels(137-141), valves(142-147), and cardiac muscle(148-150). Although the configuration of bioreactor systems have varied, the functional requirements have remained similar; to simulate physiological conditions (temperature, pH, oxygen tension, glucose levels, radial and circumferential stress) under controlled aseptic conditions to promote remodeling of cells and ECM.

We have developed a bioreactor system to allow the application of coordinated mechanical signals to bioengineered constructs during long term *in vitro* culture. The bioreactor system was designed and fabricated by Dr. Robert Dennis, Associate Professor of Biomedical Engineering at the University of North Carolina, Chapel Hill. We have utilized this system in our laboratory with our BEHM model. Each bioreactor allows up to 11 individual tissue specimens to be subjected as a group to the same mechanical stimulation protocol. The tissue culture plates are positioned on a movable platform that is controlled by a stepper motor driver circuit. The stepper controller circuit allows for direct user interface and control of each bioreactor, both for system set-up and to initiate the

experimental protocol. The actual mechanical strain protocol is programmed directly onto the embedded microcontroller prior to each experiment. The stimulation protocols can be carried out for long periods of time (weeks or months) while permitting the culture media to be changed manually, as would occur in traditional cell culture using Petri dishes. The design of the bioreactor is suitable for transforming the static engineered muscle culture for dynamic mechanical loading studies without physical transfer of the construct. Using this system, we have shown that stretching the BEHMs at a frequency of 1 Hz for 7 days results in a two-fold increase in specific force.

We have developed a micro-perfusion system in our laboratory, in collaboration with Desmond Radnoti, CEO, Radnoti Glass Inc, to support the formation of cardiac muscle (unpublished data). Our system consists of a custom biochamber designed to accommodate 11 tissue culture plates, each plate stacked vertically on an independent stage (Figure 2.6). Each plate receives an independent media flow with independent outflow manifolds for media aspiration. The internal environment of the biochamber is carefully regulated to maintain temperature and carbon dioxide. The biochamber is surrounded by a water jacket to permit flow of heated fluid for temperature control. Carbon dioxide is directly injected into the biochamber and maintained at a pre-defined level by feedback control using a solenoid valve. Cell culture media is maintained at constant temperature using a water-jacketed reservoir and is oxygenated prior to entering the biochamber. Media oxygenation is achieved one out of 2 ways; direct bubbling of oxygen in the media and perfusing

the media through a hollow chamber oxygenator. The temperature, pH, oxygen saturation and carbon dioxide values are measured and recorded in real time at multiple points within the system. The main advantage of our system is that is does not require the use of a cell culture incubator, thereby permitting complete user control of the cell culture environment.

UTILITY OF BIOENGINEERED IN VITRO MODELS FOR BASIC RESEARCH

As new layers of complexity are continually added to our understanding of basic biological processes, interdisciplinary approaches will be increasingly necessary for successful research advances. This is demonstrated by the upward tendency of funding agencies to provide substantial support for program projects involving many contributors from a variety of labs and the growing number of major publications with multiple authors from diverse scientific backgrounds. Tissue engineering combines principles of engineering and biology, leading to seemingly endless applications. However, the tremendous potential of bioengineered tissues for *in vitro* basic research applications has only recently been recognized.

Cells interpret cues from their immediate microenvironment and the growth and differentiation of many cell types is regulated by the interplay of four major signaling sources (Figure 2.7): 1) soluble growth factors, 2) insoluble ECM and growth substrates, 3) environmental stress and physical cues, and 4) cell-cell interactions(151). Given the complex mechanical and biochemical interplay,

researchers will miss biological subtleties by only considering cells grown 2-dimensionally(152). On the other hand, 3-dimensional tissue explants are complex, being composed of several different cell types that are difficult to experimentally manipulate individually. For these reasons, tissue engineering has emerged as a powerful technology where isolated cells can be bioengineered into 3-D, homogeneous tissues with characteristics similar to those observed *in vivo*. In addition, bioengineered tissues can be maintained for weeks or months in culture under physiological conditions.

Incorporation of tissue engineering components with cellular/molecular based projects has become a powerful approach for basic research. For example, researchers have used an integrated approach to examine the direct effects of microenvironment on stem cell specification(153). By varying matrix elasticity and measuring cell morphology, transcript profiles, marker proteins, and the stability of responses, they showed that naïve mesenchymal stem cells specify lineage and commit to phenotypes with extreme sensitivity to tissue-level elasticity.

Bioengineered tissues can be more easily manipulated for experiments, including transfection of cells with expression vectors to control expression of an endogenous protein or to give expression of mutated proteins. The use of tetracycline-regulated gene expression in bioengineered skeletal muscle tissues has been demonstrated. Although macroscale animal testing remains the primary method used for evaluation of toxicological and pharmacological profiles of therapeutic agent, it is possible to reduce the number of experimental animals

used in the future with the use of *in vitro* bioengineered models. Researchers have developed a microfluidic device consisting of an array of channels and 3-dimensional tissues embedded in chambers, which can be used to screen drug efficacy and toxicity in multiple tissues simultaneously. Convergence of new technologies may provide feasibility for the development and use of a microscale bioengineered animal-on-a-chip.

Researchers can manipulate their materials to control the biology of the cells growing within. The use of biological feedback mechanisms in growth factor delivery has also been explored(154). For example, a growth factor bound to the matrix and released upon cellular demand through cell-mediated localized proteolytic cleavage from the matrix, which substantially mimics the mechanism by which these factors are released in vivo from stores in the natural ECM by invading cells in tissue repair(155; 156).

The main motivation for the development of bioengineered constructs seems to be their *in vivo* utility. Although this is likely to be one very significant application of the constructs, this realization is may not occur within the short time future as cardiac tissue engineering is still in its early stages of development. However, other potential applications of bioengineered constructs exist and utility as a tool for basic research is one example.

THOUGHTS FOR THE FUTURE

There have been several important technological breakthroughs in the field of functional cardiac tissue engineering during the past decade. Using

current technology, primary isolated cardiac cells can be programmed to form functional 3-dimensional constructs *in vitro*. Several biomaterials have been shown to support heart muscle development *in vitro* and various cell sources have been utilized to study cardiac regeneration *in vivo*. However, several critical technological hurdles need to be addressed in order for the field of cardiac tissue engineering to advance.

Identification of a suitable cell source for cardiac tissue engineering still remains a challenging task. Contractile cells derived from cardiac origin are difficult to source while cells from a skeletal origin have not demonstrated the necessary fatigue resistance required for cardiac applications. Human embryonic stem cells are barred by technological advancements demonstrating fate programming in addition to the political battles that need to be overcome. Adult derived stem cells are yet to prove utility for cardiac applications. Although several approaches show promise, a clearly vision has not been established for the identification of a suitable cell source with the potential to be utilized for basic several leading to translational and finally clinical applicability.

There needs to be a new generation of biomaterials, developed specifically for cardiac tissue engineering applications. Although the current materials have adequately supported the formation of 3-dimensional cardiac tissues *in vitro*, the mechanical performance and biological properties of these bioengineered tissues do not resemble the properties of native mammalian cardiac tissue. Novel biomaterials need be developed to more closely match the properties of cardiac extracellular matrix in terms of content and orientation of

structural proteins. In addition, new biomaterials need to be programmed with feedback control mechanisms to respond to changes in the local environment. As an example of feedback control of biomaterials, generation of extracellular matrix by cardiac cells would result in programmed degradation of the material in proportion to the rate of synthesis of the extracellular matrix.

Development of a new generation of bioreactors and micro-perfusion systems presents another technological hurdle for the field of functional cardiac tissue engineering. The current body of literature clearly supports the need to utilize bioreactors for the formation of functional tissue engineered constructs. Current systems are fairly complex and involve some combination of electro-mechanical stimulation in the presence of micro-perfusion. One challenge would be to develop the ability to evaluate the changes in the functional performance outcome of the tissue construct in real time. As a simple example of real time functional assessment, it would be valuable to monitor the contractile performance of 3-dimenisonal cardiac constructs during long term culture. Another challenge for bioreactor/micro-perfusion development would be the implementation of feedback control. As an example, as maturation of tissue engineered constructs takes place, changes in the cellular environment would need to be matched by changes in the rate of micro-perfusion to adequately support the increased metabolic demands.

In conclusion, although several technological milestones have been accomplished within the field of cardiac tissue engineering, some important

challenges remain. Future studies designed to address these issues will help define the field of functional cardiac tissue engineering.

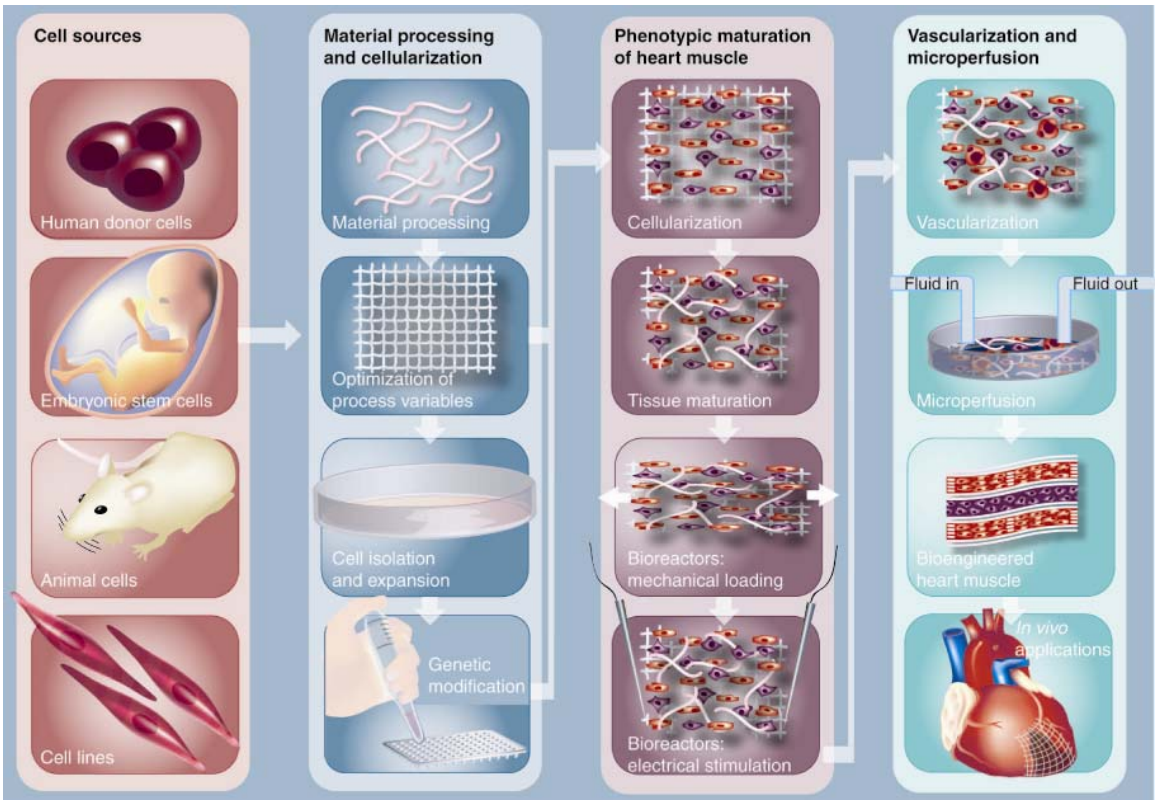

Figure 2.1 Pathway for the Formation of Bioengineered Constructs. The complex roadmap to bioengineering constructs begins with the identification of a suitable cell source; cells can be obtained from donor human tissue, derived from an embryonic origin, obtained from animal tissue and/or derived from cell lines. The cells are isolated, expanded and characterized under controlled *in vitro* conditions, manipulated to modulate the expression of specific genes and then utilized to colonize pre-fabricated biomaterials. The bioengineered constructs are then subjected to various environmental cues in the form of electrical and mechanical stimulation as well as micro-perfusion to promote phenotypic maturation of the constructs. This is followed by induction of angiogenesis, *in vitro* testing of the constructs followed by *in vivo* testing using animal models, clinical testing and development and implementation of commercialization strategies.

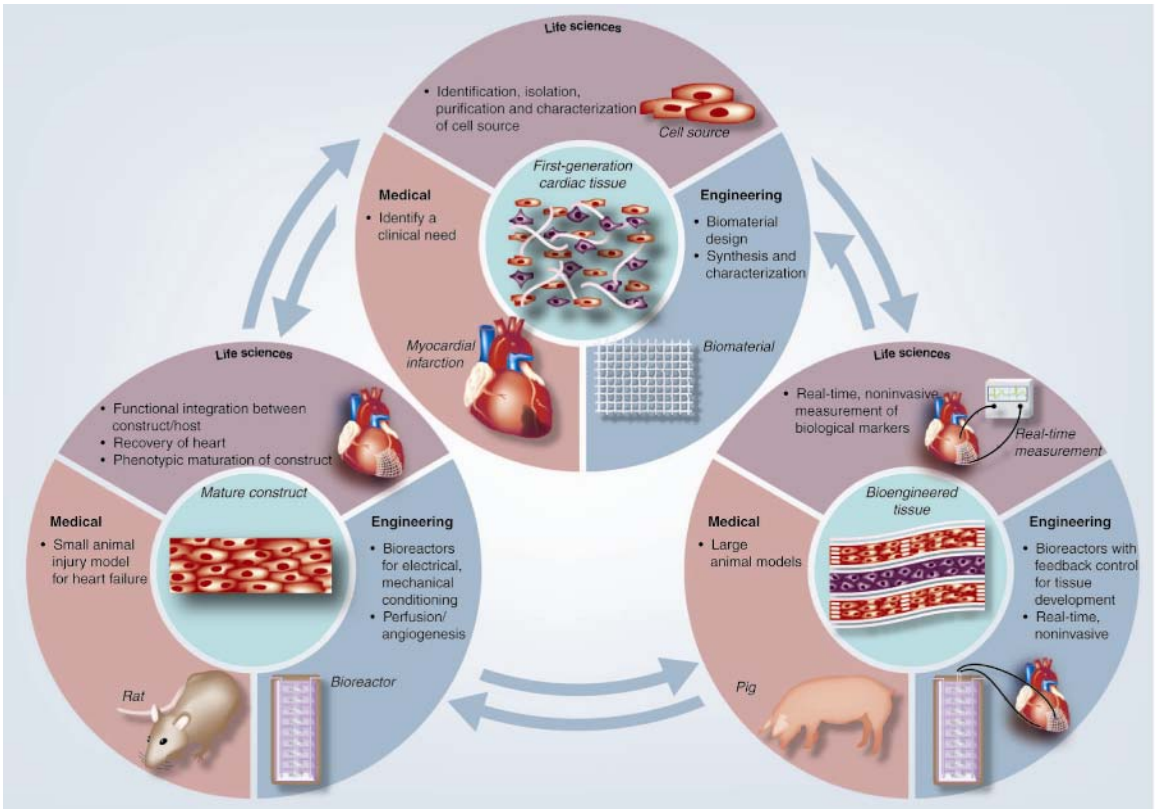

Figure 2.2 Core Technologies Required for Tissue Engineering. Implementation of a successful pathway for the formation of bioengineered constructs requires functional integration of technical expertise from several scientific disciplines; medicine, engineering and life sciences. Using an example of the development of cardiac patches, the interplay between the various cores can easily be illustrated. Identification of the need for novel treatment modalities, such as bioengineered constructs, begins within the medical discipline necessitating the identification of multi-disciplinary teams. During the early stages of the research, the engineering core works towards the development of novel biomaterials while the life science core works towards cell isolation, purification and expansion. Novel cellularization strategies lead to the formation of a premature first generation bioengineered construct. During the second stage of research, small animal models are developed by the medical experts while sophisticated biological markers are developed by experts in the life sciences. Engineering folks are generally involved in the design and fabrication of novel bioreactors and micro-perfusion systems. Later stages of research necessitate the development of large animal models, real time monitoring of functional performance with embedded feedback control and non-invasive biological markers for *in vivo* performance of bioengineered constructs. This model of functional integration of core technologies progresses intimately through every phase of technological development resulting in the development of clinically usable bioengineered constructs.

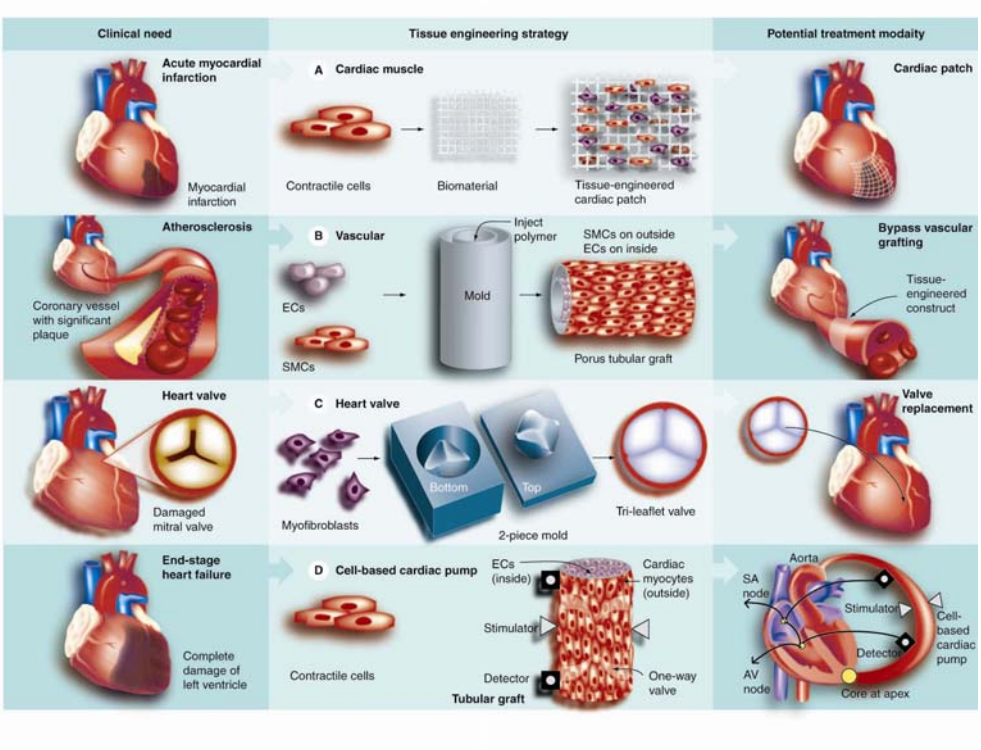

Figure 2.3 Functional Cardio-Vascular Tissue Engineering at a Glance. (A) Bioengineered Cardiac Muscle – The ability to engineer cardiac muscle by functionally integrating primary contractile cells with biomaterials results in the formation of tissue engineered cardiac patches. These cardiac patches can be utilized to treat cases of acute myocardial infarction to augment the contractile function of the failing left ventricle. **(B) Bioengineered Vascular Grafts** – The ability to fabricate tubular structures utilizing various scaffolds/hydrogels is dictated by the design of suitable molds, which is often fairly trivial. The walls of the tubular graft are cellularized with smooth muscle cells while the lumen is cellualarized with endothelial cells resulting in the formation of bioengineered vascular grafts. The utility of these vascular grafts would in cases of coronary bypass surgery. **(C) Bioengineered Tri-leaflet Valves** – The complex 3-dimensional architecture of tri-leaflet valves necessitates complex mold prototyping to fabricate tissue engineered equivalent. Once a prototype is fabricated, cellularization with myofibroblasts results in functional heart valves. **(D) Cell Based Cardiac Pumps** – The ability to engineer hollow structures with cardiac cells wrapped around the periphery can serve as a cell based devices capable of generating intra-mural pressure upon electrical stimulation. Embedded detectors on the device coupled to the SA node receive electrical impulses which are transmitted to electrical stimulators. This allows the cell based pump to contract in synchrony with the host heart. Cell based cardiac pumps can be utilized to treat cases of chronic heart failure, with mechanical left ventricular assist devices being used as a bridge to transplantation.

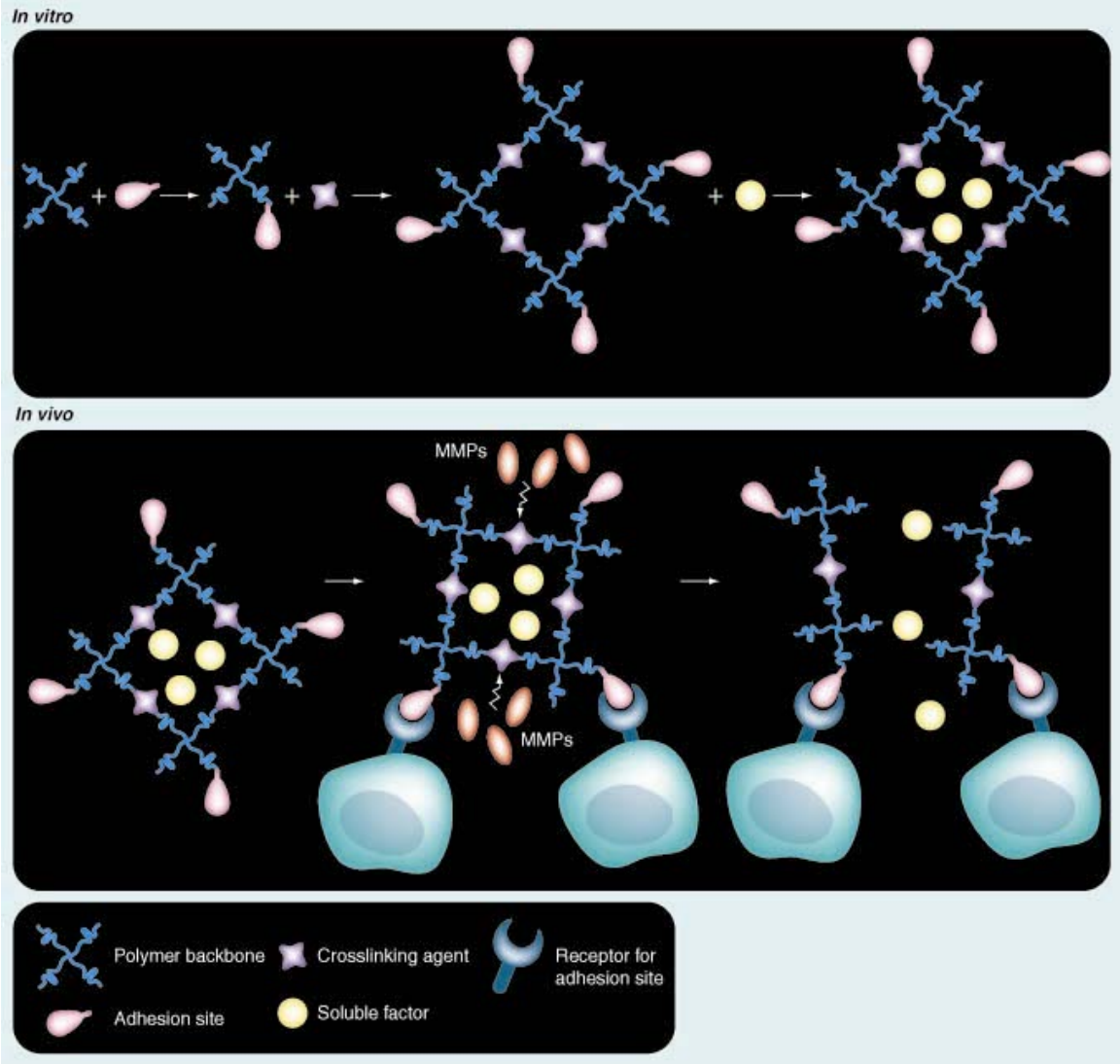

Figure 2.4 Smart Biomaterials for Tissue Engineering.
In vitro development of smart biomaterials necessitates engineering cell specific adhesion sites and cross-linking agents to trap soluble factors within the 3-dimensional architecture of the biomaterial. *In vivo*, the biomaterial is guided to specific target receptors on the surface of specific cell types promoting cell-material interaction. Matrix metalloprotein (MMP) cleavage at the cross-linking site promotes the release of the soluble factors into the local environment. This mechanism ensures the delivery of soluble factors to specifically targeted sites using the biomaterial as a carrier and the MMP as the mediator.

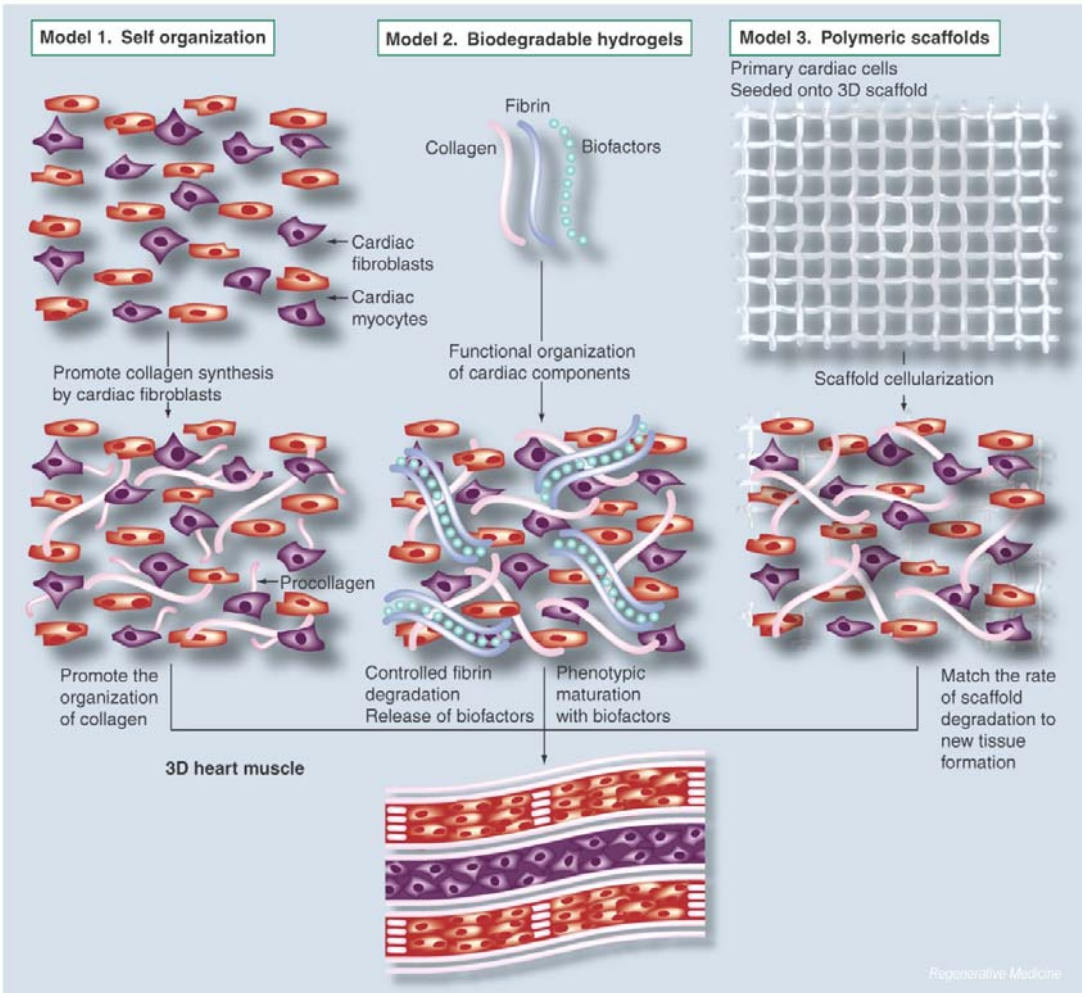

Figure 2.5 Tissue Engineering Platforms for Heart Muscle.
(A) Self Organization – Primary cardiac myocytes and cardiac fibroblasts are co-cultured on the surface of a tissue culture plate that has been coated with PDMS, natural mouse laminin and engineered with anchor points at the center. During co-culture, the cardiac fibroblasts generate pro-collagen which polymerize to form collagen fibers. The collagen provides structural support during the remodeling process. During the early stages of cell culture, the primary cardiac cells form a cohesive monolayer using the laminin as an adhesion protein. During later stages of cell culture, the spontaneous contractions of the cardiac myocytes promote delamination of the cell monolayer. The cell monolayer forms a 3-dimensional construct at the center of the plate and forms attachment points with the anchor points. These tissue constructs are termed “cardioids”. The key element of this model is that the cells form a 3-dimensional tissue construct in the absence of any scaffolding material. **(B) Biodegradable Hydrogel** – When hydrogels are utilized as a tissue engineering platform, the cells are provided with structural components to remodel around. In our case, we have utilized collagen type I as the structural element while fibrin is used as a carrier for biofactors. Primary cardiac cells are mixed with collagen and biofactors that

have been embedded in a fibrin gel. The cardiac cells utilize the collagen to form functional 3-dimensional heart muscle. During the course of construct formation, controlled degradation of the fibrin by the cardiac cells results in the release of biofactors in the environment. These biofactors act on the cardiac cells during the remodeling processresulting in significantly increased functional performance of the construct. These constructs have been termed “bioengineered heart muscle, BEHM”. **(C) Polymeric Scaffolds** – The utilization of polymeric scaffolds is to provide a pre-fabricated and pre-defined area to guide remodeling of cardiac cells to form 3-dimensional tissue constructs. The polymeric scaffolds often offer superior mechanical properties when compared with hydrogel. We have utilized chitosan in our laboratory and used phase separation methods to form porous 3-dimenional scaffolds. Cellularization of the scaffold using cardiac cells has resulted in the formation of functional heart muscle. In addition, the degradation of the scaffold is easily controlled by the addition of lysozyme. We have developed strategies to match the rate of new tissue formation with the rate of scaffold degradation. This has lead to the formation of heart muscle with high functional performance termed, “smart material integrated heart muscle, SMIHM”.

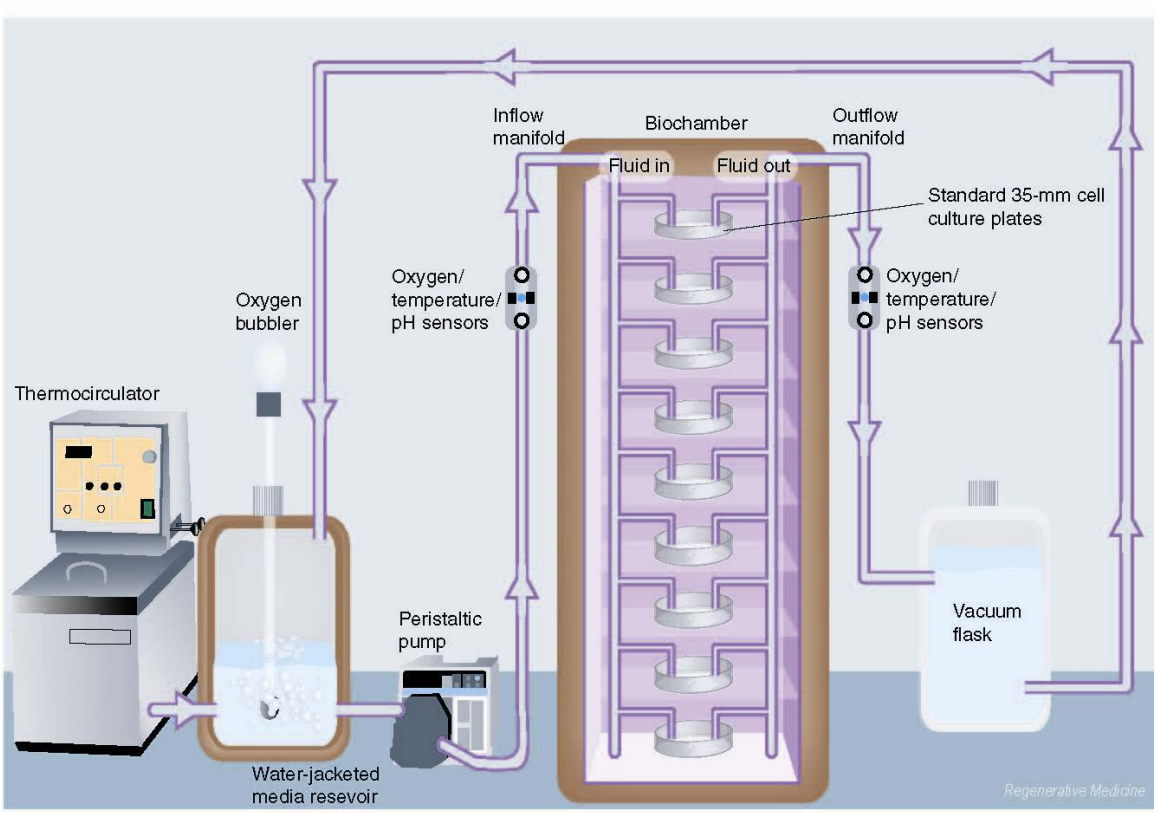

Figure 2.6 Micro-Perfusion for Heart Muscle.
The biochamber consists of multiple independent stages to accommodate tissue culture plates. Media delivery and aspiration takes place independently from each plate via manifolds that are positioned directly above the tissue culture plates. The internal environment of the biochamber is controlled to regulate temperature and CO_2 level. Media is maintained in a water jacketed reservoir to maintain temperature and is oxygenated prior to reaching the plates. The system is run on several pumps which are used to deliver media at a specified flow rate, aspirate spent media from the plates, promote mixing of the gases inside the biochamber to equalize CO_2 levels as well as promote uniform distribution of moist air. All variables (temperature, CO_2, O_2, pH, flow-rate) are monitored in real time via sensors embedded at several points throughout the system.

