1. Introduction

Cardiovascular disease is the leading cause of death in the United States (1). Following a heart attack, or a myocardial infarction (MI), the infarct region suffers extensive and irreversible cell death due to the heart's limited regenerative capabilities (Fig. 1) (2). After an initial period of necrosis where the damaged myocardium weakens, the infarct region begins to stiffen, lose its striated organization, and stop generating contractile force. In the first days after the infarction, during the necrotic phase, mechanical properties are dominated by edema. A few weeks post-MI, large fibrous collagen structures form. Finally, the tissue remodels for the remainder of the healing process primarily by collagen cross-linking (3). To compensate for the loss of contractile myocardium, cardiomyocytes (CMs) in the non-infarcted regions of the left ventricle undergo pathological hypertrophy, which often leads to heart failure (4). Currently, state of the art clinical treatment is whole organ transplantation, which is limited by a shortage of donor hearts and the need to be combined with immunosuppressant therapy (5). These

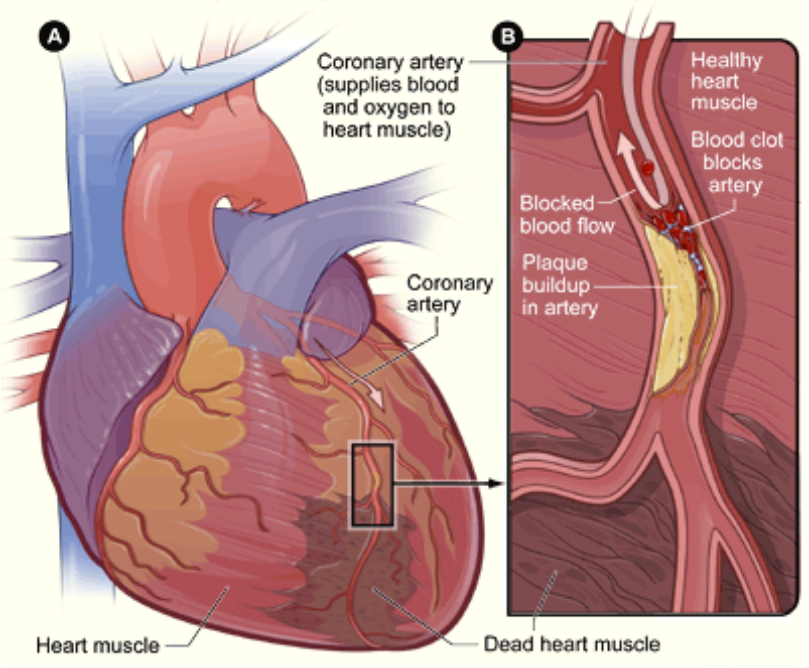

Figure 1. Acute myocardial infarction (2).

shortcomings have inspired significant research toward the development of novel treatments for patients after MI, such as stem cell therapy to regenerate dead tissue, acellular scaffolds to restore stability to the infarct region, and tissue patches to restore contractile function (6). Cardiac tissue engineering also finds applications in drug discovery and the testing of therapeutics (7).

The ability to grow functional tissue *in vitro* would open many doors in the field of regenerative medicine, particularly in the development of treatments for cardiovascular disease. The ultimate goal is to create clinically viable replacements for allotransplantations (8). The first step toward this goal is making three-dimensional (3D) *in vitro* models of cardiac tissue, which are currently being developed as platforms for testing the efficacy and safety of drugs and therapies (9). By adding an additional step to the preclinical phase, the likelihood of success in animal models and clinical trials is improved (10). Primarily, these *in vitro* models aim to replace expensive animal studies and clinically irrelevant monolayer cell cultures (11).

1.1 Designing biomaterial scaffolds for cardiac applications

1.1.1 Moving into the third dimension

Recently, two-dimensional (2D) cell culture platforms have been transitioning to more biomimetic 3D models. Using 3D model systems is more realistic because cells in that environment are exposed to mechanical and biochemical stimuli in every direction (12). Cell-cell and cell-material interactions in a 3D matrix vary in structure, localization, and function from the same interactions on a 2D surface (13). All of this can considerably affect

integrin ligation, cell contraction, and intracellular signaling (14). The 3D structure of the matrix also has an effect on solute diffusion and the interactions of growth factors and enzymes with cells (15). To mimic these complex interactions, many cardiac models have been moved into 3D by seeding cardiac cells into matrices with various physical characteristics (16-19).

1.1.2 Modulus

Mechanical properties are an important consideration when designing the scaffold component of cardiac models, as the modulus of the substrate can significantly affect the proliferation and integration of cells into the matrix (20, 21). It can also influence the differentiation of stem cells seeded in the constructs down one of the cardiac cell lineages (22, 23). In one study, human c-kit+ cardiac progenitor cells (CPCs) were seeded in cardiac extracellular matrix (ECM)-fibrin hydrogels, where the expression of endothelial and smooth muscle genes varied depending on the elastic modulus of the gel (24). Healthy myocardium has a modulus of about 20 kPa, which is an important parameter to consider in the design of these scaffolds (25).

1.1.3 Bioactivity

The composition and molecules present in native ECM depends on the tissue type (26) and disease state (27). All ECM displays unique ligands onto which integrins on the cell surface can bind to facilitate cell-ECM communication. Some polymers, like fibrin and collagen, can be used on their own to make scaffolds because they possess these ligands naturally (28-30). Other synthetic and naturally derived polymers used in 3D scaffolds, such as poly (L-

lactide-co-glycolide) (PLGA) and silk, inherently lack these binding modalities. In order for the cells to recognize and interact with these types of scaffolds, ligands must be added separately. ECM ligands typically present *in vivo,* such as fibronectin, laminin, or collagen, are often incorporated into the structures (31-34). Alternatively, rather than only exposing cells to one or a few of these ligands, decellularizing heart tissue can isolate native ECM for incorporation into scaffolds, providing cells more realistic bioactive cues (35).

Beyond the physical signaling imparted by scaffold integrins, paracrine signals can also significantly influence the action of cells, particularly in the MI environment. A few examples of cytokines present in the cardiac infarct environment include fibroblast growth factor-2 (FGF-2), transforming growth factor (TGF)-β, and tumor necrosis factor (TNF). FGF-2 is a proangiogenic factor that can provide cardioprotection following ischemia, when it has been shown to be upregulated (36). TGF-β plays a role in cardiac remodeling and fibrosis as the infarct region moves from the inflammatory phase to scar-formation (37). TNF is a factor that induces CM apoptosis and decreases contractility following ischemia (38). Inhibiting its activity improves cardiac functionality post-MI (39). These cytokines are often incorporated or controlled in models and MI treatments to improve cardioprotection following MI (40).

1.1.4 Architecture

Another important factor when developing 3D scaffolds is creating architecture that mimics that of native heart tissue. This is one of the most critical design parameters, as it directly influences the permeability and diffusion of oxygen, other nutrients, and waste throughout the model (41). The scaffold can be created with a particular porosity, surface

area, geometry, and 3D shape based on the structure of the ECM *in vivo* (42). Several attempts have been made to create scaffolds that resemble native myocardium (43), including one study by Engelmayr et al. that used a honeycomb microstructure to encourage alignment and integration of CMs like you would see *in vivo* (44). This alignment of cardiac cells is a key characteristic of native myocardium and can be encouraged by scaffolds with anisotropic structure (45). These types of features of the bulk architecture can have a major effect on cell phenotype, including influencing the differentiation of stem cells down a cardiac lineage (46-48). Pore geometry, distribution, isotropy, and size can be customized depending on the matrix materials and the fabrication procedure (49).

1.1.5 Materials

While there are a number of biomaterials that can be used to make scaffolds that mimic natural cardiac ECM, polymers are a particularly attractive option for their ability to be fabricated with particular composition and structure, depending on user need. Polymeric materials can come from either natural or synthetic sources (Fig. 2) (50). Natural biomaterials have the advantage of being biocompatible, so they only induce a mild inflammatory response *in vivo*, if any at all (51). These materials are also fairly abundant, easy to process, and biodegrade into harmless carbon dioxide and water, whereas some synthetic polymers can degrade into less benign substances (52, 53). Since they come from

Synthetic Polymers	*Natural Polymers*
Poly (glycolic acid) (PGA), Poly (lactic acid) (PLA), Poly (lactic-co-glycolic acid) (PLGA), poly (ε-carpolactone) (PCL), poly (ethylene glycol) (PEG), poly (vinylalchol) (PVA), poly (propylene fumarate) (PPF), poly(acrylic acid) (PAA), etc. *Peptide, DNA (artificially prepared natural polymers)	Collagen, Gelatin, Hyaluronate, Glycosaminoglycan, Chitosan, Alginate, Silk, Fibrin, Dextran, Matrigel, etc.

Figure 2. Polymers that can be used to make 3D scaffolds for tissue engineering (50).

natural sources, however, there are usually batch-to-batch variations based largely on the isolation procedure. Synthetic polymers avoid variability by being able to yield consistent properties, even when produced on larger scales. They also have the advantage of a finer control over mechanical properties and geometric form, although some natural materials have relatively tunable physical characteristics as well (54). Synthetic materials also lack the functionality of some natural polymers, providing only mechanical support until the cells can lay down a bioactive matrix of their own (55). Combining natural and synthetic polymers is also an option to create a scaffold with structural integrity, reproducibility, and bioactivity (56). For example, in one study by Sreerekha et al., PLGA and fibrin were electrospun together into a scaffold that promoted the differentiation of stem cells and the deposition of cardiac ECM (57).