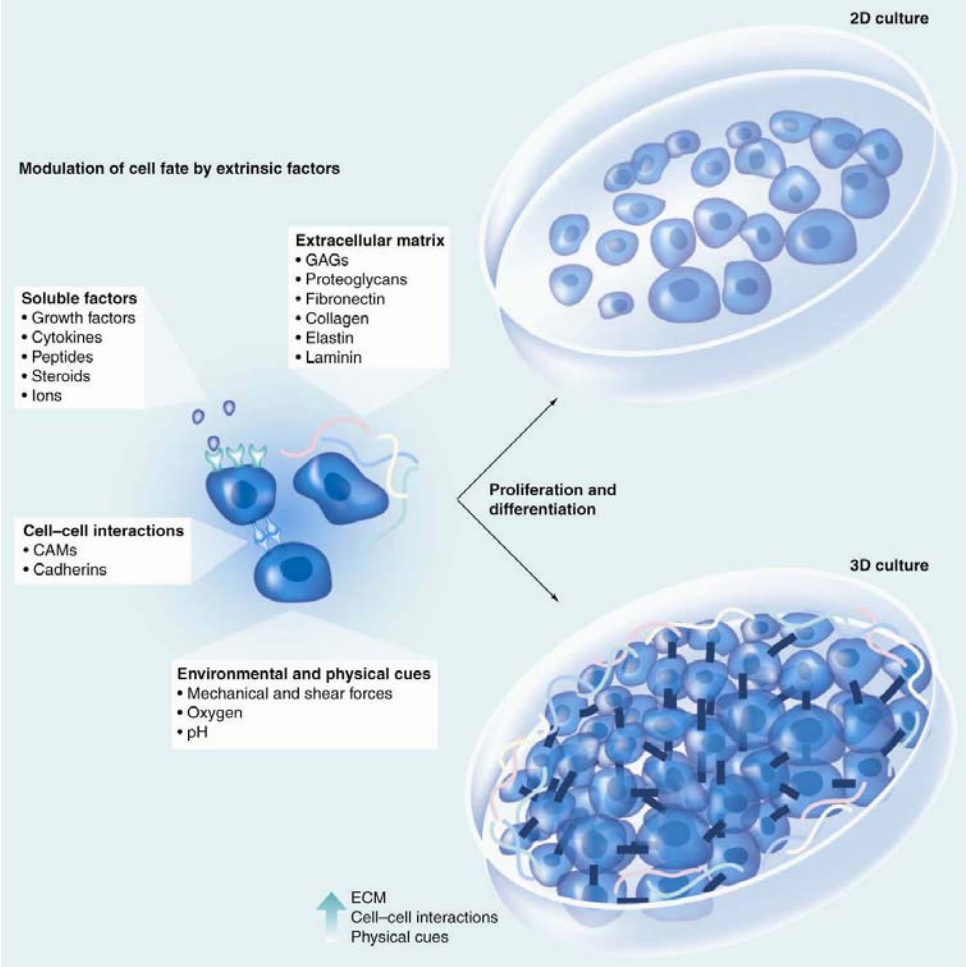

Figure 2.7 Tissue Engineering for Basic Research
Cellular growth/proliferation and differentiation is regulated by the interplay of four major signaling sources: 1) soluble growth factors, 2) insoluble ECM and growth substrates, 3) environmental stress and physical cues, and 4) cell-cell interactions. Cells grown 2-dimensionally lack the complex mechanical and biochemical interplay, which occurs naturally in vivo. By developing 3-dimensional bioengineered models *in vitro*, we can more adequately replicate tissues with characteristics similar to those observed *in vivo*. The 3-dimensional shape of bioengineered tissues allows for increased cell-cell interplay, leading to increased ECM production.

Chapter 3

Vascular Tissue Engineering:

Design Requirements and Technological Challenges

SUMMARY

The current treatment options for vascular substitution require the use of the mammary artery or saphenous vein for by-pass grafting. The major disadvantage of this strategy is the need for invasive patient surgery and the limited quantity of autologous grafting material available. Synthetic vascular substitutes like Dacron and ePTFE lack the cellular components, thereby limiting functional performance and have limited utility in small diameter applications. Tissue engineering alternatives could potentially eliminate some of these complications and may be particularly suitable for small diameter applications. Tissue engineering strategies are focused on the development of completely biological vascular substitutes with cellular components and the overall objective is to engineer a vascular graft with anatomically matched cellular and extracellular matrix components. Tissue engineering strategies have focused on fabricating vascular conduits utilizing suitable biomaterials followed by cellularization with smooth muscle cells followed by the luminal attachment of endothelial cells. This chapter provides an overview of the current state of the art

in vascular tissue engineering. Design requirements to engineer functional vascular substitutes are presented. Critical analyses of the technological hurdles which need to be overcome are also presented.

INTRODUCTION

Tissue Engineering has been defined as the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue functions. The overall objective of vascular tissue engineering is to fabricate functional vascular conduit utilizing a suitable biomaterial followed by cellularization with smooth muscle cells and the luminal attachment of endothelial cells. This chapter presents an overview of the current state of the art in vascular tissue engineering, with a focus on design requirements and technological challenges.

The first part of this chapter provides an overview of tissue engineering and the advantages of 3-dimensional culture systems as compared to traditional 2-dimensional monolayer cell culture systems. This is followed by an overview of the critical technological challenges in the field of vascular tissue engineering. We then provide a brief description of vascular anatomy and patho-physiology.

The second part of this chapter looks at the design requirements for vascular grafts. We discuss critical requirements revolving around cell sourcing, biomaterial fabrication, cell-material interaction, biocompatibility and mechanical

conditioning. This is followed by a description of current tissue engineering models of vascular grafts that have been published in the recent literature. Specific sections are devoted to a discussion of cells, biomaterials, cellularization strategies, methodology for the formation of bioengineered grafts and a description of micro-perfusion systems and the role of peristaltic flow on vascular remodeling.

In the final section, we provide an overview of our experience in vascular tissue engineering and provide a discussion of the models that are current under development within our group. We conclude this chapter with some thoughts for the future development of the field of vascular tissue engineering.

ADVANTAGES OF 3-DIMENSIONAL BIOENGINEERED CULTURE SYSTEMS

In vivo, tissues consist of cells arrayed within a complex structural and functional framework known as the extracellular matrix (ECM). The diversity observed in the morphology and composition of the ECM contributes to the properties and function of each cell and tissue type (101). Cells are also controlled by spatiotemporal cues that reside in the local microenvironment, which are presented in a 3-D context. Cells interpret cues from their immediate microenvironment and the growth and differentiation of many cell types is regulated by the interplay of four major signaling sources (Figure 3.1): 1) soluble growth factors, 2) insoluble ECM and growth substrates, 3) environmental stress and physical cues, and 4) cell-cell interactions(151). However, in order to study

cells in vitro, cells must first be removed from their native environment which provides a multitude of signals that promote their functionality. Upon isolation from their complex in vivo framework, most, if not all, cells types display changes in phenotypic, genotype, and functionality. Although some of these inputs may be lost during the cell isolation and expansion process, they can be re-induced if the correct culture environment is created. Therefore a considerable amount of research effort has been dedicated to determining the optimal culture conditions for the in vitro maintenance of cells.

The concept of maintaining live cells separated from their original tissues has been around since the 19th century and became a routinely used lab technique in the 1950s (157; 158). More recently, science historians named tissue culture one of "medicine's ten greatest discoveries" (159). The primary advantage of tissue culture is of course the ability to maintain cells in a controlled environmental under physiological conditions, including temperature and CO_2. Although traditional cell culture techniques are an immensely important and widely accepted method use to maintain cells in vitro, this system also has several limitations.

Cell isolation can be accomplished using various methods that depend on the cell type of interest. For example, cell isolation could include enzymatic and mechanical dispersal of the tissue (1; 132; 160; 161). Cells need to be kept viable while they are separated from their complex framework. The ECM not only acts as a scaffold, but also provides a multitude of signals that contribute to the regulation of a cells dynamic behavior. Cells are then sorted and isolated into a

population of cells that must be characterized. Most cells derived from solid tissues require an adherent surface for in vitro maintenance. For standard cell culture, cells are placed in a 2-D culture plate with the appropriate soluble supplements and maintained in an incubator, which provides the appropriate temperature and gas mixture (typically 37º and 5% CO_2). One of the first changes a cell undergoes is a loss of the phenotypic characteristics that are observed in the tissue from which the cells had been isolated. Cells grown 2-dimensionally display altered morphology, most notably as a flattened and more spread out appearance, which is likely to distort findings by forcing cells to adjust and adhere to flat and rigid surfaces (162; 163). Attachment and spreading of the cells onto the culture surface and is often a prerequisite for cell proliferation. In general, cultured cells become more mobile and proliferative in culture, and some cells proliferate in vitro that would not normally do so in vivo (ex: differentiated cells). Cells may be sparsely distributed in the culture dish or grown in a monolayer, which limits cell-cell and cell-matrix contacts. Differentiation characteristics are often lost, making it difficult to relate the functionality of cultured cells to the tissue from which they were derived. The 2-dimensional environment promotes cell spreading, migration, and proliferation, rather than differentiation, which inevitably leads to compromised functionality of the cells. Thus, it is not surprising that multiple studies have shown that cells grown in a 2-dimensional environment display altered gene expression patterns and cellular behavior (164; 165).

For effective in vitro testing, it is important that any measurement obtained from in vitro experiments can be interpreted in terms of the in vivo response, or at least with an understanding of the differences/limitations between the in vitro and in vivo systems. The nature of the response must be carefully considered. For example, toxicity could be measured in vitro via cell survival or metabolism assays, although the nature of the in vivo response could be at the tissue level, such as an inflammatory reaction. It has been long known that cell-specific functions are better retained when their 3D architecture is kept in tact, therefore 3-D culture systems have been developed in order to investigate tissue-level responses. Organ/tissue cultures are an alternative method, which can be used to maintain the 3-D architecture of cells in vitro. However organ cultures can not be propagated in vitro, and must instead be prepared de novo. Organ/tissue explants are complex, being composed of several different cell types that are difficult to experimentally manipulate individually. Also, to prevent rapid deterioration, organ/tissue explants are often placed in a cold (usually 4° C) bath that does not replicate normal physiological conditions. Finally, organ/tissue cultures generally have limited viability, sometimes only remaining viable for hours after their in vivo removal, and therefore long-term studies are not feasible (166).

The development of 3-D bioengineered tissue models is sometimes accomplished by culturing cells in matrices, which allows for a more accurate mimic of the in vivo architecture, compared to 2-D culture systems(81). For example, 3-D bioengineered tissues have increased cell-cell and cell-matirx

interactions, compared to cells grown 2-dimensionally. 3-D bioengineered tissues can be maintained under physiological conditions and can remain viable for several months in culture(1; 126; 127; 129; 167-171). Bioengineered tissue can also be more easily manipulated for experiments by, for example, transfecting cells with expression vectors to control expression of an endogenous protein or to give expression of mutated proteins. For all of these reasons, tissue engineering has emerged as a powerful technology where isolated cells can be bioengineered into 3-D, homogeneous tissues with characteristics similar to those observed *in vivo*.

OVERVIEW OF VASCULAR TISSUE ENGINEERING

Tissue engineering strategies are focused on the development of completely biological vascular substitutes with cellular components(172-196). The overall objective is to engineer a vascular graft with anatomically matched cellular and extracellular matrix components and mechanical properties such as structural stiffness and strength. The ability to design a vessel with suitable mechanical and biological characteristics would be required to provide a clinically viable alternative for cases of bypass surgeries. By definition, tissue engineering is the application of principles and methods of engineering and life sciences toward fundamental understanding of structure-functional relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function.

An overview of certain stages for the formation of vascular grafts is shown in Figure 3.2. In order to replicate the anatomical features of native vessels, endothelial cells, smooth muscle cells and myofibroblasts (186; 188; 197) are organized in a concentric arrangement in the presence of a suitable biomaterial(178; 198). Several sources are currently being evaluated for the vascular cells and numerous biomaterials are being evaluated to support vascular graft formation. Multiple tissue engineering platforms (self organization strategies, biodegradable hydrogels, polymeric scaffolds) are being tested to promote vessel formation.

Current models provide an important body of information about the organization of vascular cells in 3-dimensional matrices to form functional vessels. Several functional performance metrics (burst pressure, viscoelastic properties, histological/biochemical makers) have been utilized to evaluate models for vascular grafts. Several aspects of vascular tissue engineering are discussed in detail in the following sections.

PATHOPHYSIOLOGY OF VASCULAR DISEASE: ATHEROSCLEROSIS

Throughout the 20th century, as medicine rapidly developed and average life expectancy got longer, the prevalence of cardiovascular disease increased, beginning with 10% at the beginning of the century and rising to 50% of total deaths worldwide in developing countries at the start of the 21st century (199). The American Heart Association reports that in 2004, nearly 80 million people in

the United States have one or more types of cardiovascular disease, including one or more of the following: high blood pressure, coronary heart disease (myocardial infarction, angina pectoris), heart failure, and stroke (AHA). It is estimated that within the next 15 years, cardiovascular disease will be the cause of death for nearly 25 million people worldwide each year, and coronary heart disease will prevail over infectious diseases as the number one cause of death and disability (199).

Understanding the underlying mechanisms of vascular disease first requires the basic knowledge of vascular physiology and function. A normal artery is composed of three layers, the tunica intima, tunica media, and adventitia (Figure 3.3). The thin, innermost layer, or the intima, is composed of a monolayer of endothelial cells, adjacent to the basal lamina. Since the endothelial cells are in direct contact with the circulating blood, an important function of this monolayer is to secrete anticoagulative factors, keeping blood in a liquid state. The inner layer, or tunica media, is a well developed, concentric layer of smooth muscle cells, which are intertwined with many layers of extracellular matrix rich in elastin. These smooth muscles are responsible for the relaxation/contraction properties of the artery upon chemical stimulation. The outermost layer, or adventitia, contains a small number of fibroblasts and mast cells, along with a loose arrangement of collagen fibrils. This layer also supports vasa vasorum (network of small vessels that supply blood to mast cells and fibroblasts) and nerve endings (199).

The American Heart Association defines atherosclerosis as a “process in which deposits of fatty substances, cholesterol, cellular waste products, calcium and other substances build up in the inner lining of an artery” (AHA). There are many conventional risk factors that may lead to developing atherosclerosis, including smoking, hypertension, hyperlipidemia, diabetes, and obesity. Although these risk factors are common, there are individuals who develop atherosclerosis as a response to acquired, genetic atherothrombotic events, such as abnormal presence of inflammatory markers and abnormal ratios of lipoproteins.

Studies have shown that atherogenesis most likely begins with an atherogenic diet, rich in cholesterol and saturated fat. These lipoproteins then begin to accumulate within the intima of an artery, forming a lesion. Another hallmark of atherogenesis involves recruitment and accumulation of leukocytes. As these leukocytes enter and accumulate within the intimal space, they take up the modified lipids and begin to turn into foam cells. This, in turn, stimulates the migration of smooth muscle cells into the intima. There, they divide and produce an abundance of extracellular matrix proteins, hardening the fatty streak and turning it into a fibrofatty lesion. A result of these events is a decrease in the area of the intima, narrowing the space for blood to flow through, increasing blood pressure and ultimately may lead to a myocardial infarction (if lesion is within a coronary artery).

A common end-stage recovery surgery that is performed on patients suffering from severe forms of atherosclerosis is a bypass surgery. During this

procedure, a section of another blood vessel, usually acquired from the patient's leg, is used to bypass the occluded artery. Sometimes, acellularized biocompatible blood vessels can also be used to bypass the occlusion. Efforts to develop bioengineered blood vessels, especially ones with small diameters, would greatly improve the current bypass procedure. Primarily, these engineered vessels, would most likely contain autologous cells, minimizing an immune response. Secondly, the patient would be able to avoid another invasive procedure of explanting a healthy blood vessel from another body part. It is important that current vascular graft research be focused on developing small-diameter (less than 6 mm) vessels that are biocompatible with patients and also do not take a long time to develop and reorganize in vitro.

DESIGN REQUIREMENTS FOR VASCULAR GRAFTS

When considering the design requirements to engineer vascular grafts, it is interesting to think about the need to recapitulate anatomical features of native vessels versus satisfying the functional needs of the grafts(177; 184). On the surface, it may seem more likely to assume that there is indeed a need to engineer a synthetic vascular graft which mimics native vessels in terms of cellular and extracellular matrix composition, in additional to all other physiological properties. This is indeed a viable approach, since native vessels perform functionally well. An alternative path is to recapitulate the functional

requirements of vessels without the necessity for anatomical matching. Based on current scientific information, the latter approach has proven more successful with Dacron and ePTFE vascular grafts in clinical use without even the presence of cells. On the contrary, a greater proportion of current research is focused on re-engineering vascular grafts with anatomical matching. Our objective in this discussion is not to endorse any particular strategy, but rather create awareness of parallel thought processes in the field of vascular tissue engineering.

When considering the design of an anatomical matched vascular graft, the first consideration is cell sourcing, biomaterial development and interaction at the cell-material interface (Table 1). Important scientific requirements include the identification of a suitable source of cells (autologous vascular, adipose derived or embryonic derived stem cells) the ability to isolate viable cells, cell purification and enrichment strategies followed by in vitro expansion and/or differentiation as necessary, followed by cellularization and organization to form a functional tubular graft while maintaining differentiated phenotype of the cells. From a material standpoint, it becomes important to compare the advantage of utilizing commercially available (FDA approved) biomaterials as scaffolds in an attempt to expedite technological development verses considerable scientific effort required for the development of a new generation of anatomical matched biomaterials. Functional remodeling at the cell material interface, both during development in vitro and post-implantation become important considerations. Technological challenges in the design of bioreactors for mechanical conditioning (177; 192;

200-202) and micro-perfusion systems (203-207) to satisfy metabolic demands often limit in vitro development of first generation prototypes.

Another important consideration in vascular graft fabrication is biocompatibility. The formation of a continuous endothelial lining is required to limit thrombus formation and secrete growth factors to promote the vasoactivity of smooth muscle cells. Utilization of synthetic biodegradable biomaterials necessitates non-toxic degradation productions as well as the ability of safe removal from of these products from the body. In addition to being compatibly at the biological level, the vascular graft should exhibit mechanical compatibility with native vessels and withstand systematic loads without failure.

An important consideration, often overlooked, is the need to address manufacturing, distribution and sales issues during the early stages of technological development. Bulk production versus small scale on site manufacturing (greatly increasing cost) would need to be analyzed. Efficient scale –up strategies combined with cryo-perservation, transportation and product reconstitution would need to be addressed; current technological advancements would need to introduce these concepts at developmental stages of stages of development. Market segmentation, penetration and the ability to compete with existing technologies would need to be addressed. Distribution channels would need to be established taking into consideration current trends of direct sales to hospitals (where purchasing decisions are made) compared to consumer oriented marketing, which targets the end users of the technology.

MODELS OF TISSUE ENGINEERED VASCULAR GRAFTS

There are two categories of bioengineered models being developed for vascular tissue engineering models: planar 3-dimensional strip models of vascular smooth muscle cells (2; 39; 99; 208) as well as models of tubular vascular grafts (209-212). Both categories of bioengineered models are discussed here.

In addition to the development of vascular conduits, there has been some interest in the development of planar 3-D models for vascular tissue engineering applications. There have been many reports of 3-D "tissue equivalents" developed *in vitro* by culturing cells in fibrin matrices(39; 213). Fibrin-based gels have become an effective option for engineering functional muscle tissue, providing a matrix that is well suited for the development of contractile tissues . Several investigators have demonstrated the capacity to bioengineer primary isolated VSM cells into 3-D tissues(99; 214). VSM cells grown in fibrin show increased production of ECM, collagen(215), elastic fibers(216) , and also exhibit mechanical properties that are commensurate with vascular tissue *in vivo*(217). We have used fibrin gel casting to develop a 3-D human VSM strip model in vitro (unpublished data). Bioengineered "VSM strips" result from the spontaneous delamination of a confluent monolayer of primary human aortic VSM plated on a culture surface treated to control cell adhesion (Figure 3.4). Our bioengineered VSM strips are functionally similar to VSM in vivo and remained viable in culture for up to 5 weeks. VSM strips demonstrate spontaneous basal tone and can generate an active force (contraction) of up to 85.2μN upon stimulation with

phenylephrine. Bioengineered VSM strips exhibited calcium-dependent contraction and calcium-independent relaxation.

Three cell types are required for the development of tissue engineered vascular grafts: endothelial cells to coat the luminal surface, smooth muscle cells to form the medial layer and fibroblasts for the adventitial layer. Although several cell sources are being evaluated for experimental purposes, specific requirements would need to be satisfied for clinical applications. The cells would need to be non-immunogenic, functionally active, phenotypically differentiated, proliferative, and amiable to cryopreservation for transportation. Endothelial cells derived from vascular specimens(218-222), adipose tissue(223-225) or from circulating precursor cells(226; 227) have been evaluated as a potential source for vascular tissue engineering application.

Several biomaterials have been evaluated for use in vascular tissue engineering. Any materials would need to support the culture of smooth muscle cells and the formation of an endothelial cell layer on the luminal pressure. However, equally important would be the mechanical properties and the ability to withstand arterial pressure in excess of 2000 mmHg. Several biomaterials have been utilized to engineer vascular grafts and have included de-celluarized conduits(228), biodegradable hydrogels like collagen(229) and fibrin(39; 230) and polymeric scaffolds like poly-glycolic acid(231) and chitosan (232) (Figure 3.5).

There have been several models of vascular grafts reported in the literature(39; 99; 233-245). The overall objective has been to fabricate vascular

conduit utilizing a suitable biomaterial followed by cellularization with smooth muscle cells followed by the luminal attachment of endothelial cells. Different cellularization approaches have been utilized: direct layering of cell sheets around the peripheral surface or embedding the cells at the time of graft formation (Figure 3.6).

One of the first models of vascular grafts was reported by Eugene Bell and colleagues and is regarded as a seminal paper in the field of vascular tissue engineering(246). In this model, primary cells were isolated from a bovine source and mixed with collagen. A three layer structure was developed to resemble normal vessel anatomy consisting of a medial layer of smooth muscle cells, an adventitia layer of fibroblasts and with a lining of endothelial cells on the luminal surface of the vascular graft. The endothelial cells were shown to be functional active expressing von Willebrand factor, creating a barrier to large molecules like albumin and releasing prostacyclin, an inhibitor of platelet aggregation. The burst strength of the vascular graft was reported to be 323 ± 31 mmHg and was shown to be a function of collagen concentration and culture time.

Robert Nerem and colleagues utilized collagen, fibrin and a mixture of the 2 gels in conjunction with primary rat aortic smooth muscle cells to generate small diameter vascular grafts(247). One million cells were utilized per construct which were cultured for 6 days prior to mechanical testing. Gel compaction, linear modulus and the ultimate tensile stress were dependant on the gel properties with collagen shown to have the highest linear modulus. Overall, this

study served as an early demonstration of the feasibility of using different hydrogels, alone and in combination to support the formation of vascular grafts. The mechanical properties of the grafts were determined by the gel composition.

Laura Niklason and colleagues utilized smooth muscle cells derived from the medial layer of bovine aortas seeded onto tubular grafts fabricated with polyglycolic acid(248). These constructs were cultured under pulsatile flow using custom designed bioreactors for up to 8 weeks. Endothelization of the luminal surface was achieved by injecting bovine aortic endothelial cells on directly in the lumen of the graft. The presence of several histological markers was shown and indicated the formation of functional vessels. A burst pressure of over 2000 mmHg was reported. This study was valuable in showing the effect of pulsatile flow on the formation and maturation of vascular grafts.

L'Heureux and colleagues developed a multiple layered model of small diameter vascular grafts(249). Human umbilical vein endothelial cells and vascular smooth muscle cells were obtained from healthy newborns donors and fibroblasts were obtained from skin specimens from normal adult during breast surgery. Smooth muscle cells and fibroblasts were cultured independently for 30 days under conditions to promote the formation of extracellular matrix and subsequently a cohesive cell monolayer. A sequential approach was utilized for the formation of tissue engineering blood vessels (TEBVs) which consisted of physically wrapping the smooth muscle cell sheet around a tubular support structure. This was followed by physical wrapping of the fibroblast cell monolayer. Endothelial were injected in the luminal surface of the vascular graft.

The entire process for the formation of TEBV required a time period of 3 months with 3 weeks for the formation of smooth muscle cell sheets, 1 week for media maturation, 7 weeks for adventitial maturation and 1 week for endothelization. Histological data showed the presence of several vascular specific proteins and a burst pressure of over 2000 mmHg was reported.

MICRO-PERFUSION SYSTEMS

The development of 3-dimensional tissue constructs increases the metabolic requirements thereby necessitating the development of micro-perfusion systems for the continuous delivery of cell culture media and/or application of controlled fluid shear to guide functional remodeling. Micro-perfusion systems are more universally accepted as systems capable of delivering continuous fluid flow to 3-dimensional tissue constructs to support metabolic activity of the tissue constructs as well as deliver controlled fluid stress to promote functional remodeling of cells and extracellular matrices(250-253). Several studies have shown that oxygen can only diffuse through 200 μm of a tissue engineered construct, often with a thickness in excess of 200 μm (254). Thus, bioengineered tissues may require continuous media perfusion to more actively supply oxygen and soluble factors. The design of normal mammalian tissue has incorporated this rate limiting step by extensive vascularization (255)and in the case of highly metabolic tissues like cardiac muscle, every cardiac cell is within 100 μm of a capillary (256) .

Vascularization of tissue engineered constructs is a parallel strategy being evaluated to support the metabolic requirements of 3D constructs(257). Several approaches for construct vascularization have been evaluated including implantation in close proximity to an in vivo vascular pedicle(258), in vitro culture in the presence of endothelial cells(259), utilization of angiogeneic factors (260; 261)(vascular endothelial growth factor, fibroblasts growth factor) and use of solid free form fabrication for the development of micro channels in vitro(262; 263) have all shown promising results. Although not the focus of this article, conceivably, 3D constructs engineered in vitro can be vascularized and then supported by a micro-perfusion system.

The critical technological challenges in designing micro-perfusion systems are the development of micro-chambers for the culture of 3D constructs, the ability to control the temperature, pH, oxygen saturation, relative humidity and fluid flow rate and monitor/record the changes in functional outcome of tissue constructs during long term in vitro culture. In addition, the entire system needs to be maintained under sterile conditions with feedback control to change the flow variables to accommodate changes in construct phenotype (Figure 3.7). Additional functionality may include continuous measurement and control of glucose and lactate.

Several systems have been developed to support the long term culture of vascular grafts in the presence of peristaltic flow conditions(192; 264; 265). It has been demonstrated that subjecting vascular grafts to peristaltic flow conditions results in a higher degree of cell alignment, increased production of

extracellular matrix components and increase in the mechanical properties of the graft, as measured by the burst pressure(266).

VASCULAR TISSUE ENGINEERING AT THE ARTIFICIAL HEART LABORATARY

Our research in the area of vascular tissue engineering comprises three main categories; comparing different platforms for the fabrication of tubular grafts, evaluating the effectiveness of varying cellularization strategies and finally, developing system to mechanical condition the vascular grafts to promote functional remodeling.

Our efforts on fabricating vascular grafts prototypes are focused on comparing multiple platforms to support the formation of tubular structures; biodegradable hydrogels (fibrin, collagen), acellularized matrices and polymeric scaffolds. Biodegradable hydrogels provide an excellent substrate for cell differentiation while cross-linking of fibers results in excellent mechanical properties. Our preliminary work supports the utility of fibrin and collagen to promote the vascular graft formation. Acellularized matrices have the advantage of a pre-formed, often optimized extracellular matrix to promote remodeling of vascular cells. Simple detergent based acellularization methods can be utilized to completely remove all cellular components without changing the composition and properties of the extracellular matrix components; thereby providing an excellent substrate for the remodeling of vascular cells. Our third and final

strategy utilizes polymeric scaffolds; the ability to control the mechanical properties, engineer bioactivity and match the rate of scaffold degradation to tissue formation provide ample opportunity to engineer functional vascular grafts.

We have multiple strategies for cellularization of vascular grafts. In one approach, 3-dimensional patches are fabricated using vascular smooth cells, and maintained in culture to promote functional remodeling. These patches are then physically attached on the outer surface of pre-formed tubular structures while endothelial cells are injected on the luminal surface. This cellularization strategy is particular useful when utilizing polymeric scaffolds and acellular matrices. Utilization of biodegradable gels provides a unique opportunity to embed the smooth muscle cells within the 3-dimensional lattice during gel formation. This allows functional interaction at the cell-material interface thereby permitting functional remodeling. Endothelial cases can easily be injected on the luminal surface.

The third component of our vascular tissue engineering research is focused on the development of bioreactor and micro-perfusion systems to support the functional remodeling of vascular grafts. We have developed a perfusion system to support the culture of tissue engineered constructs without the need for a cell culture incubator. Several variables (temperature, pH, oxygen saturation, fluid flow rate) can easily be manipulated to study the effect on tissue formation. In addition, we are working on systems to apply cyclical loads to promote tissue maturation. Collectively, this work aims to promote tissue maturation and improvement in functional performance metrics.

THOUGHTS FOR THE FUTURE

A significant amount of progress has been made towards the development of bioengineered vascular grafts during the last decade. However, certain critical technological challenges remain to be addressed. The development of an ideal biomaterial to support cellular activity while providing mechanical stability in response to arterial pressures would be necessary. Although several biomaterials have been evaluated with varying degree of success, an ideal option has not been identified.

One critical area of technological development would be in the development of micro-perfusion systems for vascular tissue engineering applications. Although several systems have been developed, the level of sophistication is limited in terms of the degree of control over processing variables. Most of the current systems utilize a traditional cell culture incubator, which limited ability to program user defined variables. The ability to modulate processing conditions to match the phenotypic state of the vascular graft would be critical in determining long term viability during in vitro culture. In addition, the ability to delivery controlled fluid shear stresses in pre-defined directions would be necessary for maturation of the tissue engineered vascular grafts.

Functional integration at the cell-material interface and the ability to guide the developmental state of the vascular graft, specifically in response to the degree of tissue maturation, has not been given significant attention in the recent literature. At tissue engineered vascular grafts are developed, the phenotypic state of the construct will change based on several variables during in vitro

culture. As the phenotypic state of the construct changes, the culture conditions would need to accommodate these changes and modulate processing variables, eg. glucose content in media. Therefore, some degree of guided phenotypic maturation of tissue engineered vascular grafts would need to be programmed into current culture systems.

One fundamental change has to occur in the nature in which tissue engineering research is conducted. In addition to creating a greater degree of inter-disciplinary interactions, there needs to be a conscious effort to generate a high degree of public awareness as well as develop mechanisms to encourage public participation in the research. The potential impact of tissue engineering therapies can be profound. One mechanism to expedite technological developments is via cross-pollination of ideas and novel methodologies from a host of diverse disciplines.

Scientific Requirements: • Identification of a suitable cell source and development of novel biomaterials to match the anatomy of native vessels. • There needs to be functional integration of the implanted vascular graft with the host environment at the cellular and material interfaces providing continuum of vasculature. • The time required for on site engineering of vascular substitutes is critical if single stage cellularization is utilized and needs to be reduced to an absolute minimum. • Development of functional layers of endothelial cells, smooth muscle cells and fibroblasts recapitulating normal vascular anatomy.
Technological Requirements: • Development of Bioreactors and Micro-Perfusion systems to deliver controlled mechanical conditioning to modulate the functional outcome of the vascular graft.
Mechanical Properties: • The mechanical properties of the vessel wall need to exhibit viscoelastic behavior to match the mechanical properties of the native vessel. • Mechanical strength as measured by burst pressure needs to be in excess of 2000 mmHg. The mechanical strength is fairly important as the vascular substitute should be able to withstand long term hemodynamic load without failure.
Biocompatibility: • The vascular grafts need to be resistant to thrombos formation. • The extent of smooth muscle proliferation leading to neo-intimal hyperplasia needs to be limited. • The vascular substitute needs to be non-toxic and be resistant to infection. • When utilizing biodegradable materials, the degradation products need to be non-toxic and safely eliminated by the body.
Usability: • In order to simplify utilization in a clinical setting, the vascular graft needs to be readily available, either as an off the shelf option, or engineered on site by cellularization utilizing autologous cells. • From a surgical perspective, handling and suturing of the vascular substitute needs to be optimized to accommodate the requirements of the clinical environment.
Marketing and Distribution: • From a manufacturing standpoint, the vascular substitute needs to be amiable to large scale production and retain functionality during cyro-preservation, transportation and re-constitution prior to use. • From a market penetration standpoint, there needs to be a feasibly distribution network established with a clear advantage over existing therapies. • The price needs to be economically feasibly. • The vascular grafts needs to maintain longevity upon implantation to reduce the likelihood of repeat surgery thereby making the overall therapy attractive for potential patients in terms of convenience and price.

Table 3.1 Design Requirements for Vascular Grafts.

The functional requirements that need to be taken into consideration during the design of a vascular graft are identified. Scientific and technologies issues as well as mechanical properties and biocompatibility are listed. Other important issues are the usability factor for the vascular grafts as well as considerations of marketing and distribution.

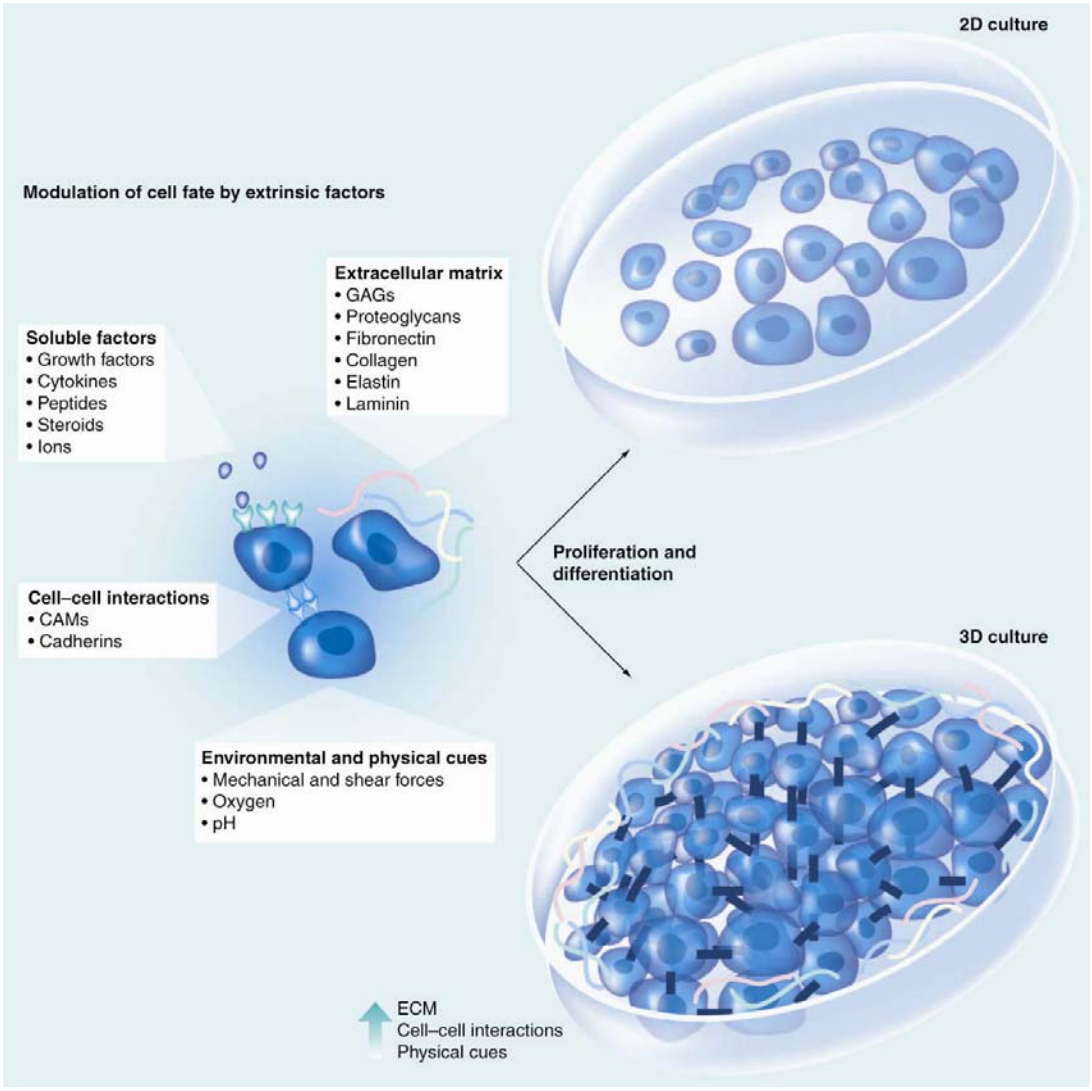

Figure 3.1 Advantages of 3D Culture in Tissue Engineering
Cellular growth/proliferation and differentiation is regulated by the interplay of four major signaling sources: 1) soluble growth factors, 2) insoluble ECM and growth substrates, 3) environmental stress and physical cues, and 4) cell-cell interactions. Cells grown 2-dimensionally lack the complex mechanical and biochemical interplay, which occurs naturally in vivo. By developing 3-dimensional bioengineered models *in vitro*, we can more adequately replicate tissues with characteristics similar to those observed *in vivo*. The 3-dimensional shape of bioengineered tissues allows for increased cell-cell interplay, leading to increased ECM production.

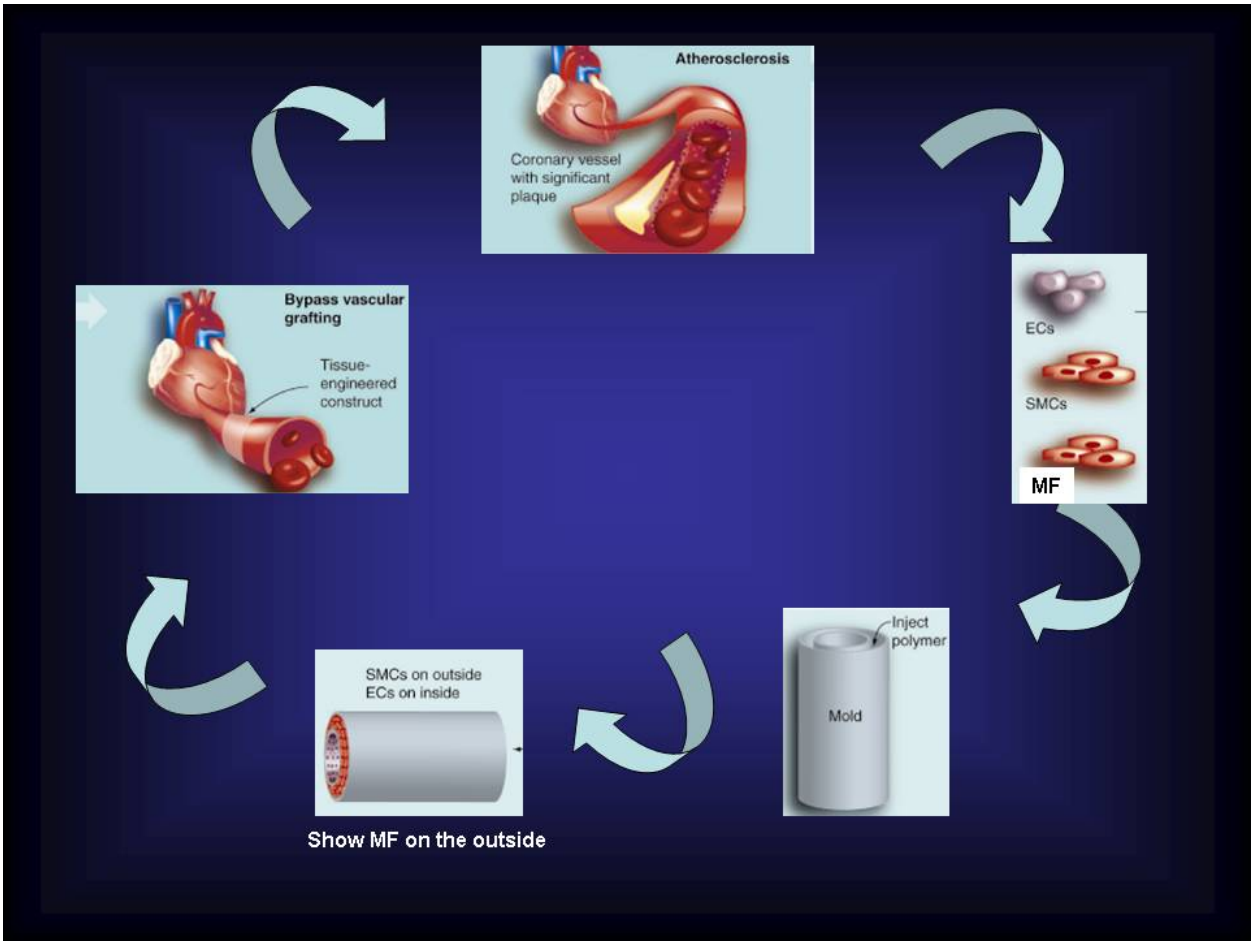

Figure 3.2 Elements of Vascular Tissue Engineering
The ability to fabricate tubular structures utilizing various scaffolds/hydrogels is dictated by the design of suitable molds. The walls of the tubular graft are cellularized with smooth muscle cells while the lumen is cellualarized with endothelial cells resulting in the formation of bioengineered vascular grafts. The utility of these vascular grafts would in cases of coronary bypass surgery.

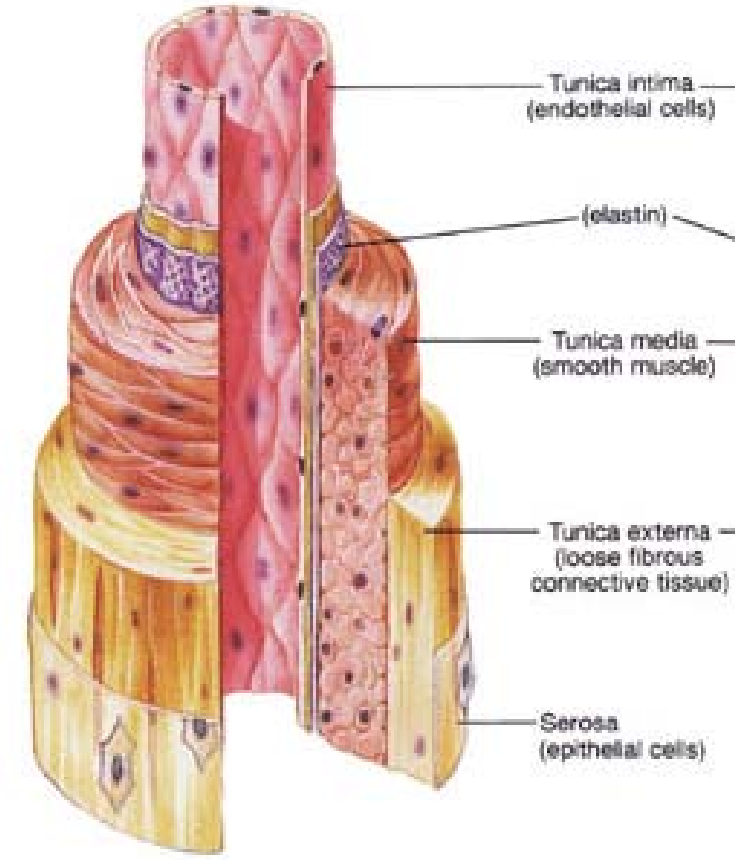

Figure 3.3 Functional Anatomy of an Artery
A normal artery is composed of three layers, the tunica intima, tunica media, and adventitia. The thin, innermost layer, or the intima, is composed of a monolayer of endothelial cells, adjacent to the basal lamina. The inner layer, or tunica media, is a well developed, concentric layer of smooth muscle cells. The outermost layer, or adventitia, contains a small number of fibroblasts and mast cells, along with a loose arrangement of collagen fibrils.

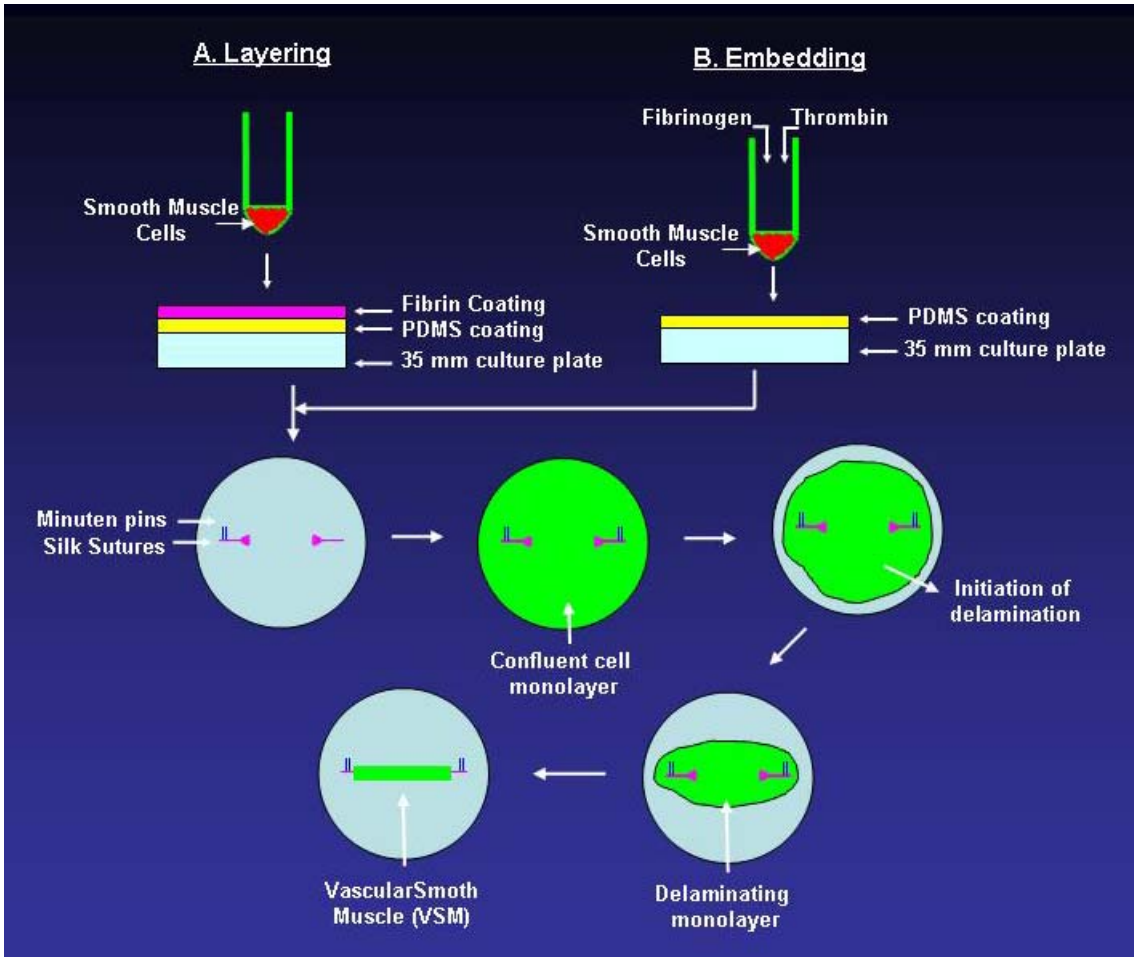

Figure 3.4 Methodology for the formation of bioengineered VSM strips
Tissue culture plates were coated with PDMS and anchor points were engineered by pinning silk sutures 12 mm apart in the center of the culture plate with four stainless steel pins. The plate was sterilized and then a thrombin/fibrin solution was mixed in the culture plate which led to polymerization. (A) Layering Approach - Following gel polymerization, primary human VSM cells were plated on the surface of the fibrin gel. (B) Embedding Approach – Cells were embedded within the fibrin gel at the time of polymerization. In both cases, cell expansion and increased cell-cell contact resulted in delamination of the cell monolayer, which was initiated at the periphery of the culture plate and progressed towards the center. The delaminating cell monolayer remained attached to anchor points that were placed in the culture dish. Bioengineered VSM strips were deemed ready for functional testing when the monolayer formed a “strip” of bioengineered tissue between the two anchor points.

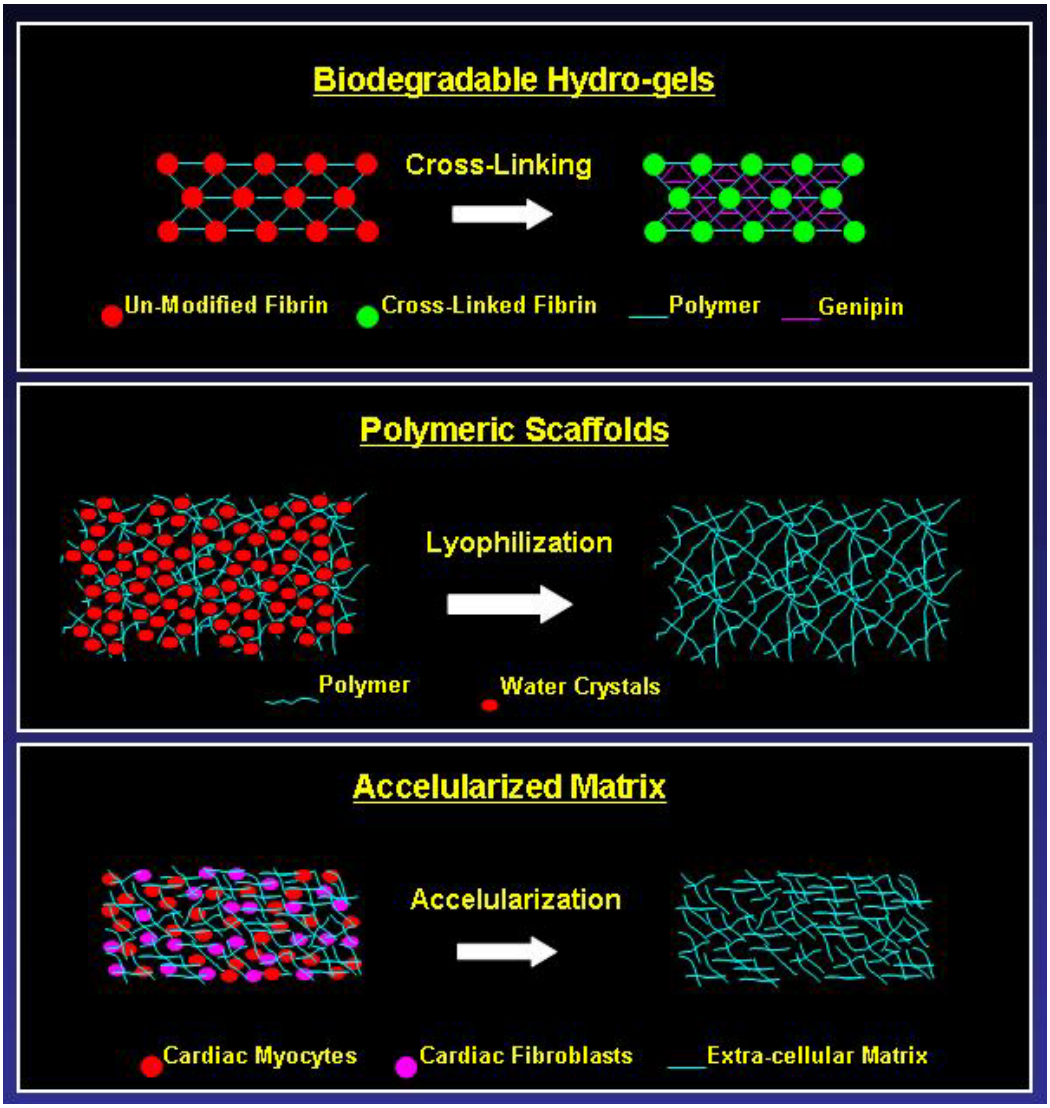

Figure 3.5 Materials Utilized for Vascular Tissue Engineering
Biodegradable hydrogels like fibrin have often been modified via cross-linking to improve the mechanical stability for vascular applications. Polymeric scaffolds, like chitosan, have been fabricated via phase separation methods to result in the formation of porous vascular grafts. De-cellularized matrices have been obtained by complete removal of all cellular components from arterial segments.

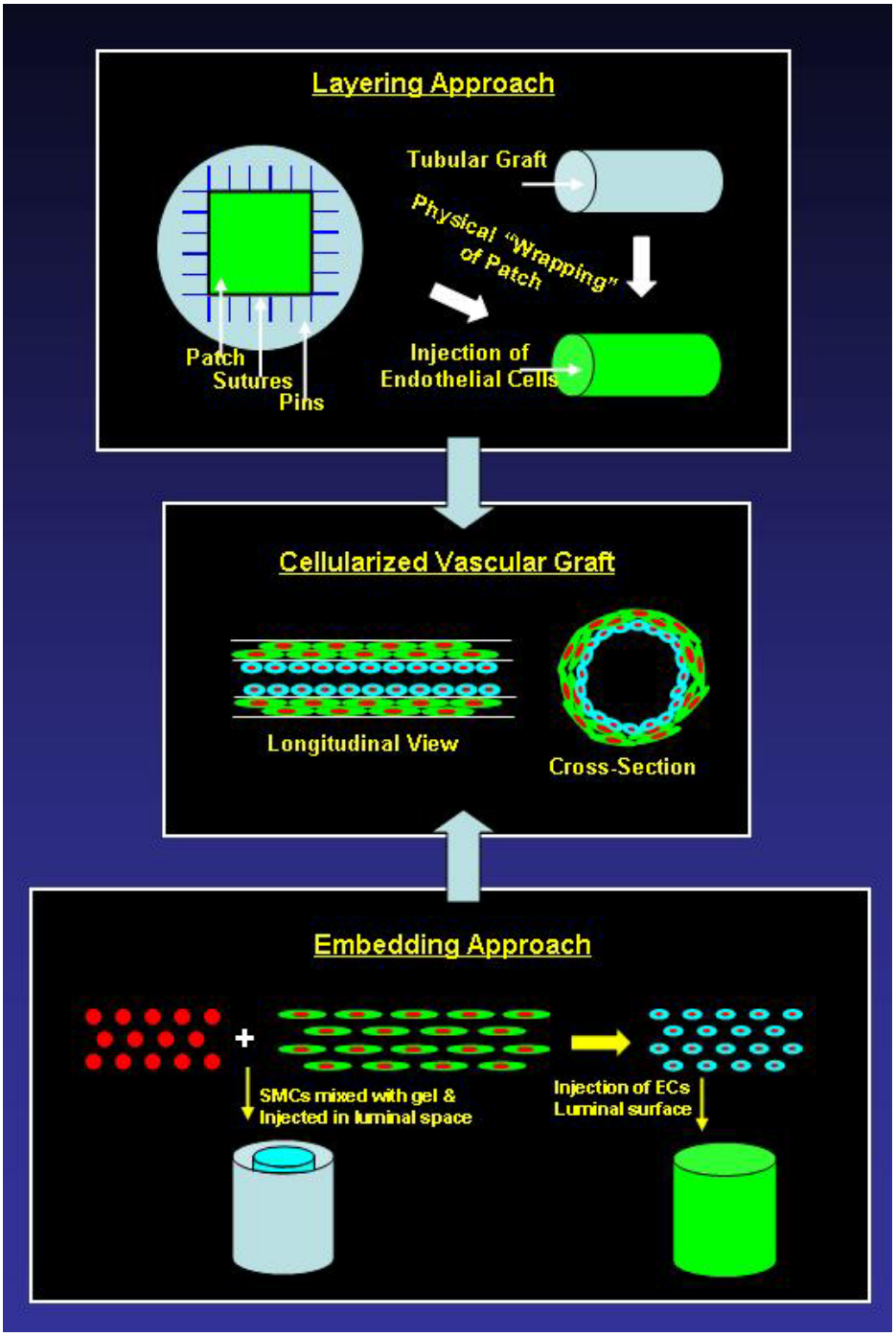

Figure 3.6 Cellularization Strategies
For the layering approach, cell sheets are fabricated with smooth muscle cells (SMCs) and physically "wrapped" around the periphery of tubular conduits. Using the embedding approach, SMCs are embedded within the material at the time of graft formation. In both cases, endothelial cells are injected on the luminal surface after in vitro culture.

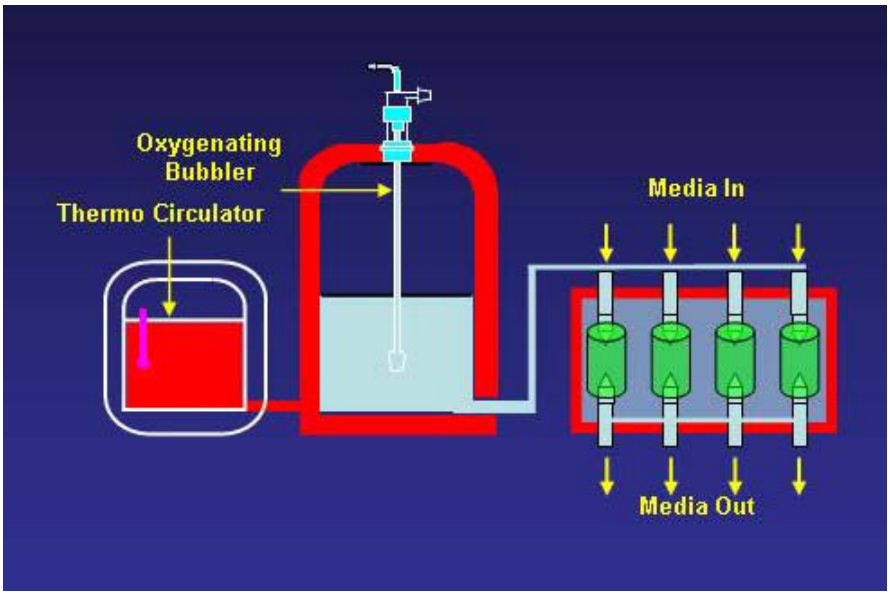

Figure 3.7 Micro-Perfusion System for Vascular Tissue Engineering
The vascular grafts are housed in a temperature regulated chamber. Media is maintained in a water-jacketed reservoir with oxygen bubblers. Utilizing peristaltic pumps, the media can be delivered to the luminal surface of the vascular grafts.

Chapter 4

Development of a three-dimensional physiological model of the internal anal sphincter bioengineered in vitro from isolated smooth muscle cells

ABSTRACT

Fecal incontinence affects people of all ages and social backgrounds and can have devastating psychological and economic consequences. This disorder is largely attributed to decreased mechanical efficiency of the internal anal sphincter (IAS), yet little is known about the pathophysiological mechanisms responsible for the malfunction of sphincteric smooth muscle at the cellular level. The objective of this study was to develop a three-dimensional (3-D) physiological model of the IAS bioengineered in vitro from isolated smooth muscle cells. Smooth muscle cells isolated from the IAS of rabbits were seeded in culture on top of a loose fibrin gel, where they migrated and self-assembled in circumferential alignment. As the cells proliferated, the fibrin gel contracted around a 5-mm-diameter SYLGARD mold, resulting in a 3-D cylindrical ring of sphincteric tissue. We found that *1*) the bioengineered IAS rings generated a spontaneous basal tone, *2*) stimulation with 8-bromo-cAMP (8-Br-cAMP) caused a sustained decrease in the basal tone (relaxation) that was calcium-independent, *3*) upon stimulation with ACh, bioengineered IAS rings showed a calcium- and concentration-dependent

peak contraction at 30 s that was sustained for 4 min, *4*) addition of 8-Br-cAMP induced rapid relaxation of ACh-induced contraction and force generation of IAS rings, and *5*) bioengineered sphincter rings show striking functional differences when compared with bioengineered rings made from isolated colonic smooth muscle cells. This is the first report of a 3-D in vitro model of a gastrointestinal smooth muscle IAS. Bioengineered IAS rings demonstrate physiological functionality and may be used in the elucidation of the mechanisms causing sphincter malfunction.

INTRODUCTION

Fecal incontinence is associated with reduced anal closure pressure and mechanical stress for maintaining tissue coaptation and closure of the anal canal, resulting in the inability to control the passage of gas or stools (feces) through the anus (267). This is largely attributed to decreased mechanical efficiency of the internal anal sphincter (268; 269). Epidemiological studies have shown that fecal incontinence affects 2–15% of the population (270-272) , including people of all ages and social backgrounds, thus making it one of the most common gastrointestinal disorders. A diagnosis of fecal incontinence often leads to devastating social, psychological, and economic consequences and a significantly lower quality of life(272). Despite the prevalence and disturbing nature of this condition, little is known about the mechanisms underlying smooth muscle sphincter malfunction. Thus there is a need for a model that provides a comprehensive understanding of sphincteric smooth muscle physiology.

From a clinical perspective, understanding sphincter malfunction in regard to fecal incontinence has been limited because traditional anal manometric measures are not able to localize specific damage or deterioration to individual sphincter structures or discern the underlying cellular or tissue processes responsible for function loss. Studies have tended to focus on symptomatic individuals and on structure or function, but not both (267). Furthermore, without a comprehensive understanding of the pathophysiological mechanisms responsible for sphincter malfunction at the cellular level, the development of

corrective strategies and treatment options for people diagnosed with fecal incontinence have been limited.

Tissue culture was developed in the early 1900s as a technique for studying the behavior of animal cells in vitro(157; 158). Advances in technology have allowed the use of tissue culture for many areas of study, including intracellular activity, intracellular flux, environmental interaction, cell-cell interaction, and genetics (273). The capacity to grow different cell types in culture and subsequently test the effects of a variety of drugs, hormones, and other interventions on cellular function is of the utmost importance. However, when force production is the primary tissue-level function, two-dimensional cell culture is an inadequate model for most experimental needs. Furthermore, cells that are propagated and tested two-dimensionally lack many of the cellular and environmental cues that play a role in differentiation and the establishment of normal physiological function. Isolation of single cells and cell suspensions has enabled biochemical and mechanical measurements at the cellular level and has advanced our level of understanding of smooth muscle function (274). Yet cell suspensions also lack the potential for cell-cell interactions and cell-matrix interactions provided by three-dimensional (3-D) tissues (273) and lack the tissue-level organization and system-level function to allow a direct study of the underlying mechanisms of clinical importance (170).

It is well known that physiological functions of tissues are retained when their 3-D structure is kept intact (39). Isolated tissues and organ preparations, such as

muscle strips, have been shown to provide researchers with a 3-D tissue that can be easily subjected to controlled changes in perfusion, oxygen availability, and agonist-induced stimulation (275-277). Despite the obvious advantages of tissue/organ explants, many limitations remain. Tissues are composed of several different cell types. For example, IAS muscle strips may be comprised of smooth muscle but also any combination of mucosal, epithelial, and/or neuronal cells. Thus it is difficult to experimentally manipulate or investigate one tissue constituent. Explanted tissues do not last indefinitely and are usually only viable for up to 4 h after removal, which prevents long-term investigation (166). Because the tissues must be manipulated for experimental purposes almost immediately after dissection, they must be prepared de novo for each experiment. Damage to the tissue and/or release of material from damaged erythrocytes occurring during dissection also inhibits the ability to produce normal functionality and environmental conditions. Last, tissue/organ explants must be placed in a cold (usually 4°C) buffer or bath to prevent rapid degradation. For these reasons, tissue engineering has emerged as a valuable tool that applies the principles of engineering and life sciences toward the development of biological models with characteristics similar to those observed in vivo. Specific cell types can be isolated and bioengineered to yield a homogenous tissue. Bioengineered tissues can be maintained in culture for long periods of time under physiological conditions (126; 127; 132; 160). Advances in tissue engineering have been clinically applied to restore, maintain, and improve tissue function, resulting in enhanced treatment for damaged tissues (278).

There have been many reports of 3-D "tissue equivalents" developed in vitro by culturing cells in special matrixes or under conditions to promote self-organization (99; 126; 127; 170). Fibrin-based gels have become an effective option for engineering functional striated muscle tissue, providing a matrix that is well suited for the development of contractile tissues (279). Fibrin gels are formed by the enzymatic cleavage of fibrinogen by the serine proteinase thrombin, allowing the fibrin monomers to spontaneously interact and form fibrils. Fibrin-based gels have advantages over synthetic polymer approaches of tissue-equivalent fabrication, specifically immediate cellularity of the construct through cell entrapment in the forming gel and subsequent fibril alignment via mechanical constraint of cell-induced gel compaction (99). Cell-mediated fibrin gel contraction occurs, mimicking alignment of muscle tissue in vivo (280). Within a fibrin matrix, cells rapidly migrate, proliferate, and digest the fibrin, replacing it with their endogenous extracellular matrix (2; 39; 99; 208). Vascular smooth muscle cells grown in fibrin have shown increased production of ECM, collagen (39; 208), and elastic fibers (19) and also exhibited mechanical properties that are commensurate with vascular tissue in vivo (19; 99). Furthermore, fibrin-based engineered tissues can be maintained for weeks in culture (99; 127). The prolonged life span of engineered tissues allows temporal investigation of the effects of cell-cell interactions, growth factors, mechanical and chemical stimuli, gene expression, and kinase action on tissue-level function.

Here we used fibrin gel casting to develop an in vitro model of the IAS from isolated rabbit smooth muscle cells. Our bioengineered "IAS rings" are 3-D

cylindrical structures resembling the IAS portion of the gastrointestinal tract. Isolated IAS cells seeded in a fibrin construct self align and form a ring in 5–10 days. The resulting IAS rings are functionally similar to gastrointestinal smooth muscle in vivo for >3 wk, including generation of spontaneous tone and agonist-induced contraction and relaxation of the basal tone with the appropriate stimuli. Stimulation with 10^{-5} M 8-bromo-cAMP (8-Br-cAMP) resulted in a calcium-independent relaxation of –16.3 ± 2.0 μN from the basal tone. When stimulated with ACh, the IAS rings produced a calcium-dependent maximal force of 15.7 ± 1.4 μN. ACh-induced stimulation was also concentration dependent, with a plateau at 10^{-6} M. Addition of 8-Br-cAMP induced rapid relaxation of ACh-induced contracted rings. Last, isolated colonic smooth muscle cells were bioengineered into 3-D rings and tested in the same manner as sphincteric rings. Bioengineered colon rings exhibited spontaneous phasic contractile behavior (amplitude: 18 ± 1.99 μN; frequency: 2.45 ± 0.21 cycles/min). Sphincter rings showed marked functional differences from colonic smooth muscle rings, including the absence of spontaneous phasic contractile behavior. This is the first report of a 3-D cylindrical, in vitro model of the IAS that is functionally similar to smooth muscle in vivo. This model provides the opportunity for a novel approach to investigate smooth muscle function and dysfunction, including the elucidation of disease mechanisms causing fecal incontinence.

MATERIALS AND METHODS

Materials and solutions. Growth media (GM) consisted of DMEM (catalog no. 12430–054; GIBCO) with 15% FBS, 3% penicillin-streptomycin, and 0.6% L-glutamine. Differentiation media (DM) consisted of 73% DMEM (catalog no. 12430–054; GIBCO), 20% Media 199, 7% heat-inactivated horse serum, and 1% penicillin-streptomycin. Krebs solution contained (in mM) 119 NaCl, 4.6 KCl, 15 $NaHCO_3$, 1.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $NaHCO_3$, and 11 glucose; 0 Ca^{2+}/2 mM EGTA Krebs solution was the same except $CaCl_2$ was not included and 2 mM EGTA was added. HEPES buffer (pH 7.4) contained (in mM) 115 NaCl, 5.7 KCl, 2.0 KH_2PO_4, 24.6 HEPES, 1.9 $CaCl_2$, 0.6 $MgCl_2$, 5.6 glucose, 0.01% soybean trypsin inhibitor, and 0.184 (wt/vol) DMEM; 0 Ca^{2+}/2 mM EGTA HEPES solution was the same except $CaCl_2$ was not included and 2 mM EGTA was added.

Cell isolation. Smooth muscle cells were isolated from the IAS of New Zealand White rabbits as described previously (274; 281). Briefly, the IAS (consisting of the most distal 3 mm of the circular muscle layer, ending at the junction of skin and mucosa) and the distal colon were removed by sharp dissection. The tissues were rapidly cleaned and stripped of connective tissue in ice-cold carbonated Krebs solution containing 2% penicillin-streptomycin. Separately, the tissues were cut into small pieces and transferred to a 100-mm plate containing 15 ml HEPES buffer with 0.1% collagenase type II (Worthington Biochemical) for digestion. The plates were placed in an incubator (37°C with 5% CO_2) for a 1-h digestion period. Fresh HEPES (15 ml) buffer with collagenase was added, and the contents of the

plates were mechanically dissociated using a 10-ml pipette and incubated for one additional hour. After the second digestion period was complete, the cells were collected and centrifuged at 800 *g* for 10 min, and the supernatant was discarded. The cells were washed and resuspended three times to ensure removal of excess collagenase and then resuspended in HEPES buffer or 0 Ca^{2+}/2 mM EGTA HEPES buffer.