When designing the scaffold, it is also essential to choose a material that meets the physiologic needs of the tissue, whether it is to ultimately be implanted in the body or sustain cells *in vitro*. For cardiac applications, this usually means that the structure needs high mechanical stability and compliance to maintain integrity through the constant pumping of the heart (58). Hydrogels can be an effective solution because of their soft and viscoelastic properties that resemble native tissue (59). However, sometimes these gels lack the mechanical strength necessary to support and integrate with the damaged myocardium (60). It has been demonstrated by Chung et al., that increasing crosslinking density in the elastin-like hydrogels can increase the modulus without affecting cell-adhesion ligand density or the phenotype of seeded cells, which has increased the applicability of hydrogels in cardiac tissue engineering applications (61). Another option would be to use a porous polymeric scaffold created by freezing (62) or electrospinning

(63), which can be tuned to have biomimetic mechanical properties, depending on the material (64). These sponge-like scaffolds have been shown to enhance angiogenesis when implanted into rat hearts *in vivo* (65).

1.1.5.1 Silk

Silk fibroin from silkworms has found many applications in tissue engineering for its biocompatibility and unique physical characteristics (66). Before it can be used, the silk protein must be extracted from *Bombyx mori* (silkworm) cocoons and the adhesive protein, sericin, removed, as it is toxic to cells (Fig. 3) (67). Once the silk fibroin is isolated, however, it elicits a minimal inflammatory response *in vivo*. Silk also has the advantage of being able to be processed to have a range of strengths and degradation rates (68).

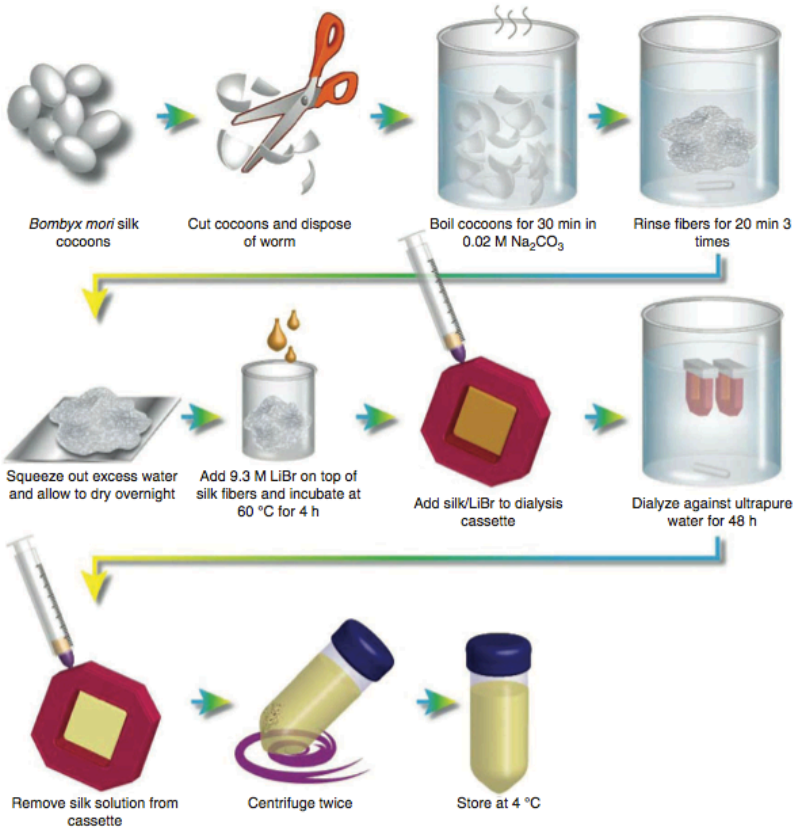

Figure 3. Preparation of silk fibroin solution from *Bombyx mori* cocoons (67).

The degummed silk protein solution may be processed into many different forms, including a porous scaffold via freeze-drying (69). Varying the freezing technique can control pore size and orientation. Isotropic freezing in a uniformly cold environment, like a freezer, results in relatively round and randomly oriented pores, whereas pores are elongated and

aligned when freezing proceeds in a single direction (anisotropic freezing). Pore size increases with decreased freezing rate, which can be adjusted by altering the freezing agent or environment (70). Once the scaffolds are created, they can be lyophilized for sterilization and to stabilize them at room temperature, thus preserving them for longer amounts of time. The silk fibroin can then be further crystallized using temperature-controlled water vapor annealing (TCWVA) to control scaffold mechanics and degradability (71).

Silk has found a number of applications in cardiac tissue engineering, but when used as a biomaterial, it is always functionalized with additional molecules, such as chitosan, hyaluronic acid, or collagen (72-74). This is because silk fibroin from *Bombyx mori* cocoons lacks cell attachment points (75). Additives, such as collagen or arginine-glycine-aspartic acid (RGD), can be incorporated into the silk scaffolds to provide bioactive ligands for cells (76, 77). Silk is a useful biomaterial for modeling the MI microenvironment at different time points post-infarction because it can recreate the physical variation seen over time during remodeling in the infarct region. Anisotropic silk scaffolds can guide cells to mimic the alignment of healthy myocardium, whereas isotropic scaffolds can represent the disorganization of the different infarct time points (78, 79).