ACh-induced stimulation and measurement of contraction in suspension of isolated IAS smooth muscle cells. To determine the effect of ACh on contraction of isolated sphincteric smooth muscle cells, ACh was added to 0.5 ml suspension of isolated smooth muscle cells for 30 s or 4 min, with untreated cells as controls. The reaction was stopped, and cells were fixed by the addition of 0.1 ml acrolein at a final concentration of 0.1%. Individual cell lengths were measured by computerized image micrometry (274; 282). The length of cells in the control state or after addition of ACh was obtained by measuring 30–50 cells encountered randomly in successive microscopic fields from each of three separate experiments. The contractile response is defined as the decrease in the average length of the cells counted and is expressed as the percent change from the control length.

Preparation of culture dishes and culture of rings. Culture plates (35 mm) were prepared as described previously (126; 170). Briefly, 1.5 ml SYLGARD [polydimethylsiloxane (PDMS)] was poured in each plate and allowed to cure for 24 h. The SYLGARD substrate provided a layer in which anchor materials could

be easily pinned in place and provided a surface to which, unless coated, the cells could not adhere. A cylindrical SYLGARD mold with an upwardly protruding 5-mm-diameter disk was placed in the center of the dish to provide a luminal space for the engineered sphincter or colon (Figure 4.1*A*). Adhesion of the mold to the SYLGARD-coated culture dish was facilitated by pressing the mold firmly against the bottom of the dish with forceps. The culture dish was sterilized with 70% ethanol for 30 min. Ethanol was then aspirated, and culture dishes were exposed to ultraviolet light for 1 h. Each dish received 500 μl GM containing 10 U/ml thrombin, and the dish was agitated until the bottom of the plate was entirely coated. Finally, 200 μl of 20 mg/ml fibrinogen was added to each plate and gently swirled. The fibrin polymerized 15 min later, and the plates were ready for cell seeding. Primary cells isolated from rabbit IAS and colon tissues were expanded separately in 75-cm^2 flasks for 1 wk. The cells were then detached from their culture flasks with 1.5 ml trypsin-EDTA, collected, resuspended in GM, and counted with a hemocytometer. The cell density was adjusted, and 5 x 10^4, 7.5 x 10^4, or 10 x 10^4 cells/ml was added to the plates in a final volume of 2 ml. Plates were returned to the incubator and removed every 48 h to change the media. After plating (5–10 days, depending on the density of the cells), GM was replaced with DM to promote the formation of adult cellular connections and smooth muscle differentiation (99; 127). The DM was replaced every 2–3 days until the ring was fully contracted and ready for testing. IAS and colon rings were fully formed in 5–10 days; however, time spent in culture for each construct varied depending on the number of cells plated and the experimental design designated

for each construct. In parallel, fibrin gel constructs were prepared normally; however, cells were not added before the incubation period to characterize the mechanical properties of the fibrin gel in the absence of cells.

Measurement of contractile properties. The protocol for measuring excitability and contractility of engineered muscle constructs was adapted from previous work (12; 127; 170). The following three variables were directly measured: torroidal diameter of the muscle construct, passive baseline force (Pb), and peak change in isometric force (P). The following two additional parameters were then calculated from the measured variables: cross-sectional area (CSA) was calculated from the diameter and specific force (sP) by dividing P by the CSA. Briefly, engineered sphincters and colons were separated from their molds (Figure 4.1*E*) using forceps, and the minimum ring diameter was measured using a calibrated eyepiece and a 5 or 10x objective lens on an inverted microscope (Axiovert 25; Zeiss, Thornwood, NY). The cross section was circular; therefore, the CSA was calculated using the measured diameter.

DM in the plate was replaced with 37°C Krebs solution, and the dish was placed on a heated aluminum platform that was maintained at a temperature of 37°C until the testing was complete. For contractility measurements, one end of the engineered sphincter or colon was anchored by a stainless steel pin (10 mm x 0.1 mm diameter) to the PDMS substrate, whereas another stainless steel pin was bent in the shape of a hook and attached by canning wax to an optical force transducer with a resolution of 1.4 μN and a range of 2 mN (Figure 4.1, *F* and *G*;

see Refs. (126; 283; 283; 284). We followed standard protocols used for measuring spontaneous basal tone of IAS muscle strips as follows: stretching of the tissue, followed by a period of equilibrium (between 20 and 60 min), where the IAS strips stabilized, resulting in the establishment of a new stable baseline of tension (275; 275; 285). Thus the bioengineered IAS and colon rings were stretched 50% of their resting length using a three-axis micromanipulator. The rings were allowed to sit for 20–30 min to reestablish a stable Pb, which was arbitrarily set at zero to allow consistent measurements of P.

Agonist-induced stimulation of bioengineered rings. All force measurements were collected at 100 samples/s for 60 s and recorded using a computer with LabVIEW data acquisition software (National Instruments, Austin, TX). A median filter of rank 2 was applied to all raw force data before being stored to minimize digitization noise without causing a phase delay for rapidly changing forces.

P was determined by subtracting the Pb from the total force values. Data analysis was done using LabVIEW and Microsoft Excel software programs. Each measurement was repeated three to eight times, and the mean value was recorded. Contractions were induced in the rings by the addition of 10^{-10} to 10^{-6} M ACh. Maximal contractions were seen 30–60 s after addition of the drug. Relaxation response in the rings was induced by the addition of 10^{-5} M 8-Br-cAMP. Maximal relaxation was achieved after 4 min; therefore, relaxation measurements are collective of four consecutive 1-min data recordings.

Preparation for histology. In preparation for light microscopy, engineered IAS rings were fixed in 4% paraformaldehyde. The rings were processed and embedded in paraffin wax according to standard histological procedures (9). All sections were made (5–8 μm thick) using a rotary microtome. Staining of the histological sections was performed to view general structure using Harris's hematoxylin and eosin.

Statistics. Tissue engineering data are presented as means ± SE for three to eight experiments per data point. Differences in mean values were compared within groups (e.g., control vs. ACh treatment), and significant differences were determined by ANOVA with the post hoc Tukey-Kramer Honest Significant Difference test. The level of significance was set at $P < 0.05$.

RESULTS

In designing an innovative model of the IAS, the following parameters were considered: three dimensionality, remodeling of in vivo structure, expression of fundamental in vivo physiological behaviors, potential for cell-cell and cell-matrix interactions, enhanced viability, and maintenance under physiological conditions in a controlled environment.

Contractility of isolated IAS cells. ACh (10^{-7} M)-induced stimulation of isolated smooth muscle cells from the rabbit IAS resulted in a sustained contractile response. Contraction was defined as the percent decrease in cell length

compared with control cell length (86.86 ± 5.29 μm, *n* = 150). ACh (10^{-7} M) induced a peak contraction at 30 s (28.85 ± 3.20% decrease in cell length, *n* = 150) and was sustained for 4 min (27.09 ± 4.43% decrease in cell length, *n* = 150; Figure 4.2). To determine the calcium dependence of this response, parallel experiments were performed in 0 Ca^{2+}/2 mM EGTA HEPES buffer. Control length of isolated smooth muscle cells preincubated in 0 Ca^{2+}/2 mM EGTA was 88.68 ± 6.41 μm (*n* = 150). As expected, ACh-induced stimulation of isolated smooth muscle cells resulted in minimal contractile response in the absence of Ca^{2+} (0.5 ± 4.47 and 4 ± 3.91% decrease in cell length at 30 s and 4 min, respectively; (Figure 4.2). These data are similar to the previously published contractile response of isolated IAS smooth muscle cells to bombesin and to exogenous protein kinase C (281; 282).

Bioengineering a 3-D IAS or colon ring. Primary cells isolated from the IAS and the circular smooth muscles of the colon were cultured separately in 75-cm^2 flasks. After 1 wk, 50,000 or 100,000 cells were transferred to plates containing a loose fibrin gel where they proliferated to confluence. As expected, cells proliferated and began to digest the fibrin gel, replacing it with endogenous ECM (21–23, 40, 46). Cells migrated and self-assembled along the line of force until they formed a parallel array of cells. As cells proliferated, they shrunk the fibrin gel, contracting it toward the center of the culture dish around a 5-mm-diameter SYLGARD mold (Figure 4.1, *B-D*). The rate of gel contraction was dependent on the number of cells initially plated, since constructs seeded with 100,000 cells consistently formed rings faster than constructs seeded with 50,000 cells (data

not shown). Tissues were considered "formed" when a 3-D cylindrical tube of sphincteric or colonic tissue contracted around the SYLGARD mold (Figure 4.1*D*). All fibrin constructs seeded with IAS or colon cells were fully formed within 5–10 days after cell seeding. The resulting 3-D IAS and colon rings remained stable in culture and were experimentally tested between 9 and 23 days. Parallel constructs of gels made without the addition of cells did not exhibit any contraction of the gel, and the gels without cells appeared unchanged after 21 days in culture. Thus contraction of the cell around the SYLGARD mold is mediated by the cells seeded in the construct.

Histological parameters. Histological analysis of the bioengineered IAS rings revealed one uniform cell type surrounded by undigested pieces of the fibrin gel matrix (Figure 4.3). The cells had many features of differentiated smooth muscle cells (fusiform, nonstriated, and uninucleated with the nucleus in the center of the cell), although the volume of the perinuclear space was small compared with cells in a normal muscle tissue. This suggests that the cells had not reached full maturation. As the gel contracted over a period of days, cells increasingly aligned in a parallel array. Histological analysis showed a gradient of cell alignment as the gel contracted and formed a bioengineered IAS ring (Figure 4.3). Fully contracted rings were composed of 20 concentric cell layers. Ring diameters averaged 91.3 ± 13.6 μm and had a range of 46.5–145.4 μm. The average CSA was calculated as 7,532.9 ± 2,010.3 μm^2 and ranged from 1,697.40 to 16,604.90 μm^2. These measurements were used to determine the specific force of each IAS ring, defined as the maximal force produced divided by the CSA.

Development and relaxation of the basal tone in bioengineered IAS rings. Elongation of an IAS ring by 50% of its length resulted in an elevation of tension followed by relaxation that was initially substantial, but gradually subsided over a period of 20–30 min as a stable baseline of tension was reached. This stable baseline was arbitrarily set at zero. Upon the addition of 10^{-5} M 8-Br-cAMP, IAS rings demonstrated a sustained relaxation response (reduction of the stable basal tone) reaching maximal relaxation 4 min after stimulation and subsequently resulting in the reestablishment of a new, stable basal tone. Average P for the rings after 4 min of incubation with 10^{-5} M 8-Br-cAMP was –14.8 ± 2.56 μN (Figure 4.4*B*). Stimulation with 10^{-5} M 8-Br-cAMP in 0 Ca^{2+}/2 mM EGTA Krebs also resulted in a relaxation response (–23 ± 4 μN after 4 min), suggesting that relaxation was calcium independent (Figure 4.4).

ACh-induced contraction of bioengineered IAS rings. Bioengineered IAS rings stimulated with 10^{-6} M ACh exhibited a peak P of 10.34 ± 3.2 μN within 30–60 s and was sustained for 4 min (Figure 4.5). Maximal P induced by ACh stimulation was 33.7 μN, resulting in a specific force (sP) of 2.8 kN/μm^2. Rings in 0 Ca^{2+}/2 mM EGTA Krebs did not contract when stimulated with ACh (0.9 ± 0.2 μN; Figure 4.5), demonstrating the calcium dependence of the contractile response. Furthermore, force generated by ACh-induced stimulation was concentration dependent (Figure 4.6), reaching a plateau at 10^{-6} M.

ACh-induced contraction of IAS rings and subsequent relaxation with 8-Br-cAMP. ACh-induced contraction of IAS rings did not appear to effect subsequent

relaxation induced by 10^{-5} M 8-Br-cAMP. Addition of 10^{-5} M 8-Br-cAMP resulted in an immediate relaxation of ACh-induced contracted IAS rings (Figure 4.7). Addition of 8-Br-cAMP induced rapid relaxation of ACh-induced contraction and force generation of IAS rings (–14.3 ± 3.8 μN; Figure 4.7*B*).

Spontaneous phasic contractile behavior in bioengineered colon rings. ACh-induced contraction and 8-Br-cAMP-induced relaxation of bioengineered colon rings showed a similar response pattern to that seen in bioengineered sphincteric rings (data not shown). However, colon rings showed one striking functional difference from IAS rings; they exhibited spontaneous phasic contractile behavior (Figure 4.8). Without the addition of any external stimuli, 100% of bioengineered colon rings demonstrated spontaneous phasic contraction. Spontaneous phasic contractions were recorded before, during, and after experimental testing (while the ring was still attached to the recording equipment), depending on the individual ring. Data were recorded at 1-min intervals continuously until contractions stopped. If uninterrupted, spontaneous phasic contractions persisted (amplitude: 18 ± 1.99 μN; frequency: 2.45 ± 0.21 cycles/min), on average, for 10.3 ± 2.19 min. Spontaneous phasic contractions were not seen in any IAS constructs at any time during data recording.

DISCUSSION

Despite the prevalence of fecal incontinence, little is known about the mechanisms underlying sphincter malfunction. Previous research attempts to understand IAS physiology, including tissue culture, cell suspensions (281; 282; 286) and tissue explants (275-277; 286), have advanced our knowledge of smooth muscle function. However, each technique has its own limitations; thus, smooth muscle function is still not clearly understood. New technological advances in tissue engineering, cell survival, and the utilization of 3-D matrixes offered new opportunities to engineer 3-D IAS sphincters. Tissue engineering incorporates three dimensionality resembling tissue explants and a homogenous population of cells similar to cell suspensions and utilizes tissue culture techniques to provide a controlled environment under physiological conditions in which tissues can survive for extended periods of time. More recently, fibrin-based tissue engineering of arterial smooth muscle has revealed a promising new technique for investigating smooth muscle (99). Using fibrin gel casting, we have developed a 3-D model of the IAS in vitro from isolated rabbit smooth muscle cells. This system uses a homogenous population of isolated sphincteric smooth muscle cells and, when seeded on a fibrin gel, they self-organize, forming 3-D functional tissues. In this study, all IAS rings formed in 5–10 days. Previous work has shown fibrin-based tissue construct viability for up to 40 days (127). Our IAS rings were only tested between 9 and 23 days in culture and therefore provide evidence for viability beyond 3 wk. Bioengineered IAS rings demonstrate spontaneous tone (force produced) that decreased (relaxation) in a calcium-

independent manner by the addition of 8-Br-cAMP or increased (contraction) in a calcium-dependent manner by the addition of ACh. Addition of 8-Br-cAMP also induced rapid relaxation of ACh-induced contracted IAS rings (Figure 4.7). These data provide evidence of physiological functionality. In addition, the absence of spontaneous phasic contractile behavior in IAS rings, which was seen in 100% of bioengineered colon rings, demonstrates tissue specificity. This model is highly reproducible and will offer an effective method for investigation of smooth muscle function because it combines the major advantages of previously reported research methods into one technique.

Bioengineered IAS rings exhibited physiological behavior functionally similar to smooth muscle in vitro. Upon seeding, cells began digesting the fibrin matrix, replacing it with their own ECM (2; 39; 127). As the cells proliferated, they self-organized along the line of force, forming a parallel array of cells analogous to what is observed in 3-D and mechanically strained cultures(287-289). The rate of ring formation was dependent on the number of cells initially plated, although this did not appear to affect force generated, since the magnitude of force generated by IAS rings was consistent regardless of the seeding number (data not shown). This was consistent with previous studies involving fibrin-based muscle constructs (127). The cells appeared to proliferate within the fibrin gel only until they reached confluence, regardless of seeding density. This suggested that normal density-dependent contact inhibition of cell division (290) had occurred during the formation of IAS rings.

Agonist-induced stimulation showed that bioengineered rings were functionally similar to smooth muscle in vivo. IAS rings demonstrated a stable, spontaneous tone until stimulated by an agonist. IAS rings showed sustained calcium-independent relaxation of the basal tone in response to 10^{-5} M 8-Br-cAMP. This is similar to smooth muscle in vivo, where relaxation is largely mediated by cAMP and is calcium independent (280; 291; 292). Upon stimulation with the same concentration of ACh, both IAS rings and isolated IAS single cells exhibited a peak P within 30–60 s after stimulation that was sustained for 4 min (Figures 4.2 and 5*A*). Contraction of both isolated single cells and bioengineered rings were calcium dependent (Figures 4.2 and 5*A*). These similarities suggest that bioengineered IAS rings have the sensitivity of isolated single cells, which may be because of the lack the barriers present in muscle strips, such as innervating nerves, epithelial lining, and serosa, and therefore provide an in vitro opportunity to experimentally manipulate one 3-D tissue constituent. ACh-induced contraction of both isolated single cells and bioengineered rings also corresponds to previously described ACh-induced contraction of human IAS muscle strips (275-277). Preincubation of engineered IAS rings with 10^{-6} M ACh did not affect the relaxation response induced subsequently with 10^{-5} M 8-Br-cAMP, since IAS rings demonstrated an immediate shift from contraction to relaxation after 8-Br-cAMP-induced stimulation (Figure 4.7). Bioengineered IAS rings have the ability to contract and relax within minutes, similar to the capacity seen in vivo. Therefore, 3-D engineered IAS constructs exhibited several physiologically functional behaviors similar to the capacity seen in vivo.

The characteristic formation of bioengineered IAS rings from isolated sphincteric cells is exclusive to the IAS. Bioengineered colon constructs demonstrated spontaneous phasic contraction in the absence of external stimuli. Bioengineered colon constructs were made from isolated colonic cells from the same animals used to make IAS rings. The frequency of bioengineered colonic phasic contractions (2.45 ± 0.21 cycles/min) was similar in frequency to that seen in colonic muscle strips (2–4 cycles/min)(293). As expected, bioengineered IAS rings did not exhibit the spontaneous phasic contractile behavior at any time. This suggests that IAS constructs, prepared using adjacent portions of the gastrointestinal tract from the same animals, exhibited physiological functionality and characteristic tissue specificity that are unique to the IAS.

Although the engineered rings described here provide an important model for studying sphincteric smooth muscle, it is important to note that these cells were not fully mature. Histological analysis of the bioengineered rings revealed a homogenous composition of differentiated smooth muscle cells that had not reached full maturation. Maturation of smooth muscle cells involves continuous regulation by a variety of environmental factors, including hormonal signals and neural and mechanical input (294; 295). Although it is known that these factors influence the maturation of smooth muscle cells, the specific role of each factor in vivo is not clearly understood (295). Optimization experiments for IAS constructs could be designed in which each physiological input is altered to maximize cell maturation. This would improve the engineered IAS rings but would also independently provide a better understanding of smooth muscle maturation in

general. Ross and Tranquillo (99) revealed increased growth of smooth muscle cells in a fibrin matrix (measured by collagen production) associated with GM (containing DMEM supplemented with FBS) vs. DM (consisting mainly of media 199). After formation of the ring, we attempted to promote differentiation by replacing the normal GM with a differentiated GM that has fewer mitogenic signals, therefore suppressing mitosis. This demonstrates the association between maturation and increased force production. Although our IAS rings were developmentally arrested and had not reached full maturation, this model is well suited for investigation of smooth muscle development, maturation, and maintenance of functional smooth muscle.

Although fibrin gel-based constructs have previously been studied, a quantitative comparison of force generation was not possible, as these labs did not have the equipment necessary for active force testing of bioengineered tissue constructs. Tranquillo and colleagues (2; 99; 208; 296) provide the necessary foundation for this area of research, demonstrating and characterizing arterial smooth muscle tissue growth and development within a fibrin matrix and the resulting tensile properties. However, fibrin-based engineering of smooth muscle has been limited to vascular/arterial smooth muscle. Furthermore, there are no data available that report the active forces generated by fibrin-based smooth muscle constructs. This is the first report of active forces, including specific force, generated by fibrin-based smooth muscle constructs. Furthermore, this paper provides the first illustration of not only gastrointestinal smooth muscle, but sphincteric smooth muscle bioengineered in a fibrin matrix. Our research on bioengineered IAS rings

advances fibrin-based engineering of smooth muscle tissue by investigating and correlating the functional properties, including measurements of P in response to agonist-induced stimulation. Specific force calculated for bioengineered IAS rings was 2.8 kN/m^2. The only available comparison of force generated by smooth muscle was explanted muscle strips from humans (10.2 kN/m^2) (275), which is 3.6-fold more force than the IAS rings. Nerem and Ensley (297) report significant variation in the mechanical strength of smooth muscle grown in artificial scaffolds among different species. Therefore, the lower force generation induced by ACh in these engineered IAS rings could be in part attributed to the different model systems being compared (human muscle vs. rabbit muscle). The difference in specific force generated in human muscle strips vs. bioengineered IAS rings may also be the result of the developmental state of the cells, since our system has not yet incorporated all of the environmental cues necessary for smooth muscle to reach full maturation. Furthermore, bioengineered rings were made using a homogenous smooth muscle population grown in a fibrin matrix. Although it has been shown that cells grown in a fibrin matrix rapidly migrate, proliferate, and then digest the fibrin, replacing it with their own ECM (2; 39; 99; 208), fibrin itself is compliant compared with the rigid ECM seen in vivo and therefore will not transmit force as well. Histology revealed that the bioengineered rings were composed of cells surrounded by pieces of undigested fibrin matrix. To address this issue in future experimentation, growth factors could be added to increase ECM production. For example, transforming growth factor (TGF) has previously been shown to increase the amount of ECM in fibrin-based engineered tissues

(99). Incorporating TGF- into our system may therefore increase ECM production and cell differentiation, resulting in a less compliant ring with enhanced force production and transmission. Furthermore, alteration of the method in which the cells are seeded may also lead to increased force generation in bioengineered constructs, for example, mixing the isolated cells directly with the fibrin before polymerization, rather than after, to increase homogeneity of the ring and reduce the percentage of undigested fibrin comprising the ring. Future research will be required to optimize this system using improved contractility as the outcome measure.

In conclusion, a fibrin-based model of the IAS has been produced. In this model, IAS cells self-organize in a 3-D fibrin matrix. The resulting 3-D rings show tissue specificity to the IAS, are highly reproducible, and are functionally similar to IAS smooth muscle in vivo. Our fibrin-based constructs provide the opportunity to test the effects of various pharmacological agents, growth factors, and mechanical interventions on smooth muscle function. This is the first report of a functional in vitro model of the IAS that may be used in the elucidation of the mechanisms causing smooth myogenic sphincter malfunction and the investigation of treatments for fecal incontinence.

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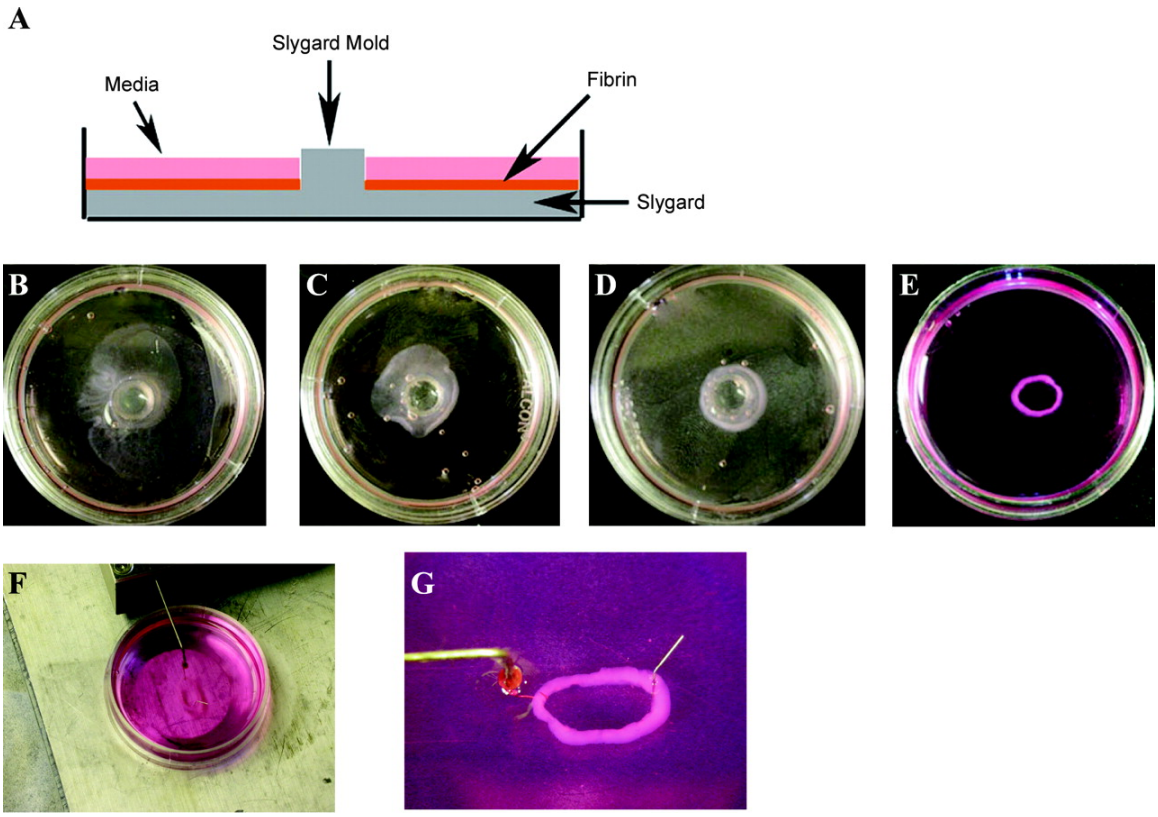


Figure 4.1 Preparation and Functional Testing Set-up
A: preparation of culture dishes; 35-mm plates were coated with 1.5 ml SYLGARD [polydimethylsiloxane (PDMS)] and were cured for 24 h. A 5-mm-diameter SYLGARD mold was placed in the center of the plate to provide a luminal space. The plates were sterilized with 70% ethanol for 30 min and then treated with ultraviolet light for 1 h. Growth media (500 _l) containing 10 U/ml thrombin was added to each plate, and care was taken to make sure the media covered the entire surface. To polymerize the gel, 200 _l of 20 mg/ml fibrinogen were added and swirled. After 15 min, the plates were ready for cell seeding, and media was added. *B-E*: formation of 3-dimensional bioengineered internal anal sphincter (IAS) constructs. IAS cells fused and contracted the fibrin gel around a 5-mm SYLGARD mold. Progression of fibrin gel contraction leading to the formation of a fully formed engineered sphincteric ring is shown (*B-D*). After ring formation, the engineered sphincter was released from its mold (*E*) before mechanical testing. *F* and *G*: mechanical testing of 3-dimensional engineered IAS rings. The construct was placed on an aluminum heating plate and prepared for mechanical testing (*F*). *G* is an enlarged view of *F*. After release from its mold, the IAS ring was secured to the SYLGARD substrate using a stainless steel pin (*G*, *right*). The other end was attached to a hook-shaped stainless steel pin and then attached to the force transducer using canning wax (*G*, *left*).

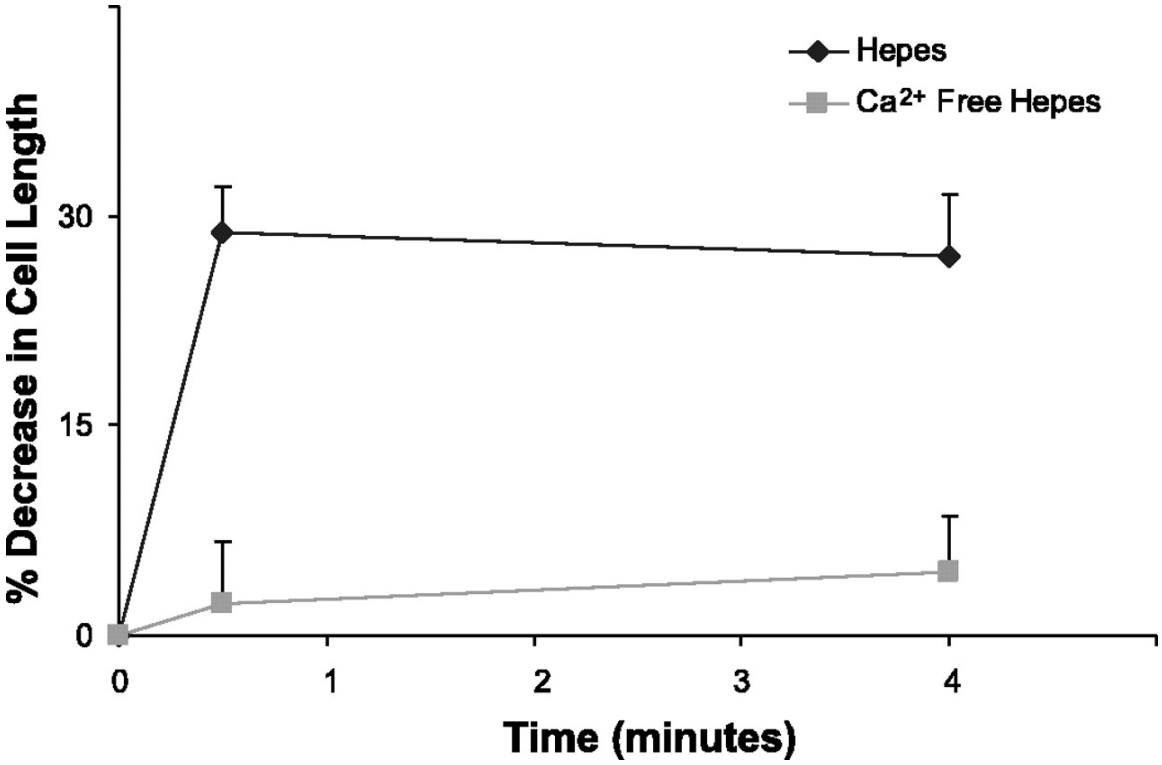


Figure 4.2 Effect of ACh on isolated IAS rabbit cells in HEPES buffer vs.0 Calcium/2 mM EGTA HEPES buffer.
ACh (10_7 M) was added to 0.5 ml of the cell suspension for 30 s or 4 min with untreated cells as controls. The reaction was stopped, and cells were fixed by the addition of 0.1 ml acrolein at a final concentration of 0.1%. Individual cell lengths were measured by computerized image micrometry. The length of cells in the control state or after addition of ACh was obtained by measuring 90–100 cells encountered randomly in successive microscopic fields from each of 3 separate experiments. Contraction was defined as the %decrease in cell length compared with control. In HEPES buffer, peak contraction (28.85 _ 3.20%) was seen at 30 s and was sustained for 4 min (27.09 _ 4.43%). Isolated cells preincubated in 0 Ca2_/2 mM EGTA HEPES exhibited little contraction at 30 s (0.5 _ 4.47%) and at 4 min (4 _3.91%). Data are means _ SE of 3 experiments.

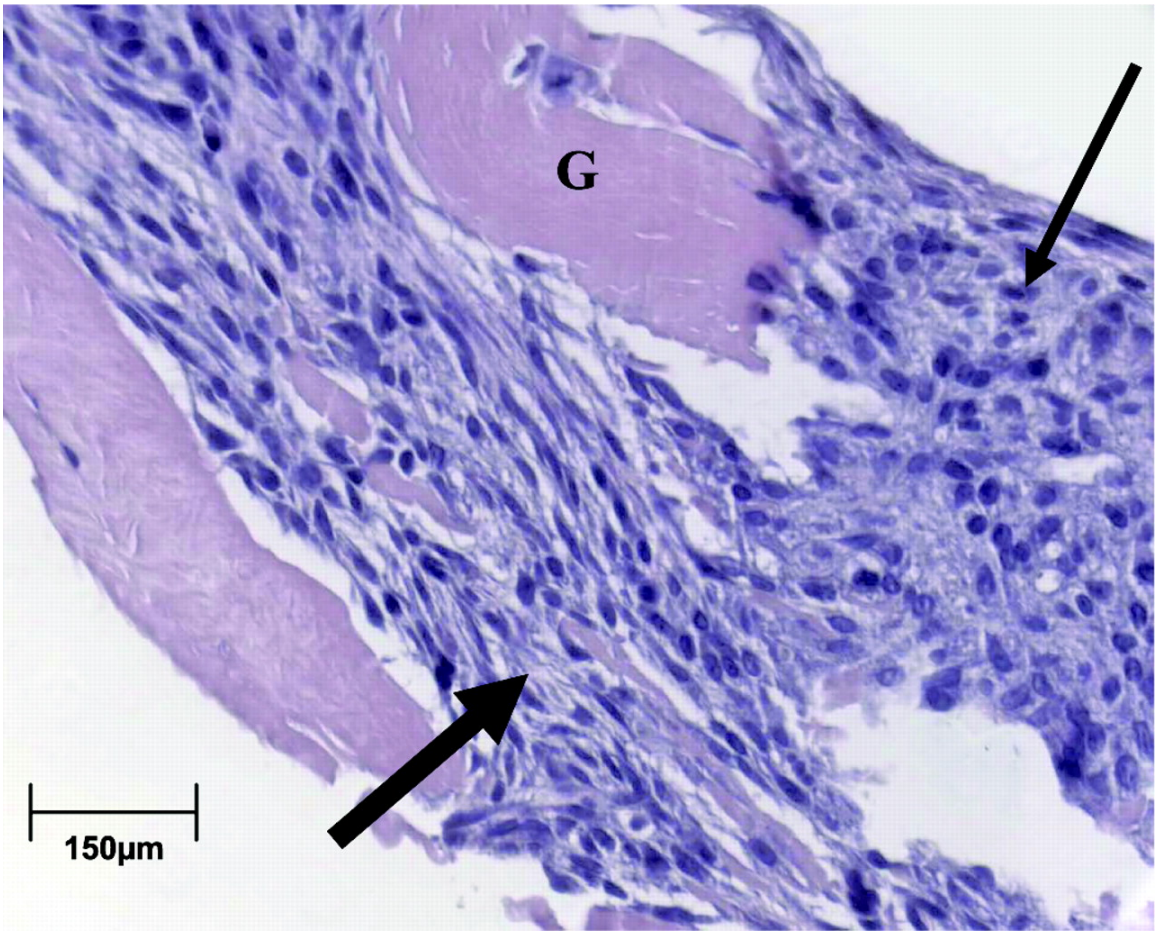


Figure 4.3 Histology of IAS rings.
Histological cross section of bioengineered IAS ring stained with hematoxylin and eosin. Photograph (magnification _400) showing a portion of an engineered IAS ring with pieces of undigested fibrin gel (G) and cells that have not yet aligned (small arrow) as well as cells that have self-organized (large arrow).

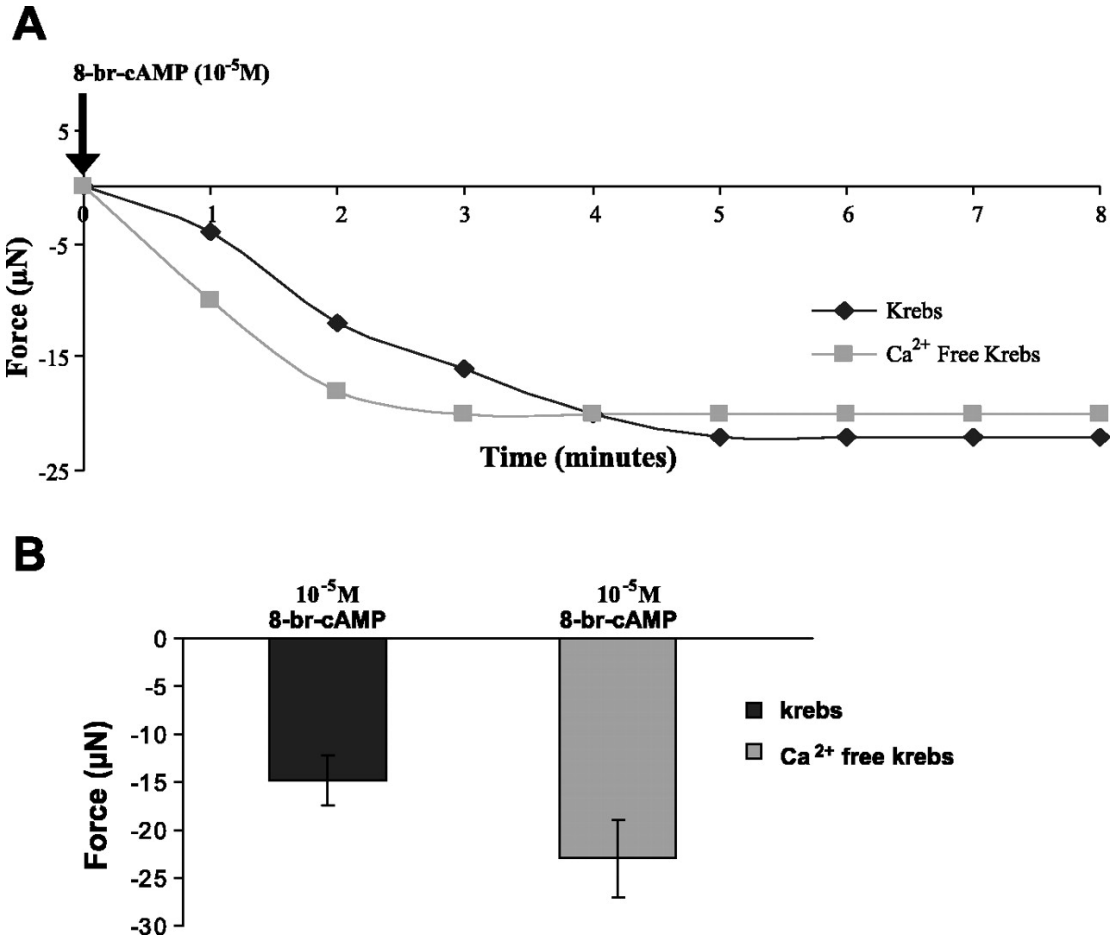


Figure 4.4 Relaxation of the basal tone in IAS constructs.
A: representative tracing of the effect of 10_5 M 8-bromo-cAMP (8-Br-cAMP) on IAS rings in Krebs vs. 0 Ca2_/2 mM EGTA Krebs buffer. Data points represent 5 successive readings of the decrease in resting force by IAS rings after the addition of 8-BrcAMP to the construct in Krebs vs. 0 Ca2_/2 mM EGTA Krebs buffer. IAS rings, in both Krebs and 0 Ca2_/2 mM EGTA Krebs buffer, showed a maximum and sustained relaxation _3–4 min after the addition of 10_5 M 8-Br-cAMP. *B*: IAS rings responded functionally to 8-Br-cAMP in a calcium-independent manner. 8-Br-cAMP (10_4 M) induced relaxation of the basal tone in both Krebs (_14.8 _ 2.56 _N) and 0 Ca2_/2 mM EGTA Krebs (_23 _ 4 _N).

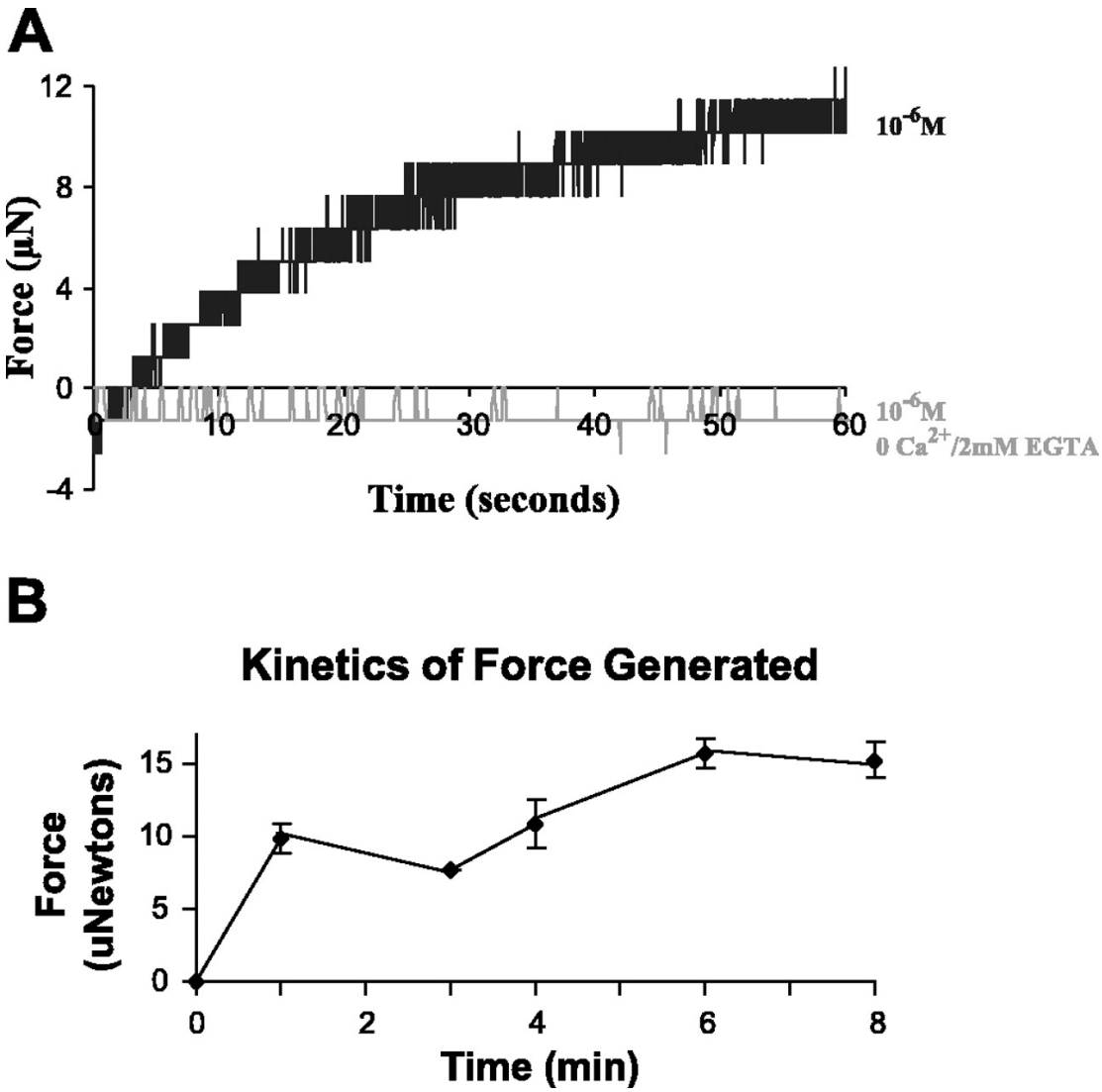


Figure 4.5 Kinetics of IAS rings in response to ACh.
A: details of the kinetics of bioengineered IAS rings in Krebs vs. 0 Ca2_/2 mM EGTA Krebs buffer 1 min after stimulation with 10_6 M ACh. Measurements were collected at 100samples/s for 60 s, and graph was generated using Microsoft Excel. *B*: kinetics of ACh-induced (10_6 M) force generation. Data points represent 5 successive readings of the force generated by IAS rings after the addition of 10_6 M Ach and indicate a sustained force generation up to 8 min.

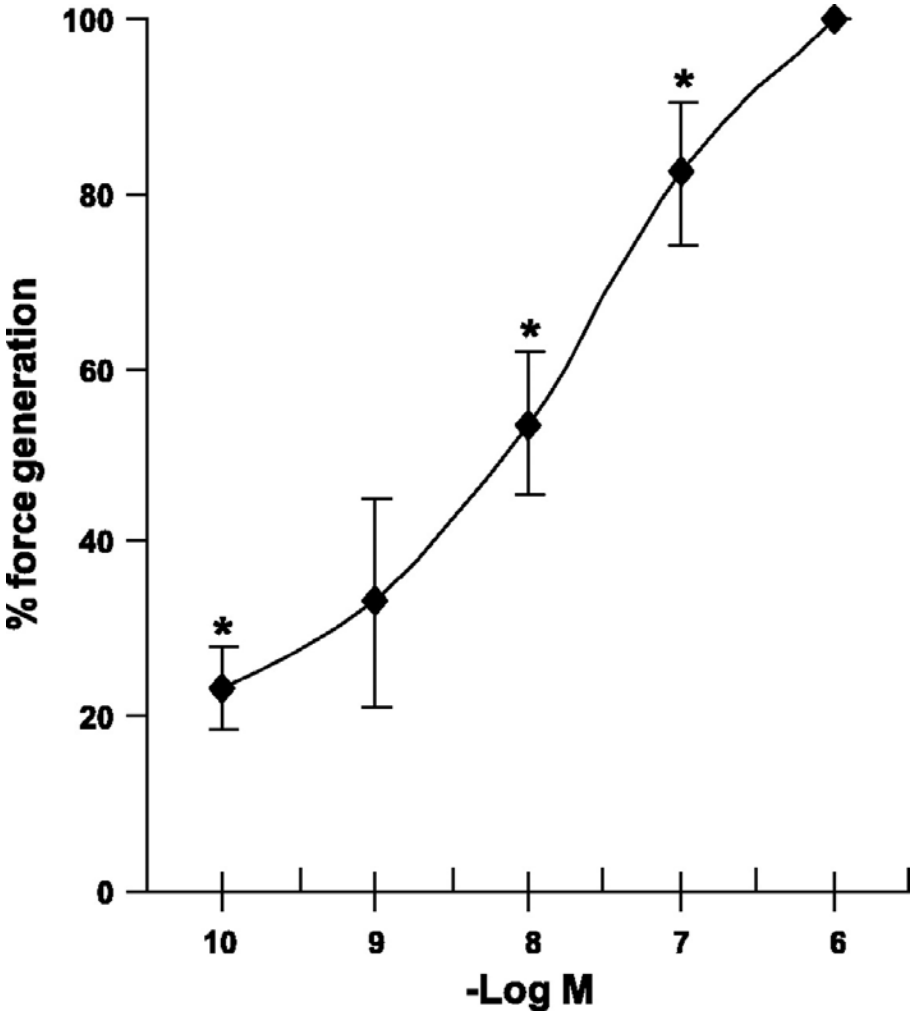


Figure 4.6 Dose-response curve for the effect of ACh on bioengineered IAS rings.
Responses are expressed as %maximal contraction, and data points are means _ SE of 3–8 experiments. Force generated by ACh-induced stimulation was concentration dependent, reaching a plateau at 10_6 M. *Significant difference between points (*P* 0.05).

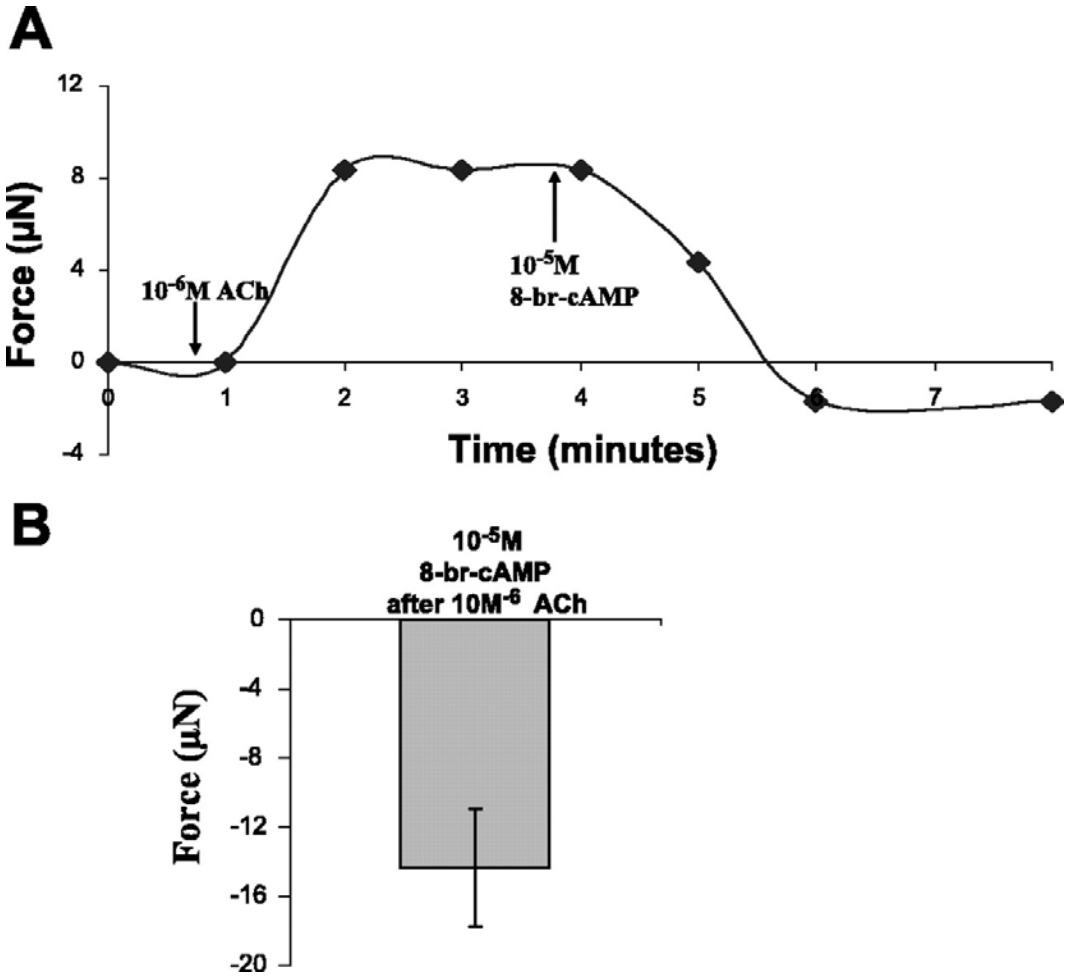


Figure 4.7 Effect of ACh and subsequent addition of 8-Br-cAMP on an IAS ring in Krebs buffer.

A: representative tracing of IAS ring response to 10_6 M ACh and subsequent addition of 10_5 M 8-Br-cAMP. Data points represent successive readings of the force generated by IAS rings after the addition of ACh followed by addition of 8-Br-cAMP. Arrows indicate the time at which the drug was added. Bioengineered IAS rings responded functionally in response to both ACh and 8-Br-cAMP. Addition of 10_5 M 8-Br-cAMP resulted in an immediate relaxation of ACh-induced contracted IAS rings. *B*:average relaxation from 10_5 M 8-Br-cAMP after addition of 10_6 M ACh-induced contraction of IAS rings in Krebs (_14.3 _ 3.4 _N).

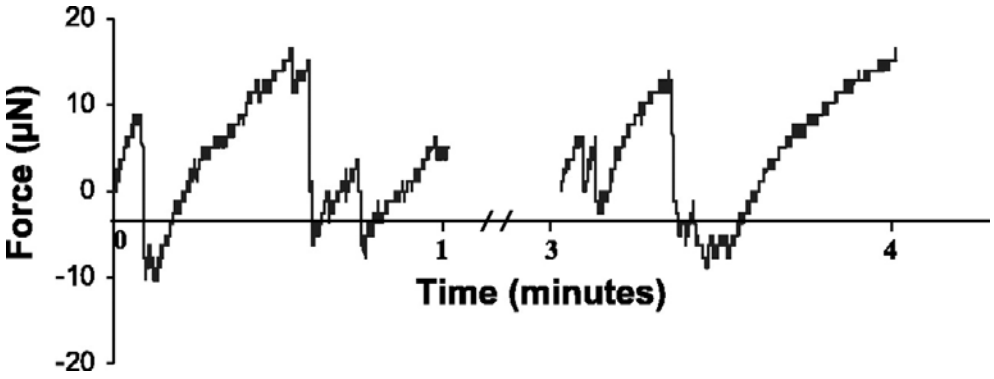


Figure 4.8 Spontaneous phasic contractile behavior in bioengineered colon rings

Bioengineered colon rings exhibited spontaneous phasic contractile behavior without the addition of any external stimuli. Bioengineered colon rings (100%) demonstrated spontaneous phasic contraction (amplitude: 18 _ 1.99 _N; frequency: 2.45 _ 0.21 cycles/min). Data represent 2 consecutive 1-min intervals. When uninterrupted and without addition of any external stimuli, spontaneous phasic contractions persisted, on average, for 10.3 _ 2.19 min.

Chapter 5

Bioengineering Functional Human Aortic Vascular Smooth Muscle Strips in vitro

ABSTRACT

The contraction and relaxation of vascular smooth muscle is responsible for the maintenance of vascular tone, which is a major determinate of blood pressure. However, the molecular events leading to the contraction and relaxation of vascular smooth muscle are poorly understood. The development of 3-dimensional bioengineered tissues provides an opportunity to investigate the molecular events controlling vascular tone in vitro. Here, we used fibrin-gel casting to bioengineer functional VSM strips from primary human aortic VSM cells. Our bioengineered VSM strips are functionally similar to VSM in vivo and remained viable in culture for up to 5 weeks. VSM strips demonstrate spontaneous basal tone and can generate an active force (contraction) of up to 85.2μN upon stimulation with phenylephrine. Bioengineered VSM strips exhibited calcium-dependent contraction and calcium-independent relaxation. The development of functional bioengineered VSM tissue provides a new in vitro

model system, which can be used to investigate the molecular events controlling vascular tone.

Introduction

Vascular smooth muscle (VSM) is a functionally critical component of various tissues in the cardiovascular system. The contraction and relaxation of VSM that resides in the tunica media of arterioles is responsible for the maintenance of vascular tone, which is a major determinate of blood pressure. According to the American Heart Association (2007), one in three adults in the United States has hypertension (high blood pressure), and when untreated can lead to stroke, heart attack, heart failure, or kidney failure. Conversely, septic shock can result in the loss of vascular smooth muscle tone, resulting in hypotension. Current strategies for controlling blood pressure in cases of both hypo- and hypertension include modifying vascular smooth muscle through cellular targets including alpha-adrenergic and calcium channel receptors. However, the molecular events leading to the contraction and relaxation of VSM are poorly understood.

Current methods used to investigate smooth muscle contractile function include isolated tissue preparations, such as explanted muscle strips, which provide researchers with a 3-dimensional (3-D) tissue that can be easily subjected to controlled changes in perfusion, oxygen availability, and agonist-induced stimulation . An advantage of this model system is that it most closely represents *in vivo* conditions. However, tissue explants are complex, being

composed of several different cell types that are difficult to experimentally manipulate individually. Also, to prevent rapid deterioration, tissue/organ explants must be placed in a cold (usually 4° C) bath that does not replicate normal physiological conditions. It is also difficult to control or manipulate expression of specific proteins in tissue explant models through transfection of specific cell types.

Tissue engineering models aim to alleviate some of the problems encountered with current model systems being utilized. In order to bioengineer functional 3-dimensional (3-D) tissue constructs, smooth muscle cells can be cultured within a suitable matrix. Isolated cells can be bioengineered to yield 3-D, homogeneous tissues, which can be maintained in culture for long periods of time under physiological conditions. Bioengineered tissue can also be more easily manipulated for experiments by, for example, transfecting cells with expression vectors to control expression of an endogenous protein or to give expression of mutated proteins. It is also possible to reduce the number of experimental animals used in the future with the use of *in vitro* bioengineered models. For these reasons, tissue engineering is a preferable approach for developing models with characteristics similar to those observed *in vivo*.

There have been many reports of 3-D “tissue equivalents” developed *in vitro* by culturing cells in fibrin matrices (13; 99; 126; 127). Fibrin-based gels have become an effective option for engineering functional muscle tissue, providing a matrix that is well suited for the development of contractile tissues . Fibrin gels are formed by the enzymatic cleavage of fibrinogen by the serine

protease thrombin, allowing the fibrin monomers to spontaneously interact and form fibrils. Fibrin-based gels have advantages over synthetic polymer approaches for tissue-equivalent fabrication. Specifically, fibrin matrices permit immediate cellularity throughout the construct by cell entrapment in the forming gel and subsequent fibril alignment via mechanical constraint of cell-induced gel compaction (1). Cell-mediated fibrin gel contraction occurs, mimicking alignment of muscle cells in tissue *in vivo* . Within a fibrin matrix, cells rapidly migrate, proliferate, and digest the fibrin, replacing it with their endogenous extracellular matrix (ECM) (99).

Several investigators have demonstrated the capacity to bioengineer primary isolated VSM cells into 3-D tissues(99; 298). VSM cells grown in fibrin show increased production of ECM, collagen (208), elastic fibers (19), and also exhibit mechanical properties that are commensurate with vascular tissue *in vivo* (19; 99). Furthermore, fibrin-based engineered tissues can be maintained for weeks in culture . This allows investigation of the effects of cell-cell interactions, growth factors, mechanical and chemical stimuli, gene expression, and protein kinase action on tissue-level function. Although fibrin gel remodeling by VSM cells have been documented (99), none of these studies have reported on the active contractile properties (force generation) of bioengineered VSM tissues.

Here we used fibrin gel casting to develop a 3-D human VSM strip model in vitro. Bioengineered “VSM strips” result from the spontaneous delamination of a confluent monolayer of primary human aortic VSM plated on a culture surface treated to control cell adhesion. Our bioengineered VSM strips are functionally

similar to VSM in vivo and remained viable in culture for up to 5 weeks. VSM strips demonstrate spontaneous basal tone and can generate an active force (contraction) of up to 85.2μN upon stimulation with phenylephrine (PE). Bioengineered VSM strips exhibited calcium-dependent contraction and calcium-independent relaxation. The development of a functional bioengineered VSM strip provides a new in vitro model which can be used to investigate the molecular events controlling vascular tone.

Methods

Proliferative capacity of primary human aortic VSM cells. We purchased primary human aortic vascular smooth muscle cells from Cambrex Inc (Cat # CC-2571, Lot # 4F1292; Walkersville, MD), which were isolated from a 57-year-old, Caucasian, male donor and obtained under informed consent. VSM growth media (fully supplemented with serum, growth factors, and antibiotics) was purchased from Cell Applications Inc (Cat # 311-500, San Diego, CA). These cells were positive for α-actin expression (see Cambrex Inc. product information: certificate of analysis), a smooth muscle-specific marker. Primary VSM cells were expanded separately in 100 x 20mm culture plates. At passages 4 and 7, the cells were then detached from their culture flasks with 1.5 ml trypsin-EDTA, collected, re-suspended in growth media, and counted with a hemocytometer. In 6-well plates, $2.5x10^3$ cells were added to each well and culture plates were in an

incubator with media changes every other day. At each designated time point, cells from 3 wells were counted, and data are presented as means ± SE.

Preparation of culture plates. Culture plates (35 mm) were prepared as described previously [3-5, 11]. Briefly, 1.5 ml SYLGARD polydimethylsiloxane (PDMS) elastomer (Dow Chemical Corporation, Midland, MI, U.S.A.) was poured in each plate and allowed to cure for 24 h. The SYLGARD substrate provided a layer in which anchor materials could be easily pinned in place and provided a surface to which, unless coated, the cells could not adhere. Anchor points were engineered on the SYLGARD surface by placing 6 mm long segments of size 0 braided silk sutures (Ethicon, Cornelia, GA, U.S.A.) 12 mm apart in the center of the culture plate. The silk sutures were anchored PDMS substrate with four stainless steel pins (10 mm x 0.1 mm diameter) (Figure 5.1). The culture dish was sterilized with 70% ethanol for 30 min. Ethanol was then aspirated, and culture dishes were exposed to ultraviolet light for 1 h. Each dish received 500 µl of growth media containing 10 U/ml of thrombin, and the dish was agitated until the bottom of the plate was entirely coated. Finally, 200 µl of 20 mg/ml fibrinogen was added to each plate and gently swirled. The fibrin polymerized 15 min later, and the plates were ready for cell seeding.

Cell seeding and culture of VSM strips. Primary VSM cells were expanded separately in 100 x 20mm culture plates. The cells were then detached from their culture flasks with 1.5 ml trypsin-EDTA, collected, re-suspended in growth media,

and counted with a hemocytometer. Different concentrations ($1x10^4$ – $1x10^6$) of VSM cells were added to the prepared culture plates in a final volume of 2.5 ml. Plates were placed in an incubator until the time of testing and culture media was changed every other day. Time spent in culture for each construct varied depending on the initial seeding density. Bioengineered tissues were grown in an incubator (37°C, 95% humidified air, 5% CO_2).

Evaluation of contractile properties. The protocol for measuring excitability and contractility of engineered muscle constructs was adapted from previous work [3-5]. For contractility measurements of VSM strips, the culture dish containing the bioengineered tissue was placed on a heated aluminum platform that was maintained at a temperature of 37°C. The culture media was replaced with 3ml of fresh growth media. One end of the bioengineered VSM strip remained anchored to the culture plate and the other end was attached to a custom-built optical force transducer with a resolution of 1.4 μN and a range of 2 mN. In order to attach the bioengineered VSM strip to the force transducer, the pins from one end of the muscle strip were detached from the SYLGARD substrate. One pin was discarded and the other pin canning was attached to the optical force transducer with canning wax (Figure 5.2).

We followed standard protocols used for measuring spontaneous basal tone of VSM strips as follows: stretching of the tissue, followed by a period of equilibrium (10-20 min), where the VSM strips stabilized, resulting in the establishment of a new stable baseline of tension. Contractions in

bioengineered VSM strips were initiated by mechanical and chemical stimuli. The active force was measured using the optical force transducer and force tracings that were digitally recorded using Lab-VIEW data acquisition software (National Instruments Corporation, Austin,TX, U.S.A.). All force measurements were collected at 100 samples/s for 60 s, and a median filter of rank 2 was applied to all raw force data before being stored to minimize digitization noise without causing a phase delay for rapidly changing forces. Standard protocols were used to measure spontaneous basal tone of smooth muscle strips(275-277; 285; 299). Using a three-axis micromanipulator, VSM strips were stretched slightly and allowed to stabilize (5-10 min). The resulting establishment of a new stable baseline of tension was arbitrarily set at zero to allow consistent measurements of active force. Contraction and relaxation in bioengineered VSM strips was induced by the addition of 10μM PE or 66mM potassium chloride (KCl) (final concentration), respectively. Solutions of KCl and phenylepherine (PE) were prepared in Phosphate Buffered Saline (PBS). All chemical were purchased from Sigma unless specified otherwise.

To determine the calcium dependence of the contractile response in bioengineered VSM strips, parallel experiments were performed in 0 Ca^{2+}/2mM ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic Acid (EGTA), a Ca^{2+} chelator, in DMEM. VSM strips were attached to the force transducer and then washed several times with 0 Ca^{2+}/2mM EGTA DMEM. Constructs were allowed to stabilize and stimulation was subsequently induced with 10μM PE.

Mechanical stimulation of bioengineered VSM strips was induced using the slack test, which was developed for VSM explants (299; 300). VSM strip length was rapidly decreased by 50 μm, which lowered the applied tension on the VSM strips enough to drop the force to zero. The response (active force) of the VSM strips after the decrease in tension was then recorded. Each measurement was repeated seven to thirteen times, and the mean value was recorded.

RNA Isolation. RNA isolation was performed using the Qiagen RNeasy Mini kit (Qiagen Inc., Valencia, CA). Three VSM strips from each group ($1x10^4$ or $5x10^5$ cells/construct) (<30g tissue) were homogenized in 600 μl of Buffer RLT using a hand-held, cordless homogenizer (Fisher Scientific Co., Pittsburg, PA) for 1 minute in a 1.5 ml microcentrifuge tube. The tissue lysate was then centrifuged at maximum speed for 3 minutes in a microcentrifuge to pellet cell debris. The supernatant was then applied to an RNeasy mini column and subjected to several washes. The total RNA was eluted with 50 μl of RNase/DNase-free water. After RNA isolation, each sample was analyzed for nucleic acid purity using the A260/A280 ratio on a spectrophotomer. Using the 260 nm absorbance value, we determined the concentration of nucleic acid within each sample. The samples were then stored at -20°C until further processing.

Preparation for histology. Bioengineered VSM strips were fixed in 10% neutral buffered formalin for approximately 24 hours and then transferred to 70%

ethanol. Bioengineered VSM strips were prepared for histology using standard histological techniques (tissue processing and paraffin embedding). Sections were obtained using a rotary microtome and all sections were made 5–8 μm thick. Sections were then stained with Harris's hematoxylin and eosin to view general structure of bioengineered VSM tissues.

Statistics. Tissue engineering data are presented as means ± SE for seven to thirteen experiments per data point. Significant differences were determined by ANOVA statistical analysis and the level of significance was set at P= 0.05. Turkey's test was used for pairwise comparisons.

Results

VSM strip formation. We determined that proliferation of VSM cells was greater at passage 4 than passage 7 (Figure 5.3). Therefore, we used cells between passages 3-5 for all experiments. VSM cells that were seeded in a fibrin gel where they proliferated and aligned to form a bioengineered "strip" of VSM tissue. Using concentrations of VSM cells between $1x10^4$ – $1x10^6$/construct, strip formation ranged from 2-35 days. Once strip formation occurred, constructs were ready for functional testing.

Seeding Density. Optimal seeding density for bioengineering VSM strip formation was determined using active force generation as the end point measured. We tested constructs with seeding densities from $1x10^4$ – $1x10^6$ cells per construct

(Figure 5.4). The time-to-construct formation varied depending on the initial seeding density, as higher seeding densities led to more rapid VSM strip formation. Following VSM strip formation, constructs were attached to the force transducer and contractions were induced by addition of 10μM PE. There were no significant differences in force between any of the seeding densities tested, however constructs initially seeded with $5x10^5$ cells exhibited the greatest magnitude of force on average (17.65±1.7μN) (Figure 5.4). Therefore we used $5x10^5$ cells per construct for all subsequent experiments.

Longevity Studies. The length of time in culture that constructs remained functional in response to 10μM PE was examined (Figure 5.5). Constructs were tested at 1, 2, 3, and 5 weeks after cell seeding. Constructs generated the greatest force at 1 and 2 weeks in culture, 41.01±4.6 and 36.71±5.1μN respectively. Although no significant difference in force generation was observed between constructs tested at weeks 1, 2, or 3, maximal force generation of 85.2μN was observed at one week in culture (Figure 5.5). Constructs remained functional for up to 5 weeks in culture. However force generation in response to PE-induced stimulation was significantly lower at the 5 week time point, compared to constructs tested at 1-3 weeks in culture (Figure 5.5).

Mechanical and Agonist-induced Stimulation of VSM strips. In order to assess the functionality of bioengineered VSM strips, contraction and relaxation was induced using chemical and mechanical stimuli. Constructs stimulated with

10μM PE displayed force generation of 41.01±4.6μN at one week in culture. At all time points, the peak contractile force from PE-induced stimulation was seen approximately 50-60 seconds after the addition of the drug. Addition of PBS was used as a control and did not generate any measurable force (Figure 5.6).

Addition of 66mM KCl to VSM strips induced relaxation (-65.8±16.9μN) from the basal tone. Maximal relaxation occurred approximately 50-60 seconds after addition of the drug. KCl-induced relaxation of VSM strips was consistently followed by sustained contraction for 4-5 minutes (Figure 5.7).

The ability of bioengineered VSM strips to exhibit myogenic force generation was examined. Mechanical-induced stimulation of VSM strips was accomplished by decreasing tension by a length of 50μm. Decreasing the applied tension on the strip caused contraction of the VSM strip, thus demonstrating a myogenic response (Figure 5.8).

Ca^{2+} sensitivity. Constructs were pre-incubated in 0 Ca^{2+}/2mM EGTA media and subsequently stimulated with 10μM PE to assess the Ca^{2+} dependence of the contractile response. Pre-incubation of bioengineered VSM strips with 0 Ca^{2+}/2mM EGTA media resulted in significantly diminished force generation upon PE-induced stimulation, thus demonstrating the Ca^{2+} dependence of the contractile response. In the absence of Ca^{2+}, constructs stimulated with 10μM PE generated an average force of 2.6 ±1.2μN compared to 27.98 ±4.1μN of force generated by constructs in the presence of Ca^{2+} (Figure 5.9).

KCl-induced relaxation of VSM strips was independent of Ca^{2+}, as VSM strips stimulated with 66mM KCl in 0 Ca^{2+}/2mM EGTA media did exhibit relaxation (-31.0 ±3.2μN) from the basal tone. However, in the absence of Ca^{2+}, no sustained contraction was seen following KCl-induced stimulation. Instead, VSM strips exhibited sustained relaxation for 4-5 minutes (Figure 5.7). Therefore, KCl-induced contraction is Ca^{2+} dependent.