1.1.5.2 Cardiac Extracellular Matrix

Native cardiac ECM is secreted and remodeled by cells throughout development, so it has been suggested that it could be used to encourage cell recruitment and regeneration in damaged myocardium (80). Gentle perfusion decellularization strategies have been optimized to largely preserve the complex structure and composition of native myocardial

ECM (81). Using these methods, the natural scaffold can be isolated and used to guide the differentiation and organization of cells in a number of cardiac tissue engineering applications (82). Culturing stem cells directly on isolated cardiac ECM encourages them down a cardiac lineage (83). When combined with other hydrogel components, such as collagen, signaling from cardiac ECM is enough to induce the differentiation of stem cells toward cardiac phenotypes without exogenous growth factors (84). Adding growth factors to a similar model, however, augments the differentiation process (85).

1.2 Biomaterials-based treatments following MI

1.2.1 Cardiac Stem Cell Therapy

A promising treatment for cardiovascular disease following MI is stem cell therapy, where stem cells are introduced to the damaged myocardium and differentiate into CMs to restore contractile function. Cells are usually administered alone, in combination with a scaffold, and/or along with growth factors (86). The goal is for the stem cells to repopulate the area

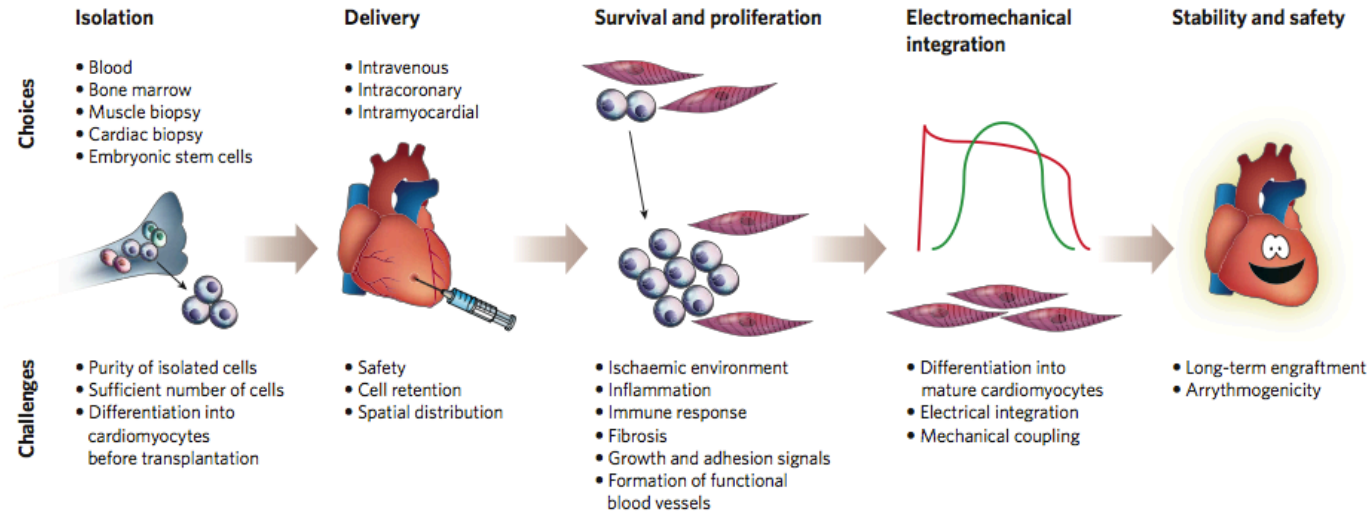

Figure 1. Challenges of cardiac stem cell therapy at every step (87).

and differentiate into contractile CMs or vascular cells to restore blood flow to the necrotic region, while also providing proangiogenic and antifibrotic paracrine signals to native cells. There are many challenges to cell therapy, however, including poor cell retention, survival, and differentiation in the harsh infarct environment (Fig. 4) (87). About 90% of cells injected into the heart are immediately lost in the circulation or leak out of the injection site. Then about 90% of the cells that are retained in the heart die within the first week, leaving only about 1% of the cells delivered initially in the infarct region (88). The injected cells may also be unable to integrate with host tissue because they cannot physically penetrate the scar tissue, which could lead to arrhythmias (89). Despite these challenges, many cardiac stem cell therapy experiments have been attempted in animal models (90-92) and clinical trials (93-95), all meeting with limited success. So far, the most commonly used cell source in these clinical trials has been autologous bone marrow cells, which provide moderate and often short-lived functional improvements.