RNA Content of Bioengineered VSM strips. Total RNA was isolated from VSM strips with seeding densities of $1x10^4$ and $5x10^5$ cells/construct. RNA was isolated 48 hours and 2 weeks after cell seeding. At 48 hours, constructs seeded with $1x10^4$ showed significantly lower RNA content (1.58±.27μg/ml) compared to constructs seeded with $5x10^5$ cells (74.76±21.34μg/ml). However, two weeks after cell seeding, there was no significant difference in RNA content between constructs seeded with $1x10^4$ and $5x10^5$, 90.78±6.66μg/ml and 68.82±5.17μg/ml respectively (Figure 5.10).

Histology. Bioengineered VSM strips were prepared for histology using standard histological techniques (formalin fixation and paraffin embedding). Sections were stained with hematoxylin/eosin to view general structure of the tissue (Figure 5.11). Histological sections revealed portions of the bioengineered strip in which cells are within the fibrin gel as well as undigested pieces of fibrin gel.

Discussion

Previous research techniques used to investigate vascular smooth muscle physiology, including tissue culture, cell suspensions, and tissue explants have advanced our knowledge of smooth muscle function (166; 299; 301). However, each method has its own limitations; thus limiting our ability to understand VSM function. Technological advancements in the field of tissue engineering, including the utilization of 3-D fibrin matrices, offered a novel opportunity to investigate VSM function via bioengineering 3-D VSM strips. Bioengineered tissues incorporates three dimensionality resembling tissue explants, a homogenous population of cells similar to cell suspensions, and utilizes tissue culture techniques to provide a controlled environment under physiological conditions in which tissues can survive for extended periods of time(1; 95; 126; 133; 170; 302). A fibrin matrix is well suited for bioengineering contractile tissues, and fibrin-based scaffolds have successfully been used to bioengineer gastrointestinal and vascular smooth muscle (1; 99). However, no previous studies have reported on the active contractile properties (force generation) of VSM cells bioengineered in fibrin matrices.

Using fibrin-gel casting, we have demonstrated the ability to bioengineer functional VSM strips from primary human aortic VSM cells. The “strip model design” was previously developed to facilitate measurement of contractile function of cardiac and skeletal constructs, which generate specific force close to

that of native tissues (161; 303). Utilizing a homogenous population of aortic VSM cells isolated from human donors eliminated the need to sacrifice experimental animals for these studies. In addition, using human VSM cells are ideal in terms of direct relevance to human health. We found that primary human VSM cell proliferation was greatly reduced at passage seven (Figure 5.3), therefore we used cells from passages 3-5 for all experiments.

When human VSM cells were seeded on a fibrin gel, they self-organized, forming a 3-D strip of tissue. The rate of VSM strip formation was dependent on the number of cells initially plated, as higher seeding densities led to more rapid strip formation. Once strip formation occurred, physiologic functionality (contractility) was examined. The culture dish containing the bioengineered tissue was placed on a 37°C heated aluminum platform and the bioengineered VSM strips were attached to an optical force transducer (Figure 5.2). Although the rate of VSM strip formation was dependent on the number of cells initial plated, the initial seeding density did not appear to effect force generation. There was no significant difference in the magnitude of force generated by VSM strips regardless of the initial seeding density (Figure 5.4), which was consistent with previous studies involving fibrin-based muscle constructs (1; 127). The cells appeared to proliferate within the fibrin gel only until they reached confluence, regardless of the seeding density. We measured total RNA content of VSM strips with initial seeding densities of $1x10^4$ and $5x10^5$ cells/construct at 48 hours and 2 weeks (Figure 5.10). Although constructs initially seeded with $1x10^4$ cells had significantly less RNA than constructs initially seeded with $5x10^5$ cells at 48

hours, there was no significant difference between the two seeding densities at 2 weeks. This suggests that density-dependent contact inhibition of cell division had occurred during the formation of the VSM strips. Alternatively, the fibrin-based scaffold may only support the metabolic activity for a limited number of cells.

Bioengineered VSM strips exhibited physiological behavior functionally similar to VSM in vivo upon mechanical and agonist-induced stimulation. Mechanical stimulation of bioengineered VSM strips was induced using the slack test, which was developed for VSM explants (166; 300). Briefly, VSM strip length was rapidly decreased enough to drop the force to zero. As the VSM strip was shortened by decreasing the applied tension, force redeveloped, thus demonstrating a myogenic response (Figure 5.8).

Agonist-induced stimulation showed that of bioengineered VSM strips were functionally similar to VSM in vivo. Previously, Morgan and colleagues demonstrated that explanted VSM strips stimulated with 10μM PE exhibited a contractile response. Our results were consistent with Morgan's findings, as bioengineered VSM strips stimulated with 10μM PE exhibited contraction (force generation of 41.01±4.6μN) at one week in culture. Induced contractions were specific to addition of PE, as addition of PBS did not induce contraction of bioengineered VSM strips (Figure 5.6). The kinetics of PE-induced contraction of bioengineered VSM strips resembled the kinetics of PE-induced contraction in VSM explants (166), and peak contractile force from PE-induced stimulation was seen approximately 50-60 seconds after the addition of the drug (Figure 5.6).

Addition of 66mM KCl to VSM strips induced relaxation (-65.8±16.9μN) from the basal tone and maximal relaxation occurred approximately 50-60 seconds after addition of the drug. KCl-induced relaxation of VSM strips was consistently followed by sustained contraction for 4-5 minutes (Figure 5.7), which was also physiologically similar to VSM in vivo (166).

Bioengineered VSM strips demonstrated the ability to contract and relax within seconds, similar to the capacity seen in vivo. PE-induced contraction did not inhibit KCl-induced relaxation or vice versa, as bioengineered VSM strips demonstrated an immediate shift from contraction to relaxation or the reverse upon addition of the appropriate drug. Further, agonist-induced contraction of bioengineered VSM strips was Ca^{2+}-dependent, as in the absence of Ca^{2+} (0 Ca^{2+}/2mM EGTA media) addition of PE or KCl did not induce a contractile response (Figures 5.7 and 5.9). However, KCl-induced relaxation of VSM strips was independent of Ca^{2+}, as VSM strips stimulated with 66mM KCl in the absence of Ca^{2+} (0 Ca^{2+}/2mM EGTA media) did exhibit relaxation (-31.0 ±3.2μN) from the basal tone (Figure 5.7). Therefore, bioengineered VSM strips exhibited several physiologically functional behaviors similar to that seen in vivo.

These studies demonstrate the feasibility of using primary human VSM cells to organize into contractile 3-D contractile tissues and provide evidence of physiological functional bioengineered VSM tissues. Our bioengineered VSM strips remained viable for at least 35 days in culture (Figure 5.5). Further, bioengineered VSM strips are highly reproducible and will offer an effective method for investigation of smooth muscle function because it combines the

major advantages of previously described research methods into one technique. This bioengineered model could be used by other investigators for various applications, including its utility as a tool for basic research to investigate the cellular/molecular mechanisms underlying contraction and relaxation of VSM. Incorporation of tissue engineering components with cellular/molecular based projects has become a powerful approach for basic research. Thus, this research may contribute to a more complete understanding of the molecular events controlling vascular tone and could lead to the development of new targets for therapeutic intervention for treating cardiovascular disease.

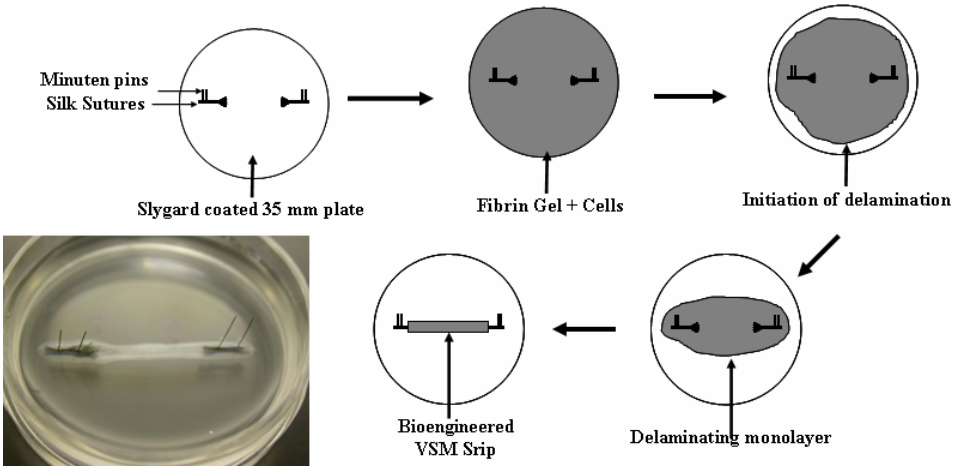


Figure 5.1 Methodology for the formation of bioengineered VSM strip. Tissue culture plates were coated with PDMS and anchor points were engineered by pinning silk sutures 12 mm apart in the center of the culture plate with four stainless steel pins. The plate was sterilized and then a thrombin/fibrin solution was mixed in the culture plate which led to polymerization. Following gel polymerization, primary human VSM cells were plated on the surface of the fibrin gel. Cell expansion and increased cell-cell contact resulted in delamination of the cell monolayer, which was initiated at the periphery of the culture plate and progressed towards the center. The delaminating cell monolayer remained attached to anchor points that were placed in the culture dish. Bioengineered VSM strips were deemed ready for functional testing when the monolayer formed a “strip” of bioengineered tissue between the two anchor points.

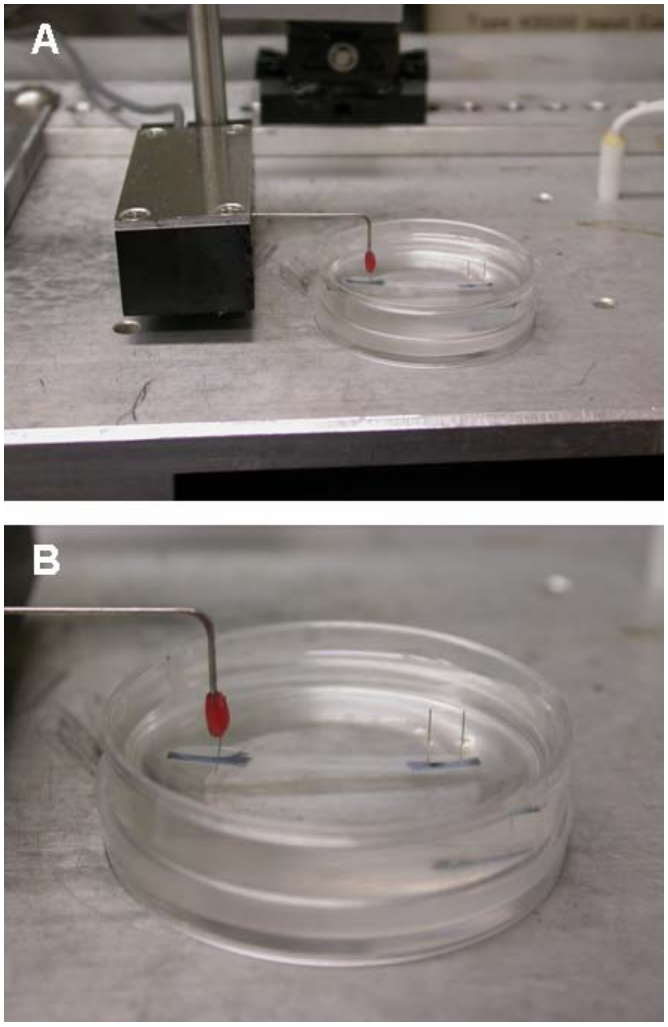


Figure 5.2 Evaluation of Contractile Properties of VSM strips.
Photograph B is higher magnification of photograph A. For contractility measurements, the culture dish containing the bioengineered tissue was placed on a heated (37°C) aluminum platform. One end of the bioengineered VSM strip remained anchored to the culture plate with two minuten pins (right side of bioengineered tissue in photographs A and B). In order to attach the bioengineered VSM strip to the force transducer, the pins from one end of the muscle strip (left side of VSM strip in photographs) were detached from the SYLGARD substrate. One pin was discarded and the other pin was attached to the optical force transducer (resolution of 1.4 μN and a range of 2 mN) with canning wax.

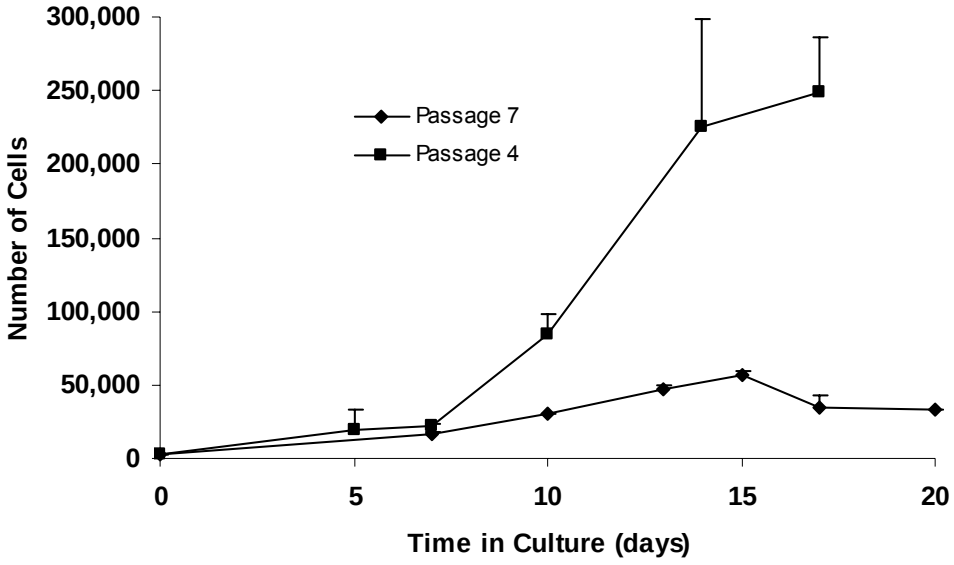


Figure 5.3 Proliferation of VSM cells
The proliferative capacity of primary VSM cells was evaluated at passage 4 and passage 7. Each time point represents the average number of cells counted from each well (n=3), and data are presented as means ± SE.

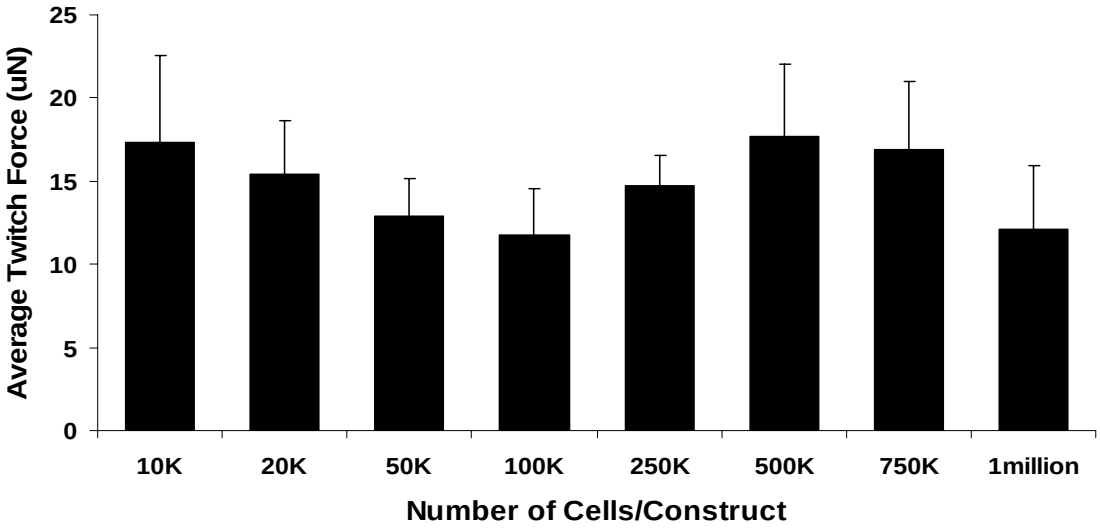


Figure 5.4 Evaluation of cell seeding density on the contractile response of VSM

Constructs were plated using varying cell densities suspended in a fibrin gel. We utilized cell densities that ranged from $1x10^4$ – $1x10^6$ cells per construct. PE-stimulation was used to evaluate active contractile force generation. There were no significant differences in force generated between any of the seeding densities tested.

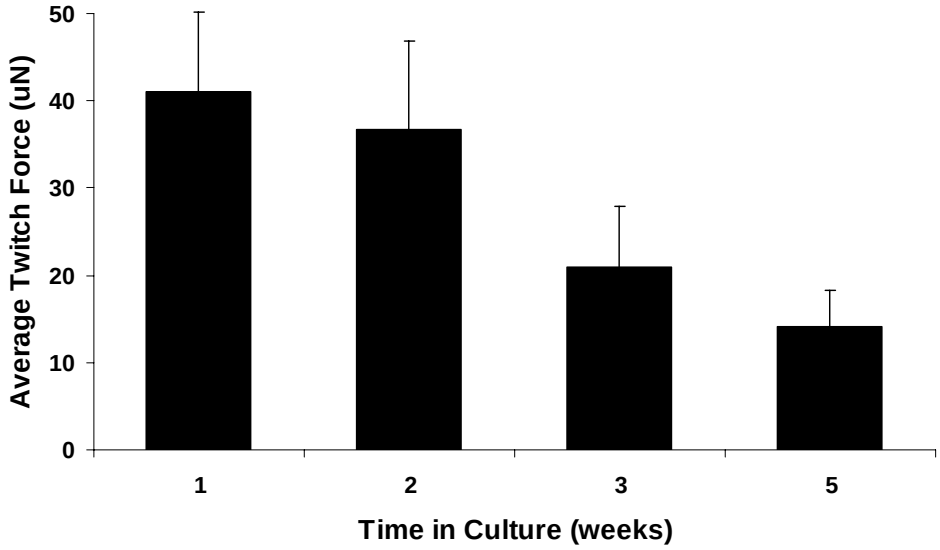


Figure 5.5 Longevity Studies.
We evaluated the stimulated active force of VSM strips over time in culture. All constructs were seeded with 500K cells/plate and force was measured at 1, 2, 3, and 5 weeks. Constructs generated the greatest force at 1 and 2 weeks in culture, 41.01±4.6 and 36.71±5.1μN respectively. Although constructs remained viable in response to PE for up to 5 weeks, force generation was significantly reduced at weeks 3 and 5.

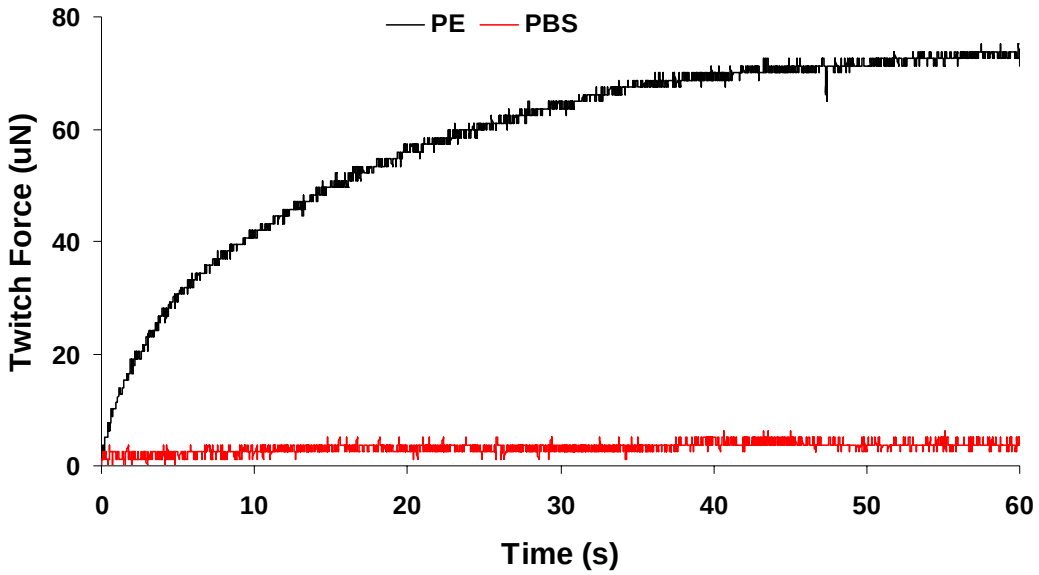


Figure 5.6 Kinetics of VSM Strips in Response to Phenylephrine
Upon 10μM stimulation with PE, measurements were collected at 100 samples/second for 60 seconds using LabView Data Acquisition software. A graph was generated using Microsoft Excel depicting the details of the kinetics of the response to PE-induced stimulation. PBS (200μl) was added to VSM strips, and served as a control.

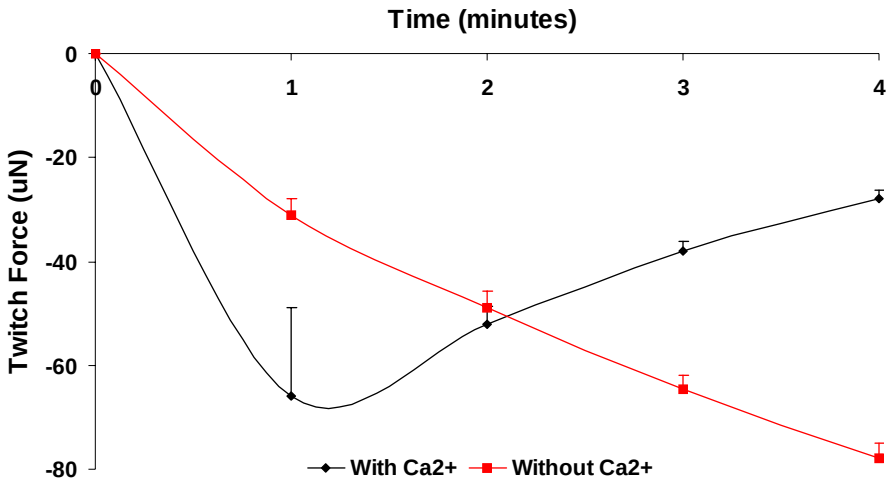


Figure 5.7 Effects of KCl-induced stimulation on VSM stirps.
Addition of 66mM KCl to constructs in growth media induced relaxation from the basal tone for 50-60 seconds followed by sustained contraction for approximately four minutes. Addition of 66mM KCl to constructs in 0 Ca^{2+}/2mM EGTA media led to sustained relaxation for approximately four minutes and no contractile response was seen. Data points represent four successive readings of changes from the basal tone of VSM strips following KCl stimulation in growth media or 0 Ca^{2+}/2mM EGTA media.

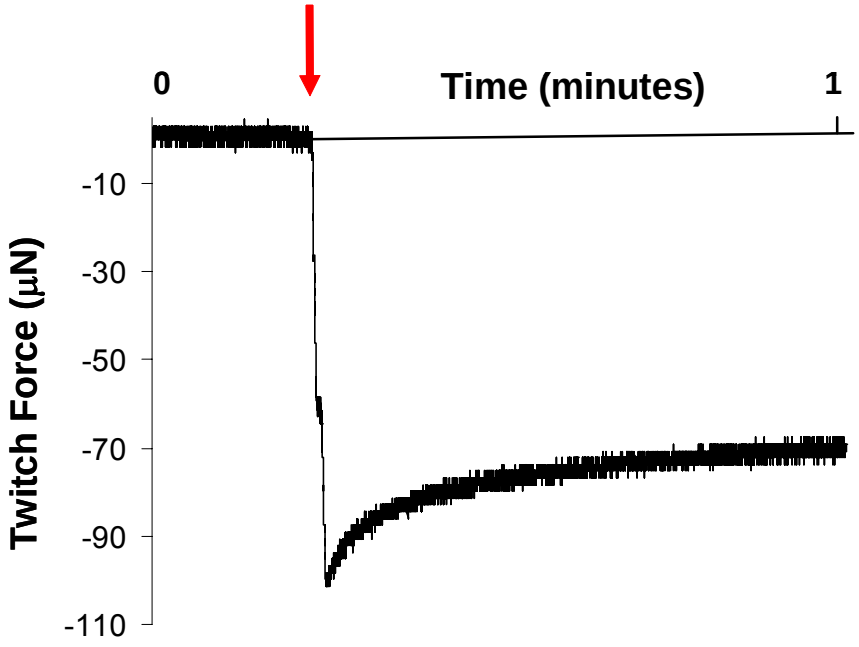


Figure 5.8 Kinetics of Myogenic Generation of Force.
Tension of VSM strips was decreased by 50µN and measurements were collected at 100 samples/second for 60 seconds using LabView Data Acquisition software. A graph was generated using Microsoft Excel depicting the details of the kinetics of the response to the decrease in tension. VSM strips consistently exhibited contraction in response to a decrease in tension.

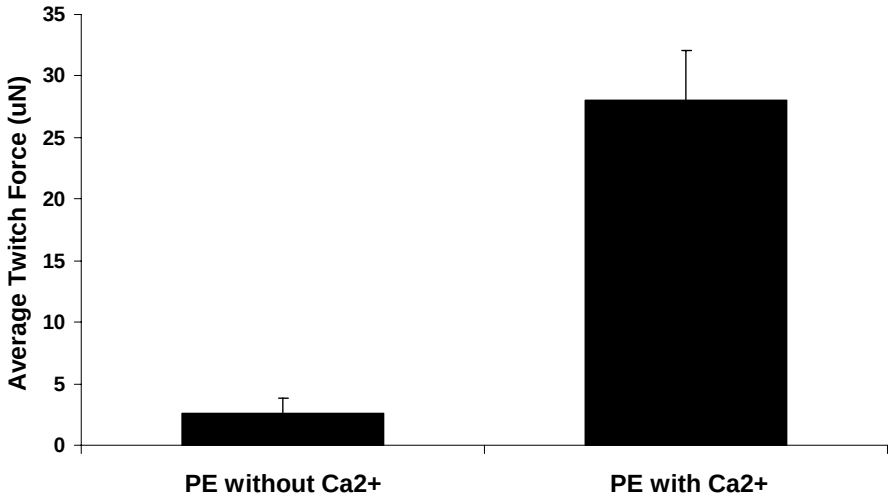


Figure 5.9 Ca2+ Dependence of Phenylephrine-Induced Contractile Response.

Pre-incubation of bioengineered VSM strips with 0 Ca^{2+}/2mM EGTA media and stimulated with 10 μM PE resulted in significantly diminished force generation (2.6 ±1.2μN) compared to force generated by constructs in the presence of Ca^{2+} (27.98 ±4.1μN). Addition of 66mM KCl induced relaxation of VSM strips from the basal tone in the presence and absence of Ca^{2+} (-65.8±16.9μN and -31.0 ±3.2μN respectively). In absence of Ca^{2+}, no sustained contraction was seen following KCl-induced stimulation. Instead, VSM strips exhibited sustained relaxation for 4minutes. Data points represent mean of force generated ±SE (n = 4-12 readings/time point)

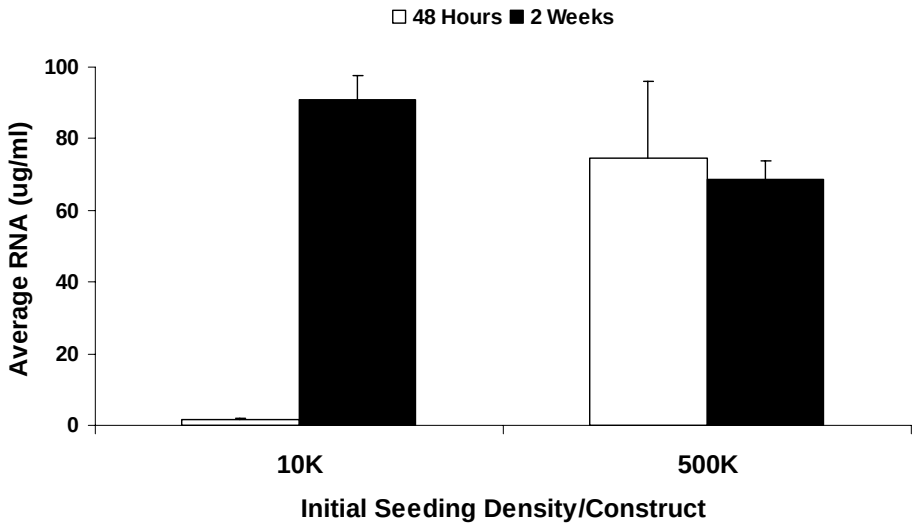


Figure 5.10 Evaluation of RNA Content in VSM Strips.
Total RNA was isolated from VSM strips with seeding densities of $1x10^4$ and $5x10^5$ cells/construct at 48 hours and 2 weeks after cell seeding. At 48 hours, constructs seeded with $1x10^4$ showed significantly lower RNA content (1.58±.27µg/ml) compared to constructs seeded with $5x10^5$ cells (74.76±21.34µg/ml). At two weeks after cell seeding, there was no significant difference in RNA content between constructs seeded with $1x10^4$ and $5x10^5$, 90.78±6.66µg/ml and 68.82±5.17µg/ml respectively. Data represent means of RNA content (µg/ml; n=3) ±SE.

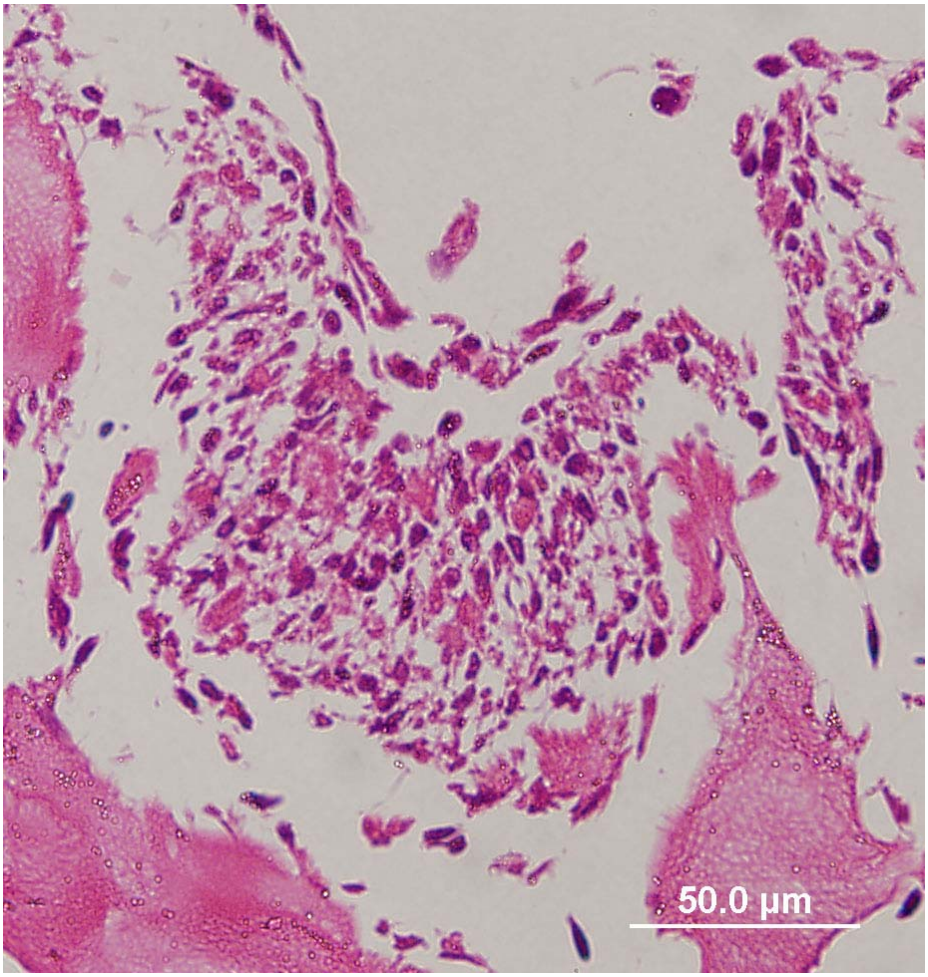


Figure 5.11 Histology of VSM strips.
Histological cross-section of VSM strip stained with Harris's hematoxylin and eosin. Photograph (600x magnification) showing a portion of the bioengineered strip in which there are cells within the fibrin gel as well as undigested pieces of fibrin gel.

Chapter 6

Development of a Micro-Perfusion System for the Culture of Bioengineered Heart Muscle

ABSTRACT

Tissue engineering strategies are being utilized to develop functional 3D heart muscle in vitro. Work within our own group has been focused on the development of bioengineered heart muscle (BEHM) utilizing fibrin gel as a support matrix. As tissue engineering models of heart muscle are developed in the laboratory, a critical technological challenge remains the ability to delivery nutrients to the entire tissue construct. In order to address this specific need, we have developed a novel perfusion system for cardiac tissue engineering applications. The system consists of a custom micro-incubator, designed to house ten 35 mm tissue culture plate on independent platforms for controlled fluid delivery and aspiration. Temperature, pH, and media flow rate and oxygenation are all regulated. In the current study, we describe the compatibility of this micro-perfusion system with BEHMs. We demonstrate that the perfusion system is capable of supporting construct viability (mitochondrial activity, total protein, total RNA) and maintaining contractile properties (twitch force, specific force, electrical pacing).

INTRODUCTION

Tissue Engineering is been defined as the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue functions (6). Tissue engineering strategies are being utilized to develop functional 3D heart muscle in vitro (6). More recent research has focused on the development of cell based cardiac pumps and tissue engineered ventricles (304): devices capable to generating intra-luminal pressure upon electrical stimulation.

Work within our own group has focused on developing 3D models of heart muscle utilizing self organization strategies (129), biodegradable hydrogels like fibrin(169) and polymeric scaffolds like chitosan (305). The overall objective has been to compare different tissue engineering platforms to support heart muscle formation. As tissue engineering models of heart muscle are developed in the laboratory, a critical technological challenge remains the ability to delivery nutrients to the entire tissue construct(306). Neovascularization of the tissue engineered construct becomes necessary to support the metabolic activity of the cells, especially within the inner core of the tissue construct(307-312).