1.2.1.1 C-kit+ Cardiac Progenitor Cells

In cell therapy, one of the most important parameters when designing the treatment is the cell type to be used. One type of cell that has shown a lot promise are CPCs, particularly those that express the stem cell surface antigen, c-kit. c-kit+ cells have already proven capable of differentiating down all three major cardiac cell lineages: SMC, endothelial (EC), and CM, to an extent (96). They can be patient-specific, since most of the endogenous population can be harvested from the right atrial appendage (97). The number of c-kit+ CPCs capable of cardiomyogenesis also increases in a failing heart, creating a greater supply from which to draw for clinical applications (98). These cells have already

undergone two phase 1 clinical trials (99). In Bolli et al., one million autologous c-kit+ CPCs were administered to patients with ischemic heart disease by intracoronary infusion, resulting in improved left ventricle systolic function and reduced scar size over time (100, 101). c-kit+ CPCs are also starting to be investigated for use in cardiac models by combining them with biomaterials such as polycaprolactone (102). *In vitro* studies have shown that c-kit+ CPCs exhibit enhanced differentiation, proliferation, and adhesion when grown on decellularized cardiac ECM over collagen I (103).

1.2.1.2 Hyperpolarization

Besides using the physical environment to influence the fate of stem cells, modulation of their membrane potential can also control their phenotype. This was originally suggested because biophysical signals play a major role in development and tissue regeneration. It has been found by Sundelacruz et al. that membrane hyperpolarization encourages the differentiation of stem cells (104), while depolarization encourages plasticity (105). This concept has also met with encouraging results when applied to neonatal CMs, whereby prolonged depolarization increased their proliferation *in vitro* (106). A recent study has characterized the most common ion channels present in adult human c-kit+ CPCs, which could be used to develop small molecule-based treatments for the differentiation of cells (107).

1.2.1.3 Cell Preconditioning

One way that researchers are trying to improve cell retention in stem cell therapies for treating cardiovascular disease is by preconditioning the cells before transplantation. A

promising method for preparing cells is to expose them to the low oxygen tension they would see in an infarct environment because it can induce protective responses before they are even administered (108). Culturing CPCs under such conditions influences their migratory, differentiation, and proliferative capabilities (109). In a study by Tang et al., CPCs conditioned under hypoxia reduced infarct size and improved cardiac function in MI mice significantly more than cells grown up in normal oxygen (110). Stem cells have also been preconditioned by exposure to various cytokines, antagonists, and small molecules to enhance viability, engraftment, and cardiac function (111-114).

1.2.2 Acellular Scaffolds

When developing patch-based treatments for heart disease, the scaffolds must provide necessary mechanical support as well as physical and biological cues to encourage cell engraftment, differentiation, and maturation. They must also be non-toxic and biodegradable, so that cardiac ECM secreted by the new or recruited cardiac fibroblasts can eventually replace it (115). One way biomaterials are being used to restore cardiac function *in vivo* is by implanting acellular scaffolds to stabilize the damaged ventricular wall. Acellular biomaterial patches have been tested in a number of small and large animal preclinical trials (116-118). Growth factors can also be incorporated into these scaffolds to augment healing and cell survival (119-122). For example, in Miyagi, et al., vascular endothelial growth factor (VEGF) was covalently incorporated into a porous collagen scaffold, which led to increased angiogenesis in the patch when implanted over an infarct *in vivo* (119).

Injectable matrices are also being investigated due to the minimally invasive delivery techniques and their ability to reinforce damaged myocardium (120-124). While some native smooth muscle cell (SMC) populations can infiltrate these scaffolds, they still lack beating CMs. For that reason, their long-term regenerative potential is limited, as they are meant mainly to stabilize damaged tissue rather than replace lost cells.

1.2.3 Cardiac Tissue Patches

Scaffolds can be seeded with autologous progenitor cells or CMs and implanted over the infarct region of the heart in an attempt to recellularize and restore contractile function to that area. A major limitation in using differentiated CMs in constructs is that they can only be implanted in immunodeficient animals, making that technique clinically impractical. Cardiac tissue patches are typically made *in vitro* and can be functionally evaluated for their ability to generate contractile force and propagate electrical signals, without generating arrhythmias. They must be highly elastic while enhancing cellular integration, remodeling, viability, and differentiation (125). A number of cardiac patches have been tested *in vivo*, demonstrating that they can integrate into host tissue and improve cardiac function when implanted (126, 127). There has been a phase I clinical trial, Myocardial Assistance by Grafting a New Bioartificial Upgraded Myocardium (MAGNUM), to demonstrate the feasibility of delivering stem cells using a collagen patch (128). In this study, autologous bone marrow stem cells were isolated and both injected into the MI region and seeded into a scaffold. The scaffold was then affixed over the scar region and covered with another acellular scaffold. While this study has proven the safety and feasibility of such a treatment, it did little more than improve the stability of the damaged

myocardium. Sheets of CMs can be cultured and directly transplanted into rat hearts without the use of scaffolds, and still functionally integrate into host tissue (129, 130). This solution is limited by its immunogenic cell sources and its inability to be scaled up into multiple layers without running into nutrient transport problems.

A major challenge associated with seeding 3D scaffolds in any tissue engineering context is the need for vasculature to supply oxygen and nutrients to cells within the construct. Adding channels to the cardiac patches enhances perfusion both *in vitro* (131) and *in vivo* (132). It is also possible to encourage vascularization using custom perfusion chambers to deliver oxygen and nutrients to developing tissue (133). Seeding endothelial cells along with cardiac cells can enhance neovascularization in tissue constructs (134). These techniques increase the critical size of patches that could ultimately be used clinically, promising more effective patches for humans.

1.2.4 Cardiac Model

These cardiac tissue patches could also be used *in vitro* to model native myocardium as a platform for testing drugs and optimizing therapies (135). Differentiated cells can be used in this case because the tissues are not meant to be implanted *in vivo.* These tissues are typically scaled down to less than 500 μm to increase the quantity that can be tested at once and to avoid the need for special vascularization (136). Although these models are still in early stages of development, Park et al. has developed a poly(glycerol sebacate) (PGS) scaffold with biomimetic anisotropic mechanical properties and molecular and biophysical cues to study cell signaling in cardiac tissue development (137). Other models have been developed as well to study various characteristics of myocardium *in vitro* (138). One

current challenge in engineering cardiac tissue is determining how biomimetic the systems have to be to model relevant tissue function (139).

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2. Objectives

In this study, the complexities of the cardiac infarct microenvironment were modeled with 3D silk scaffolds to understand how CPC proliferation and differentiation are affected by infarct composition and oxygen tension. These models were also used to evaluate how preconditioning and implantation strategies augment the regenerative potential of CPCs. Finally the ion channels of human CPCs were characterized to understand their prevalence and variation for the development of hyperpolarization differentiation strategies.

2.1 Develop healthy and infarct model systems by changing implantation, oxygen tension, and scaffold ECM type

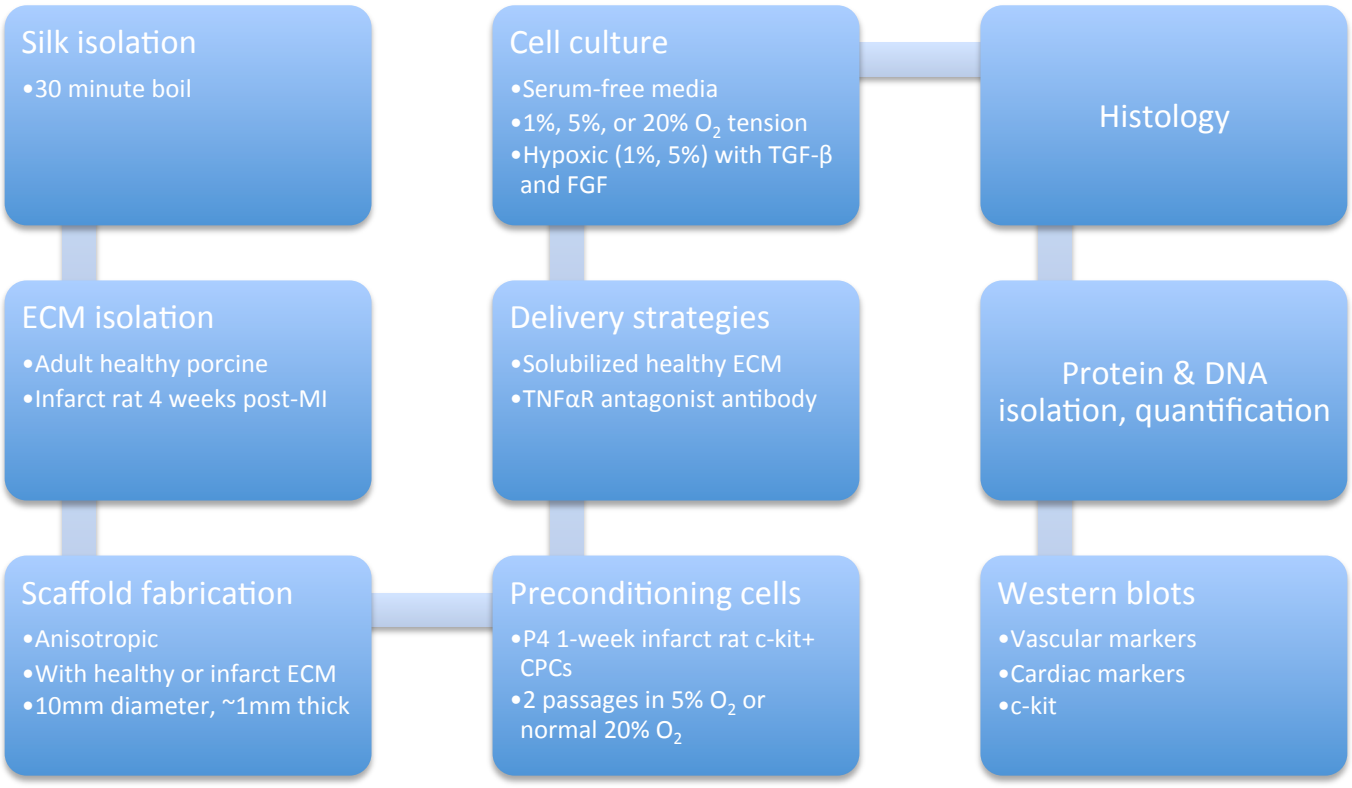

Figure 1. Experimental design for developing the models in Aim 1.