Micro-perfusion systems are being developed to satisfy the high metabolic requirements of bioengineered heart muscle (207; 313-316). Micro-perfusion systems are generally accepted as systems capable of delivering continuous fluid flow to 3-dimensional tissue constructs to support metabolic activity of the tissue constructs as well as deliver controlled fluid stress to promote functional

remodeling of cells and extracellular matrices (317-319). The critical technological challenges in designing micro-perfusion systems are the development of micro-chambers for the culture of 3D constructs, the ability to control the temperature, pH, oxygen saturation, relative humidity and fluid flow rate and monitor/record the changes in functional outcome of tissue constructs during long term in vitro culture. In addition, the entire system needs to be run under sterile conditions with positive feedback control to change the flow variables to accommodate changes in construct phenotype.

Several perfusion systems have been described in the literature to support the formation and culture of 3D bioengineered heart muscle(320-322). However, one of the major limitations of the current systems is the inability to exhibit a high degree of control on the process variables. Current perfusion systems utilize a standard cell culture incubator to control the processing variables. Although cell culture incubators have been valuable in support 2D monolayer systems, there have not been as efficient for the culture of tissue engineering constructs. Three-dimensional constructs have significantly higher metabolic requirements, which vary based on cell/tissue type and the degree of functional development of the 3D constructs. It is unlikely the current perfusion systems could adequately satisfy the multitude of functional requirements for tissue engineered constructs.

In order to address this specific need, we have partnered with a commercial vendor (Radnoti Glass Inc, Monrovia, CA) to develop a novel perfusion system for tissue engineering applications. The system consists of a custom micro-incubator, designed to house ten 35 mm tissue culture plate on

independent platforms for controlled fluid delivery and aspiration. Cell culture media is maintained in a temperature regulated reservoir and independently delivered to every tissue culture plate via a peristaltic pump with electronic flow regulation. Temperature, pH, and media flow rate and oxygenation are all regulated. One of the main advantages of the current system is that all processing variables are user defined and controlled, thereby eliminating the need for a cell culture incubator. A second advantage of the current system is the ability to partner with a commercial vendor, thereby expediting the time required to commercialize this technology to ensure availability to all researchers.

In the current study, we describe the compatibility of this micro-perfusion system with 3D bioengineered heart muscle, formed by culturing primary cardiac myocytes in the presence of a fibrin gel. We demonstrate that the perfusion system is capable of supporting construct viability and maintaining contractile properties (twitch force, electrical pacing). We provide additional support (cell viability, total protein, total RNA) to demonstrate the compatibility of the perfusion system with bioengineered heart muscle.

METHODS

NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been observed. All materials were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Description of the Perfusion System

The system consists of a custom micro-incubator designed to accommodate 10 tissue culture plates, each plate stacked vertically on an independent stage (Figure 6.1). Cell culture media is maintained in a temperature regulated reservoir and is delivered to the tissue culture plates via a peristaltic pump. Media oxygenation is accomplished by both direct bubbling of oxygen in the media reservoir or utilization of a membrane oxygenator. Media is aspirated from each plate utilizing custom designed manifolds attached to a vacuum line. The aspirated media is recycled to the media reservoir. High relative humidity is maintained in the micro-incubator by delivering moist air from a humidification chamber. Temperature, pH, oxygen saturation and fluid flow rate are monitored are controlled and recorded.

Fluid entering the micro-incubator is divided into individual channels using luer lock connections thereby permitting independent flow to each tissue culture plate. Fluid aspiration was accomplished via custom designed manifolds. The consisted of a single harness with independent nozzles for each tissue culture plate. The diameter of the nozzle is 0.22 mm and the distance between each nozzle is 1.3 mm.

The micro-incubator consists of an independent stage for each of 10 tissue culture plates (35 mm diameter) with 3 metal supports and 2 wings on either side to position the plates. The lid of micro-incubator has been designed to accommodate ports for fluid inlet and outlet, delivery of moist air as well as ports for sensors to measure processing variables.

Isolation of Primary Cardiac Cells

Cardiac myocytes were isolated from 2-3 day old F344 rat hearts using an established method(323). Hearts were cut into fine pieces and suspended in a dissociation solution (DS) which consisted of 0.32 mg/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) and 0.6 mg/ml pancreatin dissolved in a buffer consisting of 116 mM NaCl, 20 mM HEPES, 1mM Na_2HPO_4, 5.5 mM glucose, 5.4 mM KCl and 0.8 mM $MgSO_4$. Digestion was carried out in an orbital shaker for 5 minutes at 37^{o}C, after which the supernatant was replaced with fresh DS and the digestion process was continued for an additional 30 minutes. At the end of the digestion process, the supernatant was collected in 5 ml of horse serum (Invitrogen Corporation, Auckland, New Zealand), centrifuged at 1500 rpm for 5 minutes and the cell pellet was resuspended in 5 ml horse serum. Fresh DS was added to the original, undigested tissue and the digestion process was repeated an additional 2-3 times. Cells from all the digests were pooled, centrifuged and then suspended in culture medium (CM) consisting of 320 ml M199, 100 ml F12k, 50 ml fetal bovine serum, 25 ml horse serum, 5 ml antibiotic-antimycotic (Invitrogen Corporation, Auckland, New Zealand), hydrocortisone 40 ng/ml and insulin 100 ng/ml.

Fabrication of Bioengineered Heart Muscle

The detailed method for preparing the culture surface for engineering skeletal muscle has been described in detail [21, 22]. This procedure was

modified to engineer cardiac muscle [23]. Briefly, 35 mm culture plates were coated with 1.5 ml of a polydimethylsiloxane (PDMS) elastomer (Dow Chemical Corporation, Midland, MI). An elbow connector was attached to one end of the 35 mm plate to accommodate fluid delivery (Figure 6.2A). Anchor points were 6 mm long segments of size 0 braided silk sutures (Ethicon, Cornelia, GA) pinned 12 mm apart in the center of the culture surface (Figure 6.2B). Five hundred micro-liters CM containing 10 U/ml thrombin were plated on the surface of 35 mm cell culture plates coated with SYLGARD (polydimethylsiloxane - PDMS, type 184 silicone elastomer). After this, 200 µl of 20 mg/ml fibrinogen was added to the 35 mm plate. The solution was mixed to promote the formation of a fibrin gel within 10-15 minutes in a 37°C incubator. Primary cardiac cells were diluted in CM and plated at 1 million cells in 2 ml of CM/35 mm plate after complete gel formation (Figure 6.2C). Gel compaction resulted in the formation of bioengineered heart muscle (BEHM) approximately 6 days after initial cell plating (Figure 6.2D). At the time of formation, BEHMs were positioned directly in front of the elbow connector (Figure 6.2E, F). The cells were cultured in an incubator at 37^{o}C and 5% CO_2 with medium changes every second day.

Media Delivery and Aspiration to BEHMs

In order to accommodate BEHMs within the micro-incubator, the 35 mm tissue culture plate was transferred from the cell culture incubator. This eliminated any need to physically handle the BEHM thereby reducing the likely of any damage to the construct. Individual 35 mm plates were placed on

independent platforms within the micro-incubator. Fluid channels for media delivery and media aspiration were positioned for each plate (Figure 6.3). Media delivery was accomplished by utilization of a peristaltic pump which delivered oxygenated media to the micro-incubator (Figure 6.3A). Prior to entering the micro-incubator, fluid flow was divided into independent channels for delivery to each tissue culture plate utilizing multiple leur locks connectors in series. Media was independently delivered to each BEHM via the elbow connector attached to each 35 mm tissue culture plate. The media flow rate was set at 10 ml/min. For media aspiration, we utilized a custom designed harness which consisted of a single manifold with independent nozzles leading to each tissue culture plate (Figure 6.3B). The output of the harness was attached to vacuum for media aspiration. Aspirated media was recycled to the media reservoir and re-circulated through the perfusion system.

Placement Bioengineered Heart Muscle within the Micro-Incubator

The micro-incubator was designed with independent stages to accommodate 35 mm tissue culture plates, surrounded by a water-jacketed reservoir for temperature regulation (Figure 6.4A). In order to accommodate placement of tissue culture plates, the micro-incubator was transferred to a cell culture incubator. Once the plates were securely positioned within the micro-incubator they were sealed and returned to the perfusion system (Figure 6.4B). Media delivery to each plate was accomplished via an elbow connector while media aspiration was accomplished via individual nozzles (Figure 6.4C).

Running the Perfusion System

In order to sterilize the system, 70% ethanol was perfused through all components at a flow rate of 50 ml/min for 30 minutes. The system was than perfused three times with DPBS for 30 minutes each to ensure complete removal of the ethanol. After sterilization and washing, cell culture media was transferred to the media reservoir via a fluid injection port using 60 ml syringes. This mechanism of media delivery ensured that the fluid reservoir remained sealed thereby assuring sterility. The media was perfused for one hour prior to introduction of BEHMs to ensure stabilization of system variables (temperature, oxygen saturation, and pH).

After stabilization of the system variables, 35 mm cell culture plates with BEHMs were placed in the micro-incubator. In order to transfer the plates, the micro-incubator was separated from the perfusion system and placed in a cell culture hood. The plates were positioned on the independent stages and secured in place with the wings. Individual ports for media delivery were attached to each tissue culture plate and the outflow manifold was positioned. The micro-incubator was sealed and returned to the perfusion system. Once the culture plates were secured in the micro-incubator, it was sealed and returned to the perfusion system, limiting any risk of contamination. The entire process for the placement of the tissue culture plates took 10-15 minutes. During this time, media perfusion was continued, however, completely bypassing the micro-incubator.

Once the BEHMs were secured positioned in the micro-incubator, media flow to the 35 mm tissue culture plates was initiated. BEHMs were maintained in the perfusion system for one hour. After the one hour run, the BEHMs were recovered and assessed for contractile activity, cell viability, total protein and total RNA and expression of myosin heavy chain α/β, SERCA2 and phospholamban. As controls, we utilized BEHMs that were maintained in a standard cell culture incubator for the same duration of time. The controls were maintained in 35 mm tissue culture plates which were modified to accommodate perfusion.

Sterility Test

The cell culture media was tested for contamination after every perfusion run by recovering spend media at the end of the run and culturing in a cell culture incubator for 7 days. The media was microscopically evaluated for any contamination during the 7 day culture period.

Cell Viability

To examine the cell viability of BEHMs cultured in the perfusion system as well as under standard culture conditions, we used a WST-1 Assay protocol (Roche Applied Science, Indianapolis, IN). Briefly, 100µl of WST-1 solution was added to culture dishes containing BEHMS and 2ml of fresh media. After an overnight incubation, absorbance was read on a standard plate reader at 485nm

and compared to a standard curve. The incubation time was optimized in prior pilot studies. Three plates were utilized for the perfusion run and three plates as controls. A total of 3 absorbance readings were acquired for each plate.

Evaluation of Contractility

The method for evaluating the contractility of tissue engineered constructs has been described in detail(126; 170). Briefly, the BEHM was placed in CM at 37^{o}C between parallel platinum electrodes. One end was fixed to the plate and the other end was attached to a custom-built optical force transducer. Stimulated active force (response to a single electrical impulse) measurements were then recorded at 5 volts, with a frequency of 1 Hz and a 10 ms pulse width. The length of each BEHM was adjusted to obtain maximum stimulated active force using a multi-axis micromanipulator. This optimal length was designated as L_o and was recorded. The cross-sectional area of the BEHMs was calculated from the construct diameter which was determined by a calibrated eyepiece reticle with a resolution of 5 μm. The construct was assumed to be cylindrical for this calculation. The specific force (kN/m^2) of each BEHM was determined by normalizing the stimulated twitch force to the total cross-sectional area. The BEHM was electrically paced at frequencies between 1 and 5 Hz, with all other stimulation parameters remaining constant. The contractile properties of 10 BEHMs was evaluated; 5 after perfusion and 5 controls.

Total Protein

A total of 6 plates were analyzed for the total protein content, 3 after a 1-hour perfusion system culturing and 3 after standard culturing conditions. To extract total protein from each plate, we used M-PER® Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with Halt™ Protease Inhibitor Cocktail Kit (Pierce, Rockford, IL), following the manufacturer's instructions. The media in all tissue culture plates was aspirated and plates were washed with DPBS. Briefly, the Halt™ Protease Inhibitor Cocktail and supplied EDTA Solution was added at 10 µl/ml to the M-PER® Mammalian Protein Extraction Reagent. Next, 400 µl of the solution was added to each culture dish and allowed to gently rock for 10 minutes. Each lysate was then collected separately and centrifuged down for 10 minutes at 14,000 x g. The supernatant was collected and analyzed for protein concentration using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA).

RNA Isolation

RNA isolation was performed using the Qiagen RNeasy Mini kit (Qiagen Inc., Valencia, CA). Briefly, the cell monolayers were disrupted with the addition of 350µl of Buffer RLT (RNeasy Lysis Buffer), collected into a microcentrifuge tube, homogenized using a hand-held rotor homogenizer, and centrifuged at 300 x g for 5 minutes. The supernatant was discarded and 70% ethanol was added to each sample which was then loaded into RNeasy mini columns and subjected

to various washes, according to manufacturer's protocol. The total RNA was eluted with 30µl of RNase/DNase-free water.

After RNA isolation, each sample was analyzed for nucleic acid purity and concentration of nucleic acid using the A260 and A260/A280 ratio on a spectrophotomer. The samples were then stored at -20°C until further processing.

A total of 6 tissue culture plates were utilized for RNA extraction, with 3 plates each for perfusion and controls. Every plate was treated independently.

Statistical Analysis

We used one way ANOVAs combined with the Tukey's test for all pair-wise comparisons. Minitab V13.31 (State College, PA) was used for statistical analysis.

RESULTS

Usability of the Perfusion System

The perfusion system consisted of several components, some of which were custom fabricated for this specific application. Initial prototyping, assembly and validation required a significant investment of time. Multiple configurations were often evaluated and repeated prototyping was required. Placement of the sensors required the design of custom holders, configured to accommodate the specific dimensions of the probes and were water-jacketed for temperature

regulation. However, after initial design, prototyping and validation, the system was fairly easy to use. Individual components (except the micro-incubator) were not separated from the system; sterilization, washing and transfer of media were all possible without physical removal of any component.

Fabrication of BEHMs

Our methodology for the fabrication of BEHMs has been previously described (reference). In order to accommodate placement in the micro-incubator, the tissue culture plates had to be fitted with an elbow connector at one end; the lids did not completely cover the plates as a result. However, there were no signs of media contamination resulting from this. The entire process of BEHM formation was fully reproducible. Spontaneous contractions of the primary cardiac myocytes were visible within 24 hours after plating. A cohesive cell monolayer was seen to be contracting in synchrony with 48 hours after plating and BEHM formation was complete within 6 days after initial cell plating.

Placement of BEHMs within Micro-Incubator

In order to transfer the BEHMs to the perfusion system, the micro-incubator was placed within a cell culture hood. The 35 mm tissue culture plates in which the BEHMs had formed were then placed on individual stages within the micro-incubator. Channels for fluid delivery and aspiration were then positioned. During this process, there was no physical handling of the BEHMs as the entire tissue culture plate was transferred to the micro-incubator. There was no

physical damage to any constructs during the physical transfer to the micro-incubator. Since transfer of the BEHMs to the micro-incubator occurred within a cell culture incubator, there were no signs of media contamination during perfusion. This was validated by culturing spent media in a cell culture hood for 7 days.

Stabilization of System Variables

Temperature was regulated throughout the perfusion system by circulating hot water from a thermo-circulator through the water-jackets of all perfusion components. Maintaining a temperature of 38.1^{o}C in the thermo-circulator was sufficient to maintain a media temperature of 37.4^{o}C. Temperature stabilization required approximately 30 minutes. Media pH was maintained by adding HEPES buffer at a concentration of 25 mM and the pH was maintained at 7.4 for the duration of the perfusion run. In addition to regulating temperature and pH, the oxygen saturation of the media was maintained utilizing a membrane oxygenator. Temperature and pH values were digitally recorded while oxygen saturation was not.

Media Delivery and Aspiration

In order to accomplish media delivery to the BEHMs within the micro-incubator, the flow was divided into independent channels prior to entering the micro-incubator. This ensured independent media delivery to each BEHM. Media aspiration was accomplished via individual nozzles attached to a single

harness leading out of the micro-incubator. Although multiple configurations were evaluated for media delivery and aspiration (e.g. individual ports for media aspiration), we found this configuration to be most suitable, based on the amount of tubing required, the overall usability and the ability to achieve uniform fluid delivery/aspiration. Media flow was perpendicular to the BEHMs. However, this did not result in any physical damage to the BEHMs resulting from fluid stress. There were no incidences of physical damage to the BEHMs resulting from media flow in the micro-incubator.

Ability of BEHM to Tolerance Fluid Flow

Upon recovery of the BEHM after perfusion, we visually evaluated the overall integrity of the constructs. There were no signs of separation of the tissue mass from the sutures or any signs of physical movement of the sutures. We did not observe any breakage of the BEHM at any point along the tissue construct. Under microscopic evaluation, we observed spontaneous contractions of the BEHMs after perfusion. The frequency of the spontaneous contractions was in the range of 1-2 Hz, similar to the controls. Overall, the BEHMs were able to tolerate the fluid flow very well with no visible signs of physical damage to the tissue constructs.

Cell Viability, Total Protein and Total RNA

The cell viability, evaluated utilizing the WST assay (which measures mitochondrial activity) was maintained during BEHM perfusion. The average absorbance at 580 nm was found to be 0.863 ± 0.056 for control BEHMs and 0.840 ± 0.040 for BEHMs after perfusion (Figure 6.5). We utilized N=3 for both groups. There was no significant difference between the absorbance values of the BEHMs after perfusion, therefore validating construct viability.

The total protein of control BEHMs was found to be 472.9 ± 19.3 μg/BEHM and 387.3 ± 37.9 μg/BEHM for constructs after perfusion (Figure 6.6A). The total RNA content of control BEHMs was found to be 3.84 ± 1.73 μg/BEHM and 3.25 ± 0.20 μg/BEHM for constructs after perfusion (Figure 6.6B). N=3 for both groups and there was no significant difference between the total protein and total RNA between control BEHMs and BEHMs after perfusion.

Diameter and Cross-sectional Area of BEHMs after Perfusion

We evaluated the diameter, cross-sectional area, twitch force, specific force (twitch force normalized to total cross-sectional area, and pacing characteristics of the BEHMs. As an initial indicator of contractile function, the BEHMs were subjected to single electrical impulses and found to generate twitch force (Figure 6.7). The magnitude of the twitch force for the BEHMs after perfusion was comparable to the twitch force of control BEHMs (Figure 6.7). By evaluating individual force tracings, we also found that the rate of contraction and the rate of relaxation of the BEHMs after perfusion had not changed (Figure 6.7).

The average diameter of the control BEHMs was found to be 992.9 ± 69.8 μm and 797.2 ± 46.7 μm (Figure 6.8A). The cross-sectional area of the BEHMs after perfusion was calculated to be 79.0 ± 11.8 x 10^5 μm^2 and 50.6 ± 6.0 x 10^5 μm^2 for control BEHMs (Figure 6.8B). We utilized N=5 for both groups. There was a statistically significant difference between the diameters and the cross-sectional of the BEHMs after perfusion with p=0.048 for the diameters and p=0.038 for the cross-sectional area.

Twitch Force and Specific Force of BEHMs after Perfusion

The average twitch force for control BEHMs was found to be 444.3 ± 133.85 μN and 426.1 ± 50.96 μN for BEHMs after perfusion (Figure 6.8C). N=5 for each group with p=0.90. There was no significant variation in the twitch force of the BEHMs after perfusion, when compared to control BEHMs. The degree of variability of the twitch force for control BEHMs was considerable greater than that of BEHMs after perfusion.

The average specific force of the control BEHMs was calculated to be 0.60 ± 0.17 kN/m^2 and 0.89 ± 0.14 kN/m^2 for BEHMs after perfusion. We utilized N=5 for the specific force. There was no statistical difference between the specific force between the two groups, with N=5. Although the difference in specific force was not statistically significant, evaluating the actual numbers for both groups provides useful insight. The specific force for the control BEHMs were calculated to be 0.52, 0.68, 0.38, 0.19, 1.2 kN/m^2. The specific force for the BEHMs after perfusion were calculated to be 1.2, 1.0, 0.55, 1.1, 0.56 kN/m^2.

Electrical Pacing of BEHMs

We evaluated the ability of the BEHMs to be electrically paced at frequencies of 1-4 Hz (Figure 6.9). At all the pacing frequencies we tested, the BEHMs were able to elicit repetitive contractions that correlated directly with the number of electrical impulses. In addition, the BEHMs were able to relax to baseline conditions between successive contractions, even at the higher pacing frequencies. The ability of the BEHMs to respond to electrical pacing after perfusion was similar to the response of control BEHMs.

DISCUSSION

Our previous work has focused on the development of tissue engineering platforms to support the formation of functional 3-dimensional heart muscle in vitro. We have evaluated the feasibility of utilizing self organization strategies, biodegradable hydrogels and polymeric scaffolds for cardiac tissue engineering applications. Technological advancements have permitted the formation of tissue constructs which exhibit a high degree of phenotypic resemblance to normal mammalian heart muscle. As tissue engineering models are developed, it becomes necessary to define physiological culture conditions in vitro which more closely resemble in vivo conditions. This often includes mechanical stretch, chemical stimulation with growth factors and the micro-perfusion systems. The focus of the current study has been to describe and validate a novel micro-

perfusion system that has been developed in our laboratory to support the culture of 3-dimensional tissue constructs.

The central element of the perfusion system is a micro-incubator, custom designed to accommodate culture of 35 mm tissue culture plates. Independent platforms have been fabricated to allow spatial distribution of the tissue culture plates. The micro-incubator is the only component of the perfusion system which needs to be removed to accommodate the placement of the plates. This accommodates ease of operation of the system. Physical transfer of BEHMs to the micro-incubator is accommodated by transferring the entire 35 mm tissue culture plate from the cell culture incubator to the micro-incubator. The transfer takes place under sterile conditions in a cell culture hood, which minimizes any risk of contamination to the BEHM. Therefore, with the current configuration, we were able to ensure physical transfer of the BEHMs to the perfusion system without any physical handling of the constructs thereby reducing damage and contamination.

In order to accommodate compatibility of BEHMs with the micro-incubator, we need to modify the tissue culture plates by attachment of an elbow connector to one end. Though a simple modification, our concern was the introduction of contamination, as the lids of the tissue culture plates were not completely covering the plates (due to the elbow connector). This was, however, not a problem. In the process of this study, we have fabrication over 50 BEHMs with no incidence of contamination.

We configured independent flow channels to accommodate media delivery to each tissue culture plate. Cell culture media was maintained in a water jacketed reservoir and delivered to the micro-incubator in a single channel. Prior to entering the micro-incubator, the fluid flow was divided into multiple outflows utilizing leur lock connectors in series. Although this arrangement required a significantly large amount of tubing, media easily delivered to each tissue culture plate. In our configuration, fluid flow was perpendicular to the orientation of BEHMs. Although it is likely that fluid stresses would have a significant impact on 3-dimensional tissue remodeling and that parallel versus perpendicular flow regimes would result in very different physiological outcomes, this factor was not incorporated into our current experimental design. Rather, this would need to be a focus of follow up studies. In order to accommodate media aspiration, we utilized custom fabricated manifolds. Our design was based on glass Pasteur pipettes routinely utilized in cell culture applications. The manifold consisted of multiple nozzles attached to a single harness, analogous to having multiple Pasteur pipettes in series. This system proved simple and effective for media aspiration. An alternative configuration for media aspiration would have been independent fluid channels, replicating the configuration utilized for media delivery. The only limiting factor in this alternative configuration was the excessive amount of tubing required, making is difficult to work with. This was the motivation for fabricating the manifolds, which accomplished the same task, but was significantly easier to work with.

Overall, the perfusion system was easily to utilize and very compatible with BEHMs. Fabrication of BEHMs, physical transfer to the micro-incubator and establishment of flow regimes were accomplished without any difficulty.

After validating the compatibility of the perfusion system with BEHMs, our next objective was to support culture of BEHMs within the micro-incubator. We selected a one hour time point to determine the functional performance of BEHMs in response to media perfusion. We found that there was no detachment of the BEHMs from the sutures and there were no visible signs of physical damage of the constructs in response to perfusion. Spontaneous contractions of the BEHMs were visible under examination utilizing an inverted microscope. These initial metrics severed well to demonstrate physical compatibility of the BEHM with the perfusion system. This is often a challenge as 3-dimensional tissue constructs (like BEHMs) are at any early state of development and vulnerable to physical damage in response to any external stimuli. This is even more important when working with custom components (like the micro-incubator) which have not been tested in to support the culture of tissue constructs.

In order to evaluate the functional outcome of BEHMs in response to perfusion, we evaluated cell viability, total protein and total RNA. All three metrics showed that the BEHMs retained functionality after being subjected to perfusion.

The contractile properties of the BEHMs were also evaluated. Interestingly, while we found that there was no significant difference between the twitch force of the BEHMs after perfusion, there was an increase in the specific

force. Though the increase in specific force was not statistically significant, possibly due to the small utilized in this study combined with the variability in the twitch force, there was a general trend showing this increase. Correspondingly, we observed a significant decrease in the diameter and total cross-sectional area of BEHMs in response to perfusion; this difference was even statistically significant.

The increase in specific force was therefore a result of a decrease in diameter of the BEHMs, rather than an increase in twitch force. Though speculative, this decrease in diameter could be due to decreased water retention of BEHMs. The BEHMs were fairly large at the time of perfusion, in excess of 700 μm, due to the presence of a large amount of fibrin gel. Fibrin gel degrades within 2 weeks after BEHM formation resulting in a diameter of 200-300 μm. Hydrogels, like fibrin, have high water retention. It is likely that fluid stress resulted in a decrease in water content of the BEHMs thereby resulting in decease in diameter and a subsequent increase in specific force. Though non physiological, the change in geometry of BEHMs in response to perfusion would be an interesting phenomena to evaluate. Does it indeed impact the physiological of the 3-dimensional tissue construct and can this be manipulated to enhance functional performance in response to controlled perfusion variables?

Our final performance metric was the ability of the BEHMs to respond to electrical pacing. We found that there was no change in the pacing characteristics of the BEHMs after perfusion, indicative of physiological calcium handling properties.

Collectively, the performance metrics demonstrated that BEHMs were able to sustain culture in the micro-incubator. Additional metrics, histological and biological, would be necessary to further validate the compatibility of BEHMs with the perfusion system. Follow up studies would be required to evaluate the effect of perfusion variables (e.g. media flow rate) on the functional performance of BEHMs. The ability to support long term culture of BEHMs would also be an important variable. However, the current study does provide the necessary framework to evaluate the effect of controlled perfusion variables on the functional outcome of BEHMs.

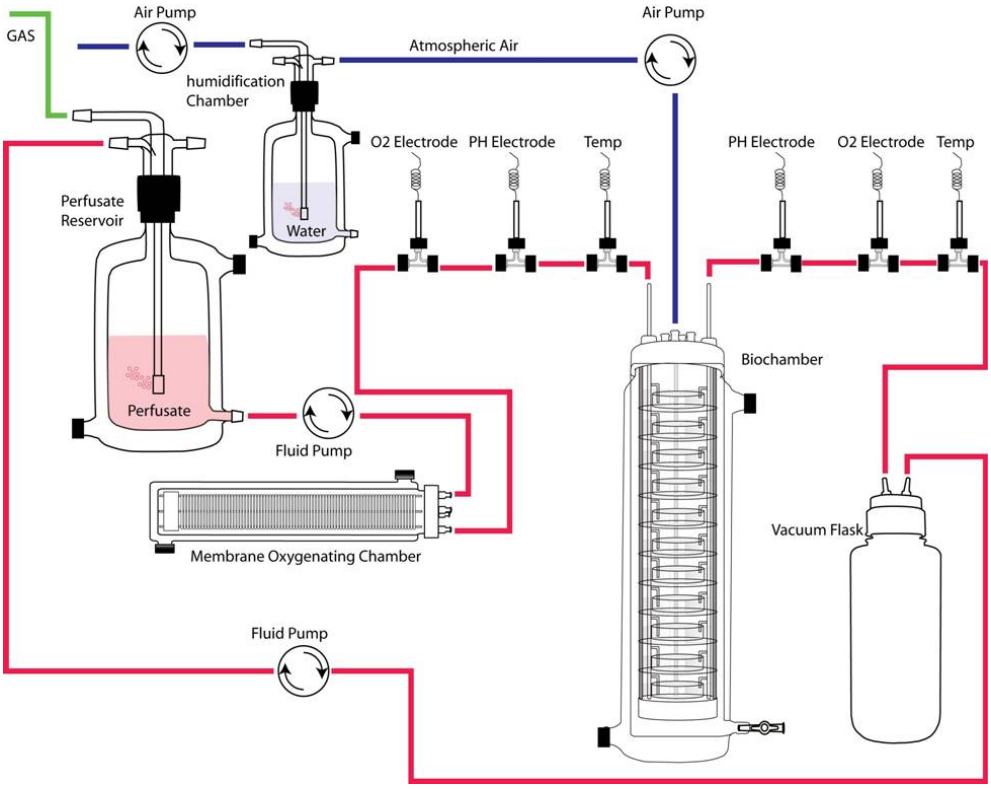


Figure 6.1 Schematic of Perfusion System
A compact design Water Jacketed for precise temperature control the bio-chamber incorporating a series of 10 shelves with wings that allow for easy placement and removal of standard 35mm disposable sample dishes. Two specialized manifolds were precision fabricated to allow simultaneous placement of the fluid flow ports to each chamber. In the initial design a single peristaltic pump drives the inflow, drawing from a water jacketed temperature controlled reservoir. A single vacuum line drives the outflow. Outflow is collected in a vacuum flask and recycled through the system. The inflow and out flow fluid manifolds are configured with inline measurement of O_2, pH and temperature. Threaded luer-lock ports at the top of the chamber in conjunction with single direction check valves allow for gas in flow and out flow without the possibility of reverse flow contamination.

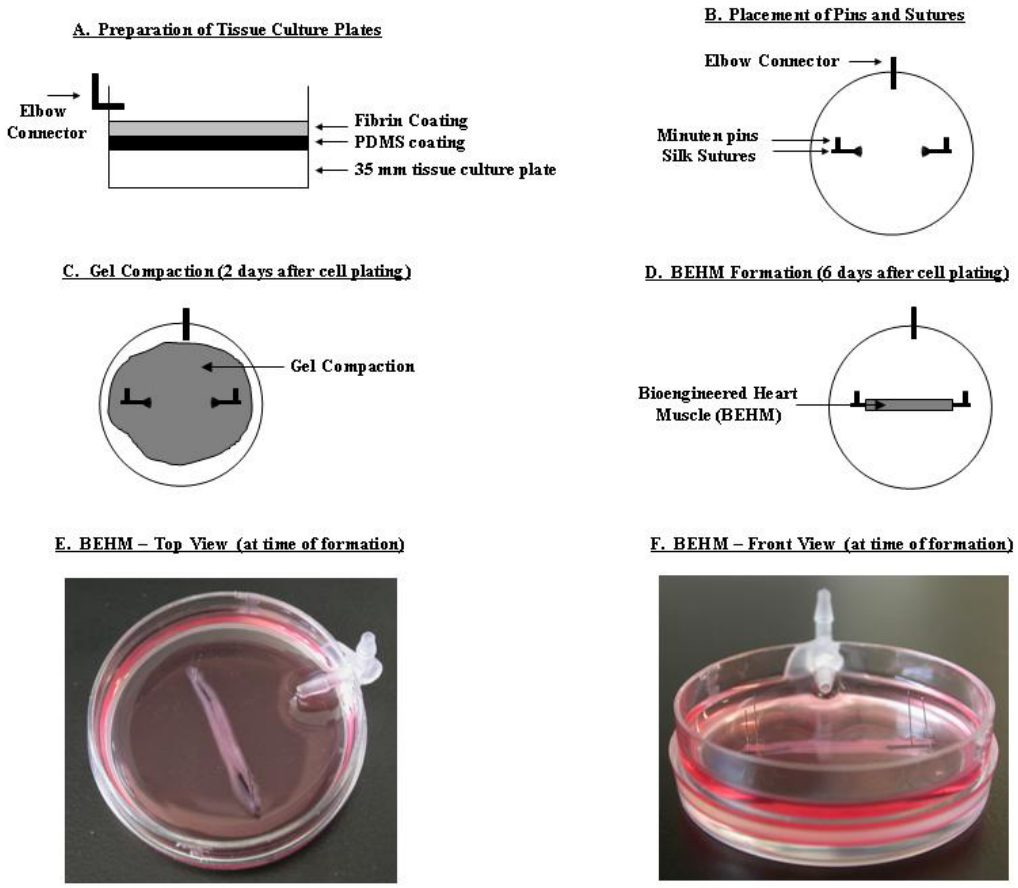


Figure 6.2 Methodology for BEHM Fabrication
(A) Preparation of the Tissue Culture Plates – 35 mm plates were coated with PDMS and then with fibrin. An oxygen probe was positioned in the center of the plate. (B) Placement of Pins and Sutures – size 0 silk sutures were placed in the center of the tissue plated and positioned with 0.1 mm diameter minuten pins. (C) Gel Compaction – primary cardiac cells were isolated from 2-3 day old neonatal rat hearts and plated on the surface of the fibrin gel ($1x10^6$ cells/plate). Within 2 days of cell plating, the cardiac cells promote the compaction of the fibrin gel. (D) BEHM Formation – Gel compaction continues, promoting BEHM formation. (E) Orientation of the elbow connector relative to the BEHM, as seen from a top view. (F) Orientation of the elbow connector relative to the BEHM, as seen from a front view.

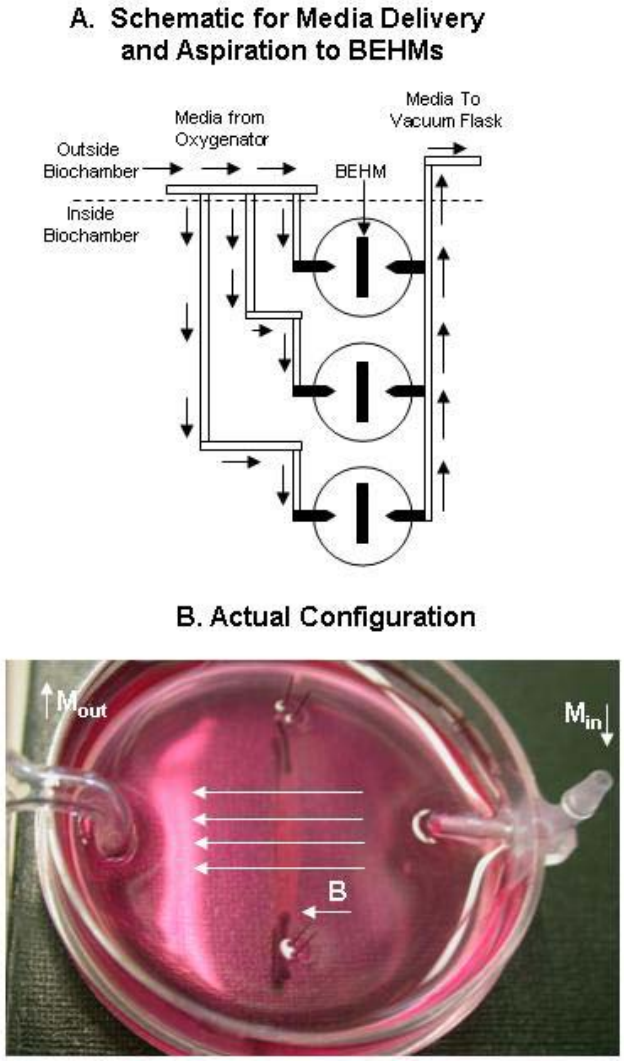


Figure 6.3 Media Delivery and Aspiration
(A) Schematic for Media Delivery and Aspiration to BEHMs - Independent fluid flow was delivered to every tissue culture plate while a single harness was utilized for fluid aspiration. Fluid flow was perpendicular to the BEHMs. (B) Actual Configuration – Image shows a single BEHMs (B) placed within a 35 mm tissue culture plate with an elbow connector for media delivery (M_{in}) and independent nozzles attached to a single outflow manifold for media aspiration (M_{out}).

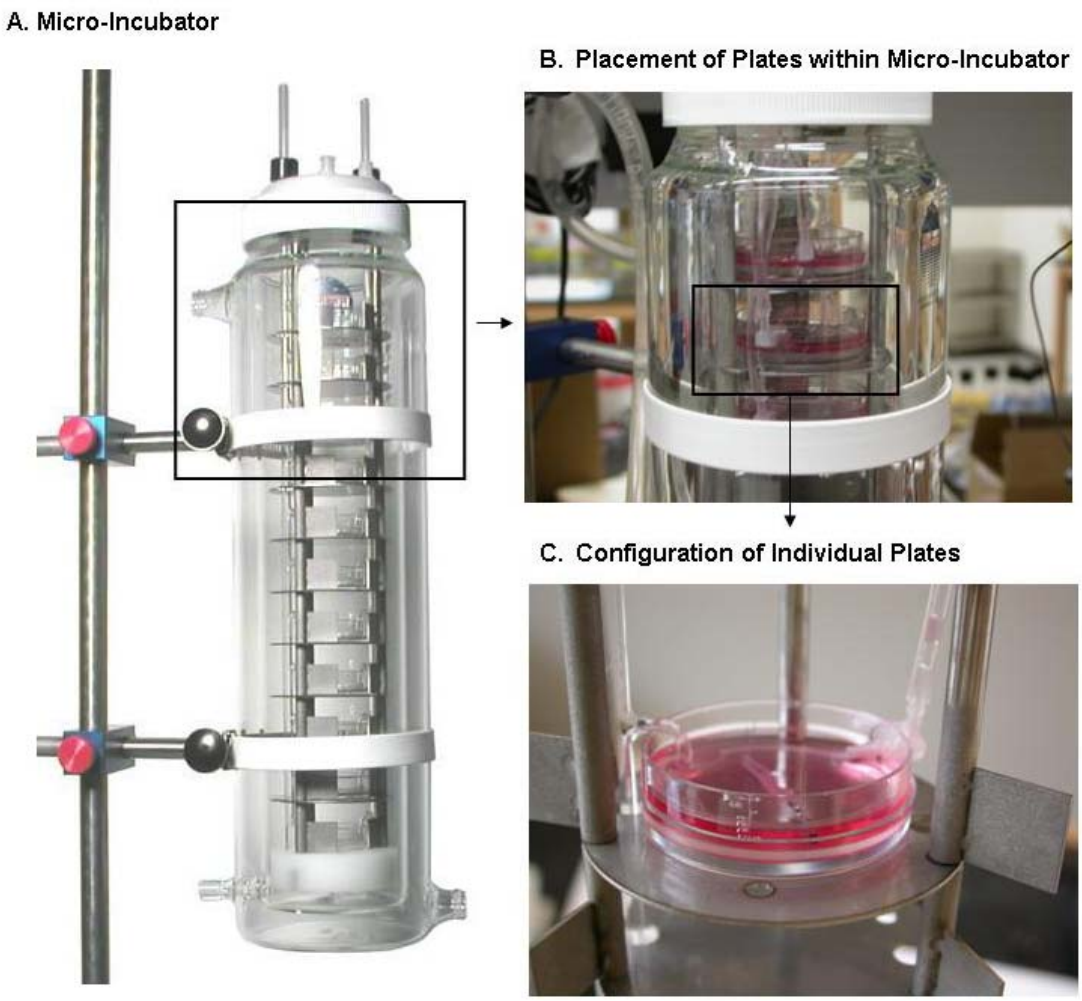


Figure 6.4 Placement of BEHMs within Micro-Incubator
(A) Micro-Incubator **-** The micro-incubator consisted of independent stages for placement of 35 mm tissue culture plates surrounded by a water jacket for temperature regulation. (B) Placement of Plates within Micro-Incubator - Tissue culture plates containing BEHMs were easily transferred to the micro-incubator, without the need to physically handle the constructs. (C) Configuration of Individual Plates – Media was delivered via an elbow connector while media aspiration was accomplished via individual nozzles.

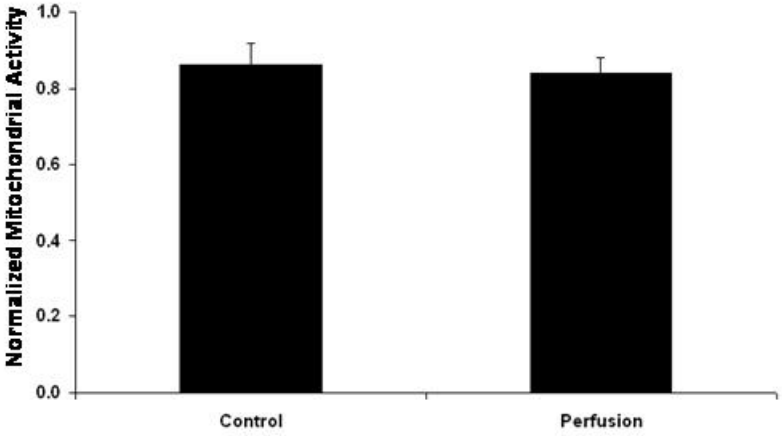


Figure 6.5 BEHM Viability
The cell viability was evaluated using the WST assay. The normalized mitochondrial activity for the controls was found to be 0.863 ± 0.056 and 0.840 ± 0.041 for BEHMs after perfusion. N=6 for each group and error bars represent standard error of mean. There was no statistical difference between the 2 groups using one-way ANOVAs.

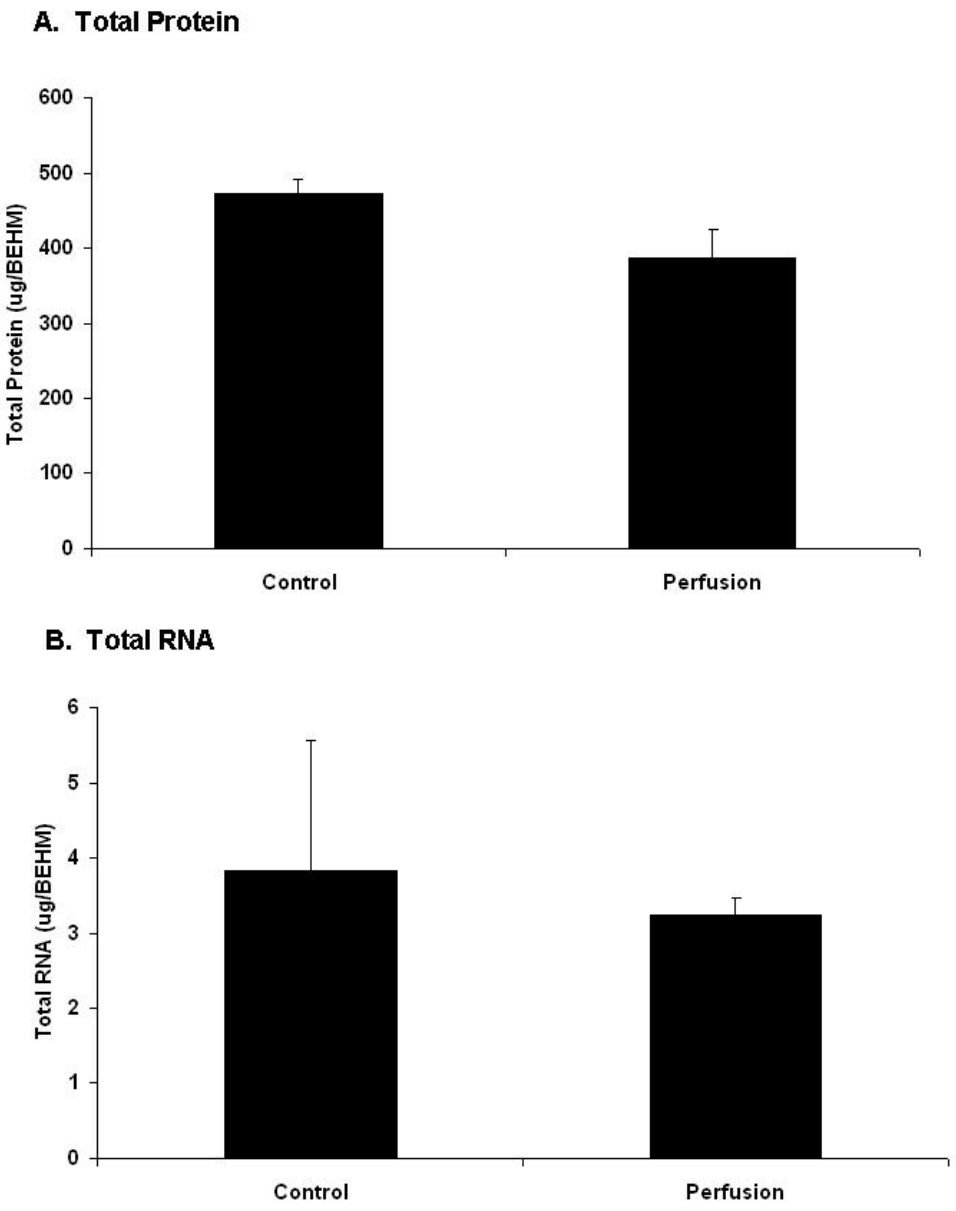


Figure 6.6 Total Protein and Total RNA
(A) Total Protein – the average protein for control BEHMs was found to be 472.9 ± 19.3 µg and 387.3 ± 37.9 µg for BEHMs after perfusion. (B) The total RNA content of control BEHMs was found to be 3.84 ± 1.73 µg and 3.25 ± 0.20 µg for BEHMs after perfusion. N=3 for both groups and the numbers refer to total protein and/or total RNA per BEHM. Error bars represent standard error of mean. There was no statistical difference between the 2 groups for total protein and total RNA. .

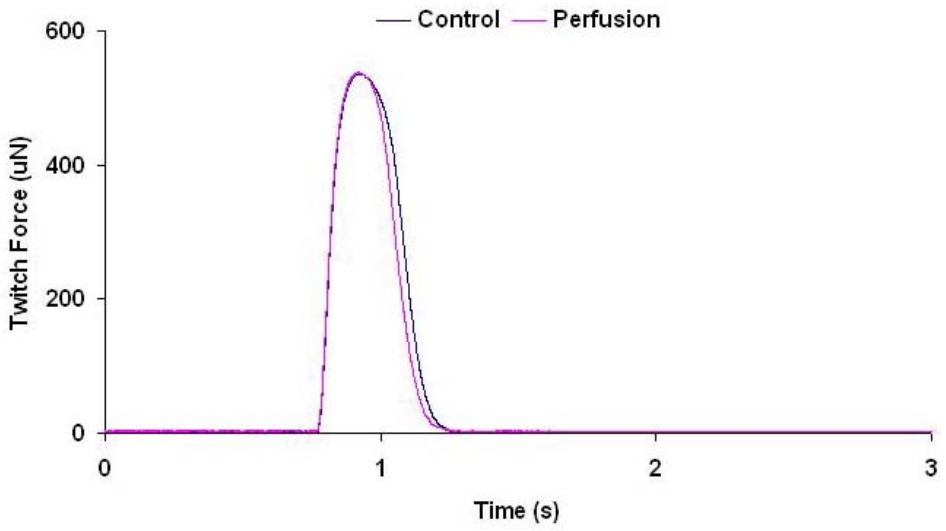


Figure 6.7 Representative Tracings of Twitch Force
BEHMs were electrical stimulated with a single impulse of magnitude 10 V, frequency 1 Hz and a pulse width of 10 ms. The twitch force was recorded using an optical force transducer. Representative tracings for control BEHMs and BEHMs after perfusion are shown.

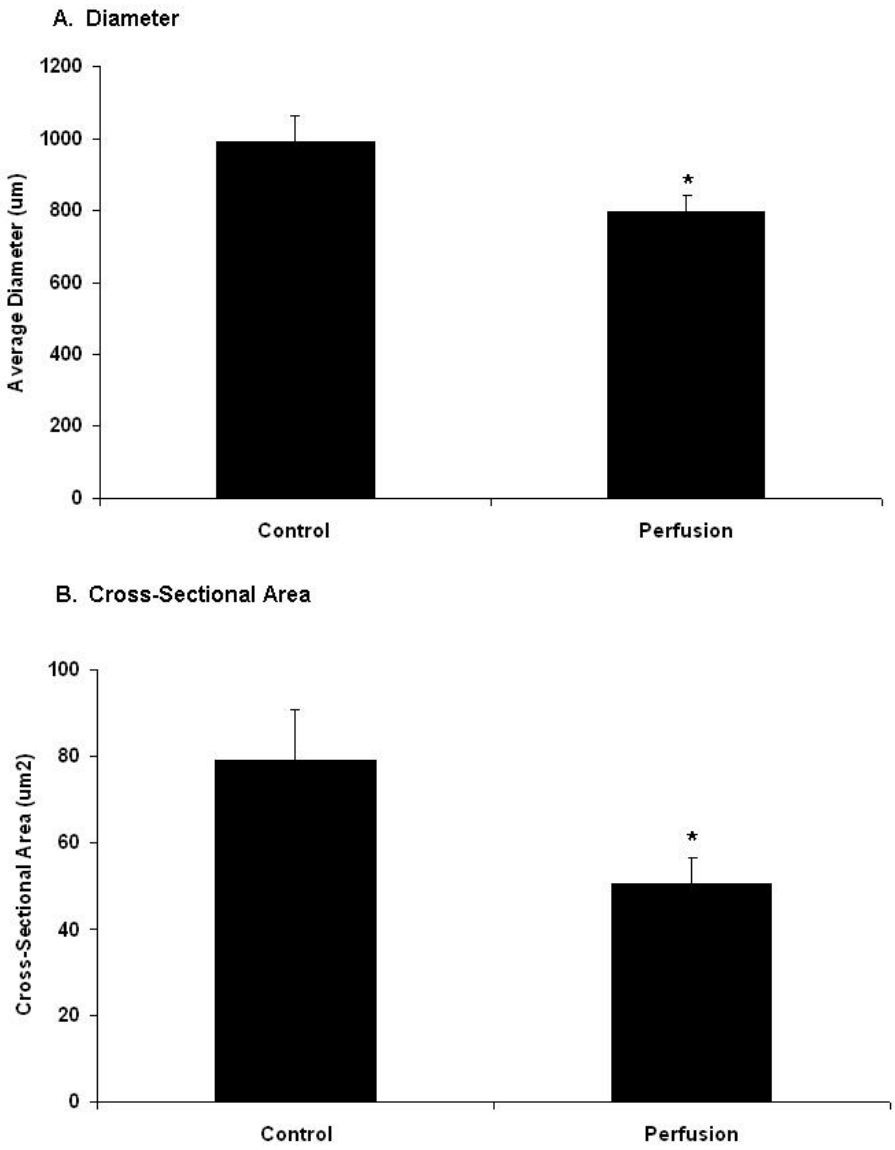
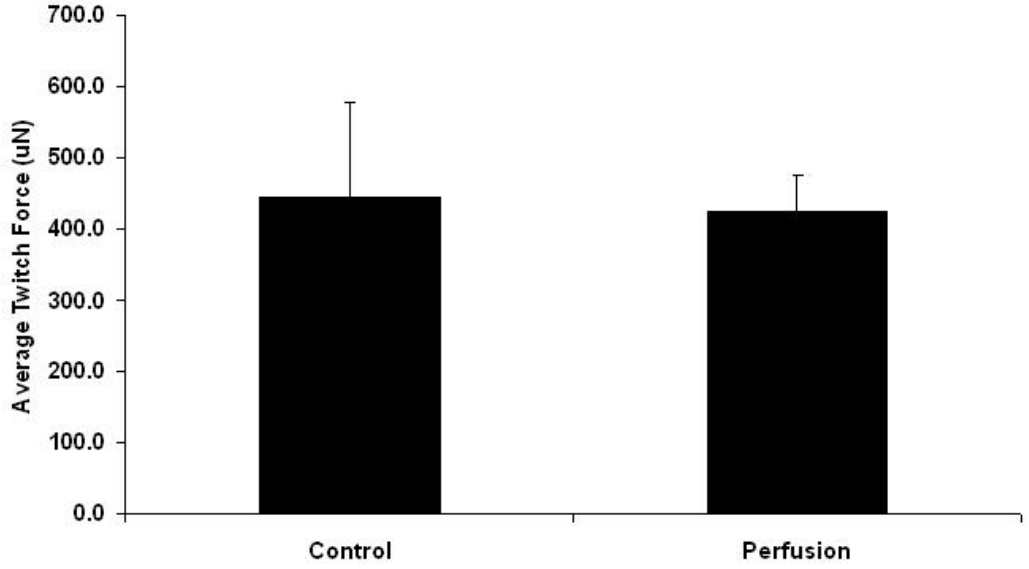
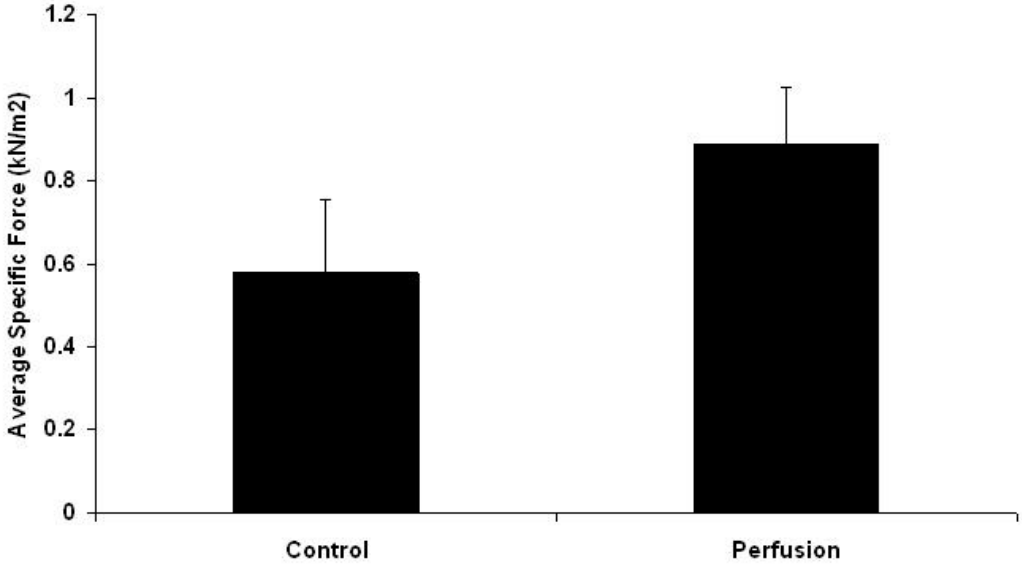


Figure 6.8 Contractile Properties of BEHMs
(A) Diameter – The average diameter of the control BEHMs was measured to be 992.92 ± 69.83 μm and 792.22 ± 46.70 μm for BEHMs after perfusion. (B) Cross-sectional area – The average cross-sectional area of BEHMs was calcuated to be 78.97 ± 11.75 $x10^5$ and 50.61 ± 6.03 $x10^5$ for the BEHMs after perfusion. (C) The average twitch force of control BEHMs was measured to be 444.3 ± 133.9 μN and 426.1 ± 51.0 μN for BEHMs after perfusion. (D) The specific force of the BEHMs was calculated to be 0.58 ± 0.18 kN/m2 and 0.89 ± 0.14 for BEHMs after perfusion. For all groups, N=6 and error bars represent standard error of mean. P=0.048 for (A) and 0.038 for (B). There was no statistical difference between the twitch force and the specific force between the control BEHMs and the BEHMs after perfusion.

C. Twitch Force



D. Specific Force



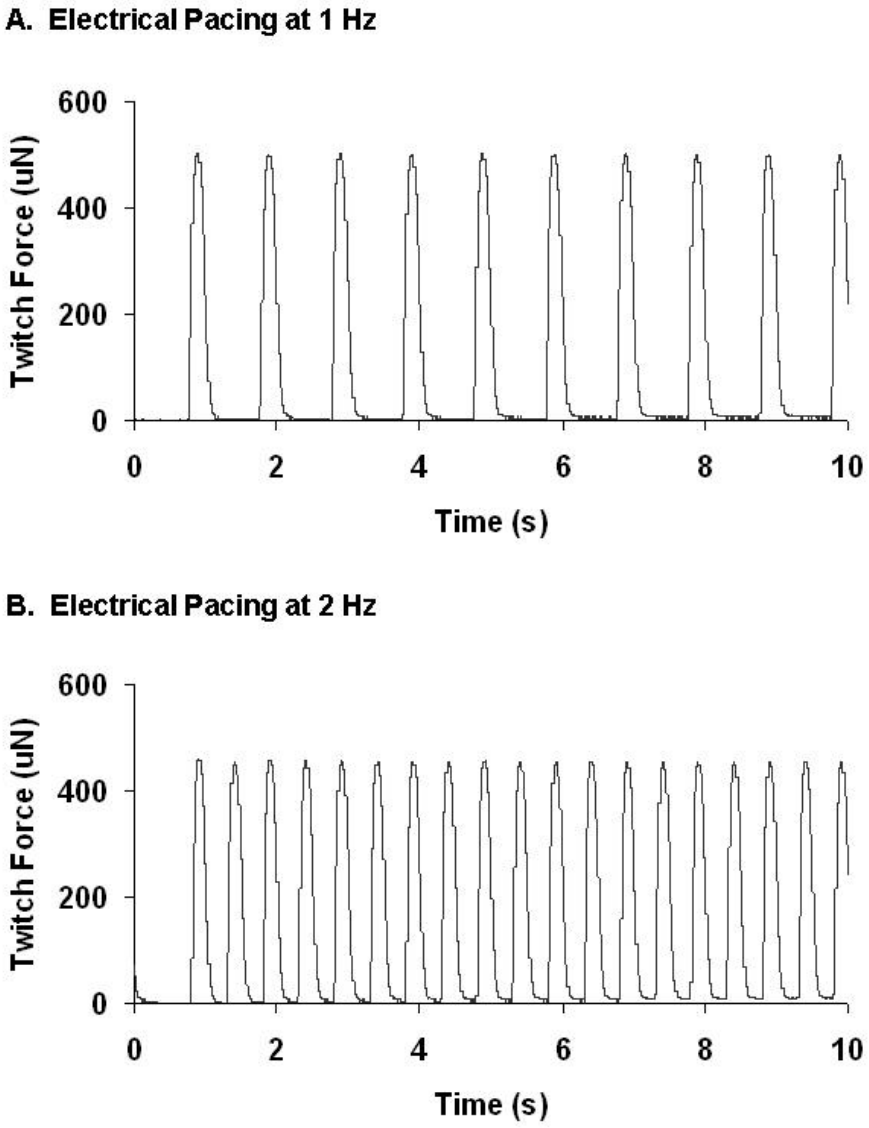
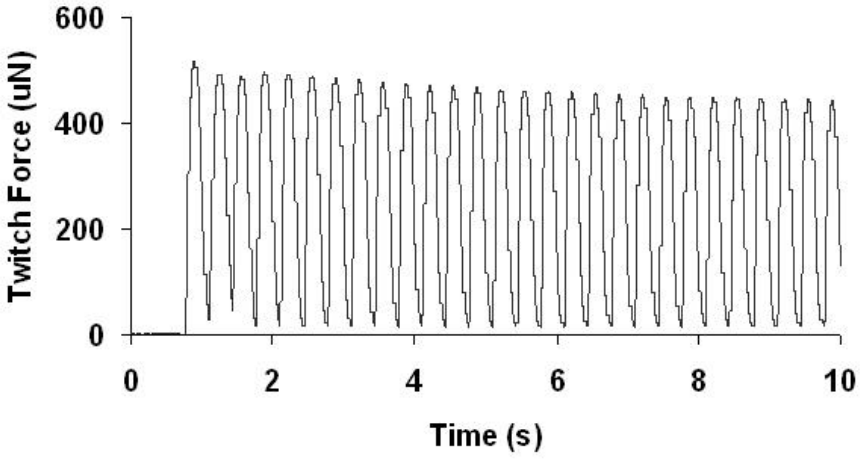
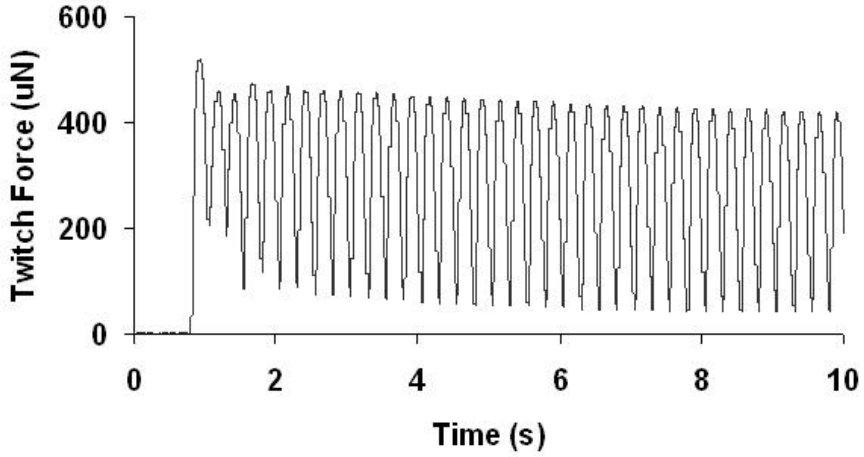


Figure 6.9 Electrical Pacing of BEHMs after Perfusion
BEHMs were electrically paced at varying frequencies utilizing a 10 V impulse with a pulse width of 10 ms. (A) 1 Hz, (B) 2 Hz, (C) 3 Hz and (D) 4 Hz.

C. Electrical Pacing at 3 Hz



D. Electrical Pacing at 4 Hz



Chapter 7

Conclusions

The work described in this thesis is divided into three broad areas; overview of cardiovascular tissue engineering, development of functional 3-dimensional models of smooth muscle, and micro-perfusion for bioengineered cardiac muscle. Collectively, the work described in this thesis bridges the scientific and technological gap in several aspects of functional tissue engineering.

OVERVIEW OF CARDIOVASCULAR TISSUE ENGINEERING

In the first part of the thesis, a critical overview of the field of cardio-vascular tissue engineering is provided. Current status of the field is provided with an emphasis on cell sourcing, biomaterial fabrication, bioengineered models of cardiac and vascular tissue, and bioreactors/micro-perfusion systems. In addition to providing a snapshot of the field, technological challenges which need to be addressed for the development clinical usable of cardio-vascular tissue have been provided.

The last decade has seen the development of several models of bioengineered cardio-vascular structures. Collectively, the body of work

demonstrates the feasibility of culturing primary cells derived from cardio-vascular sources within 3-dimensional matrices to form functional tissue engineered models. Several critical technological challenges remain, particularly in the fabrication of contractile models of vascular tissue and the development of novel micro-perfusion systems to support the long term culture of tissue engineered constructs. The work described in this thesis addresses both of these challenges.

BIOENGINEERED MODELS OF SMOOTH MUSCLE

The second part of this thesis is focused on the development of bioengineered models of smooth muscle cells, both from gastrointestinal (GI) and vascular tissue. The field of GI physiology has relied on the utilization of traditional models like 2-dimensional monolayer for basic research, with limited access to the advantages of 3-dimensional tissue systems. In order to bridge this knowledge gap, novel bioengineered models for the anal sphincter and colon were developed. To date, the models described in this thesis are the only published in vitro bioengineered models for GI structures. This provides researchers with a powerful new tool to study GI physiology in isolated 3-dimensional tissue constructs; something which was simply not possible before.

To further expand the work in smooth muscle tissue engineering, the field of vascular tissue was evaluated. Although 3-dimensional tissue models had been developed for vascular applications, there was no data available on the contractile behavior of the bioengineered constructs. Rather, biochemical

characterization was substituted for the lack of contractile data. It is a well known fact that the primary function of vascular tissue is to regulate the flow of blood flow through vascular tone. Therefore, in order to address this critical knowledge gap, the work described on GI tissue engineering was extended into the vascular arena. The primary objective was to use the experience gained with GI smooth muscle cells to develop a 3-dimensional model for vascular tissue engineering, with the primary objective of characterizing the contractile properties.

In order to develop a model for vascular tissue engineering, human aorta smooth muscle cells were cultured in a fibrin matrix. The contractile properties of the resulting vascular tissue constructs were characterized. We determined that the vascular constructs exhibited spontaneous tone, were responsive to both mechanical and chemical-induced stimulation.

The work presented in this thesis provides insight into the contractile behavior of bioengineered vascular constructs. Combined with the published data on the biochemical properties, this represents a more complete characterization of bioengineered vascular constructs, thereby providing researchers with an expanded knowledge base.

MICRO-PERFUSION FOR BIOENGINEERED HEART MUSCLE

In the third and final part of this thesis, a novel micro-perfusion system is described and shown to support the culture of bioengineered cardiac constructs. Three dimensional tissue constructs have higher metabolic requirements compared to traditional 2-dimensional monolayer culture. This is due to the

increase in mass and cellular density of bioengineered constructs. There are currently no commercially available perfusion systems for tissue engineering applications. Therefore, we sought to design, fabricate and test our own perfusion system.

Our system consists of a novel micro-incubator with independent stages for tissue constructs. Media flow and aspiration are independently regulated for each plate and system variables (temperature, pH and oxygen saturation) are controlled. The entire perfusion system can be maintained without the need for a standard cell culture incubator. This increases user control over processing variables, providing an opportunity to modulate culture conditions to match the metabolic requirements of the tissue constructs.

Utilizing this system, we have demonstrated the feasibility of support the culture of bioengineered cardiac constructs for up to 24 hours. We have shown that the cardiac constructs maintain twitch force, spontaneous and electrically induced pacing, with an increase in cell viability and expression of cardiac specific genes. In addition, we have partnered with a company (Radnoti Glass Technology, Inc, Monrovia, CA) with an interest in commercializing this technology. This will allow researchers to have access to standardized systems for the long term culture bioengineered constructs.

FORCE GENERATION OF BIOENIGNEERED TISSUES

The specific force generated by bioengineered tissues compared to whole muscle explants is summarized below (Table 7.1).

Bioengineered Model	Specific Force ($kN/\mu m^2$) of Bioengineered Tissue	Specific Force ($kN/\mu m^2$) of in vivo Tissue
Rabbit Sphincter Ring Model	2.8	10.2 (human smooth muscle explant)
Human Aortic Smooth Muscle Strip Model	0.66	26 (ferret aortic smooth muscle explant)
Rat Cardiac Muscle Strip Model	0.98	44.4 (rat papillary muscle explant)

Table 7.1 Force Generation of Bioengineered Tissues

Specific force calculated for bioengineered tissues were lower than the available explant comparisons. However, some of the only available comparisons of specific force were in different model organisms. The lower force generated by bioengineered tissues could be in part attributed to the different model systems being compared (human muscle versus rabbit muscle), as there may be variation in the mechanical strength among different species. The difference in specific force generated in bioengineered tissues versus explants may also be the result of the developmental state of the cells, since our system has not yet incorporated all of the environmental cues necessary for bioengineered muscle to reach full maturation. Furthermore, bioengineered tissues were made using a homogenous population of muscle cells grown in a fibrin matrix.

Although specific force of bioengineered tissues was lower than muscle explant comparisons, all bioengineered tissues exhibited striking tissue-specific functionality. For example, the frequency of bioengineered colonic phasic contractions (2.45 ± 0.21 cycles/min) was similar in frequency to that seen in colonic muscle strips (2–4 cycles/min). The bioengineered tissues described here represent an early, proof-of-concept demonstration of the feasibility of bioengineering functional muscle tissue in vitro. Future advances in meeting the various technical challenges, including the continued development of bioreactors and micro-perfusion systems to introduce all of the electrical, chemical, and mechanical stimulation required for tissue maturation, will surely lead to increased force generation of bioengineered tissues.

FUTURE DIRECTIVES

The work described in this thesis forms the foundation of a significant amount of ongoing research at the Artificial Heart Laboratory at the University of Michigan in Ann Arbor. The research on vascular tissue engineering has generated significant interest within the group and has led to an initiative to fabricate functional models of vascular grafts. The cell culture techniques, fabrication of 3-dimensional models and evaluation of contractile properties for the vascular graft project are all derived from the work described in this thesis.

The work described on perfusion has generated a significant amount of interest, both for scientific research and commercial potential. Researchers at

the Artificial Heart Laboratory are utilizing the perfusion system to define conditions for the long term culture of bioengineered heart muscle. The effect of oxygen saturation, fluid flow rate, and fluid shear stress are currently being evaluated. In addition, an aggressive effort to develop the commercial potential of this technology is being evaluated.

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