Model	Oxygen tension	Cytokines	Implantation	Time points
Healthy	20%	None	• Hypoxic precond. (5% O_2) • TNFαR antagonist • Solubilized healthy ECM • None	• 5 days • 2 weeks • 4 weeks
Infarct	5% (low) 1% (very low)	FGF, TGF-β		

Figure 2. Parameters observed in each cardiac model, including the oxygen tension and cytokines in the culture environment, implantation strategies used, and time points. The appropriate ECM was used in each type of model scaffold.

c-kit+ CPCs were cultured in specific microenvironments indicative of both healthy and infarcted myocardium (Fig. 1). Alterations in adhesion, expansion, and differentiation potential were evaluated and both preconditioning and implantation strategies were compared for their ability to enhance cellular phenotype and thus promote the regenerative potential of CPCs (Fig. 2). The three strategies evaluated included: (i) hypoxic preconditioning to slowly introduce the cells to a reduced oxygen tension to minimize oxidative stress (culture at 5% oxygen for two passages as compared to standard 2 0% O_2), (ii) delivery with soluble TNF-α Receptor (TNF-αR) antagonist to minimize cellular apoptosis due to cytotoxic stress, and (iii) delivery with solubilized cardiac ECM, which has demonstrated the ability to promote cell survival and adhesion following shear stress (1). The preconditioning control for this experiment was CPCs grown in a normal environment in the absence of an implantation strategy. Models were then cultured under low oxygen tension (5% O_2), to mimic native myocardial oxygen tension, or very low oxygen (1% O_2), to mimic the infarct environment. The culture control scaffolds were maintained at standard *in vitro* cell culture oxygen (20% O_2).

Learning how different preconditioning strategies affect these cells could improve cell therapy strategies by encouraging them down a particular lineage before introduction into

the infarct environment. Knowing how cells would then respond in an *in vitro* infarct model would provide more clinically relevant and controlled results than an animal model.

2.2 Assess the variability between lines of human c-kit+ CPCs to aid in the development of a novel preconditioning strategy

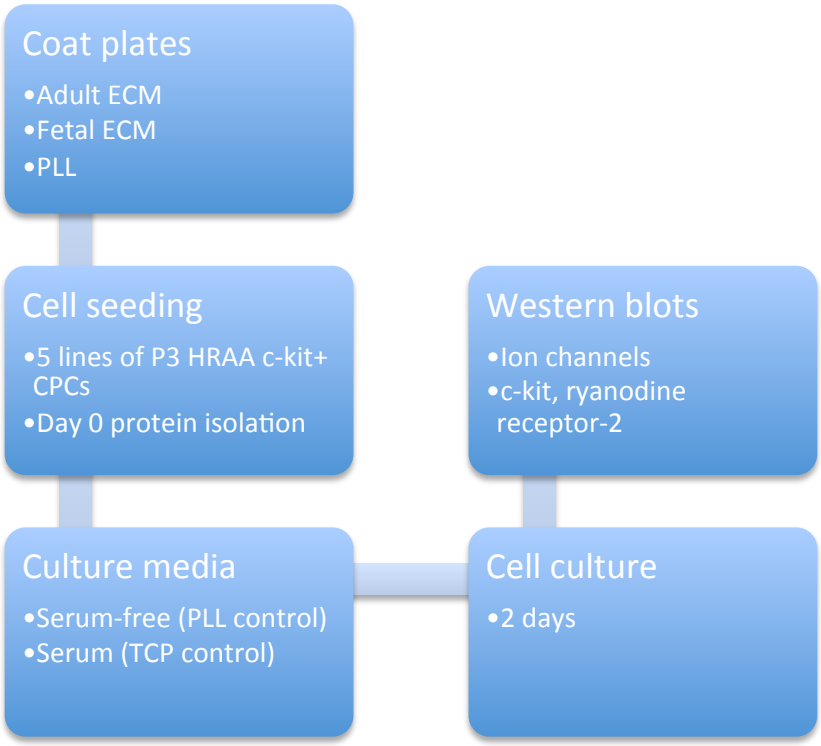

Figure 3. Experimental design for evaluating cell line variability in Aim 2.

In this objective, c-kit+ CPCs isolated from human right atrial appendages (HRAAs) of human patients were cultured on different 2D substrates in serum and serum-free medias to compare the expression of ion channels across individuals and between culture conditions (Fig. 3). The CPCs were cultured on plated adult or fetal porcine ECM, with the serum-free media control on poly-L-lysine (PLL) and the serum media control on tissue culture plastic (TCP). PLL was used to aid cells in adhering to the surface in serum-free conditions. Histological assessment and western blotting were performed two days after

culture to qualitatively and quantitatively assess how both line and culture conditions impacted the expression of various ion channels including: Nav1.2, HCN4, c-kit, Slo1BK, and ryanodine receptor-2 (RyR-2) (Fig. 4). It is important to characterize how ion channel expression varies across *in vitro* culture conditions and between individual patients in order to identify the optimal cell culture protocol and determine whether the differentiation protocol must be patient-specific.

Ion Channel	Channel Type	Description
Nav1.2	Voltage-gated sodium channel	Heart excitability and conduction
HCN4	Potassium/sodium channel	Expressed in pacemaker region of the heart
Slo1BK	Potassium channel	Electrical signal propagation in heart
Ryanodine receptor 2	Calcium channel	Cardiomyocyte contraction

Figure 4. The cardiac ion channels quantified in this study.

2.3 References

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3. Methods

3.1 Cardiac Models

3.1.1 Silk isolation

Silk fibroin solution was prepared from *Bombyx mori* cocoons as previously described (1). Briefly, the cocoons were boiled in a 0.02 M sodium carbonate solution for 30 minutes (Sigma-Aldrich, St. Louis, MO), then rinsed with distilled water and dried overnight. The silk fibers were then broken down in 9.3 M lithium bromide at 60°C for four hours (Sigma-Aldrich, St. Louis, MO). The resulting solution was loaded into Slide-A-Lyzer Dialysis Cassettes (3,500 MWCO, ThermoScientific, Rockford, IL) and dialyzed against deionized water (diH_2O) to remove the lithium bromide. The silk solution was then centrifuged twice (9,700 RPM, 20 min, 4°C) to remove insoluble particulates. The concentration of silk in the solution was calculated by drying and massing a known volume of the solution. This technique yielded a 6-8% wt/vol silk solution, which could be stored at 4°C for up to 3 weeks.

3.1.2 Cardiac ECM

Adult porcine hearts were obtained from the abattoir and the left ventricles separated for ECM isolation. The tissue was decellularized as previously reported (2, 3). Briefly, the ventricles were sectioned into 3mm^3 pieces, immersed and stirred in a 1% sodium dodecyl sulfate (SDS) solution in phosphate buffered saline (PBS) until the tissue was translucently clear. The decellularized ECM was rinsed in diH_2O overnight, lyophilized, and milled into a fine powder with a small tissue mill and 40mm mesh strainer. The ECM powder was then

pepsin-digested (1 mg/mL) in 0.1 M HCl for at least 48 hours. The pH of the solution was adjusted to 7.4 with NaOH and lyophilized again. The ECM powder was reconstituted in diH_2O for incorporation into silk scaffolds or cell culture media.

Infarct rat myocardium was isolated 4 weeks post-MI as previously described (4). MI was induced in Sprague-Dawley rats by permanently ligating the left coronary artery with a suture. Animals with significant infarcts (40% or more of the left ventricle) were allowed to recover for 4 weeks, at which point the hearts were isolated and decellularized via adapted Langendorff perfusion with 1% SDS until the tissue became translucently clear. The hearts were then rinsed with 50 mL of diH_2O, followed by 50 mL of 0.5% triton X, and then rinsed again with diH_2O. They were then washed a final time with 1x PBS using a peristaltic pump for 72 hours, with the PBS changed every 12 hours. The scar region of the heart was then isolated, minced, and frozen at -20°C overnight. These samples were then lyophilized, weighed, and then solubilized in an acidic pepsin solution as described above.

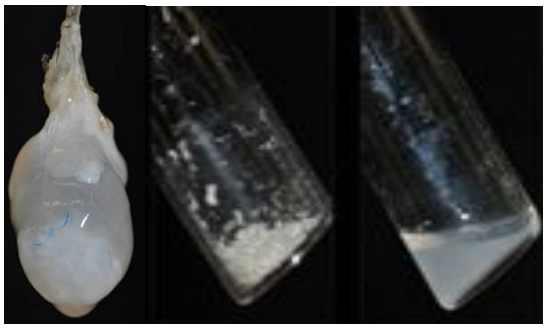

Figure 1. Decellularizing and preparing infarct rat myocardium (4).

3.1.3 Scaffold fabrication

Silk scaffolds were frozen as described previously (5). First the silk and ECM solution was prepared by combining 3% silk (wt/vol) solution and 30 mg/mL reconstituted healthy porcine or 4 week infarct rat ECM. The solution was then loaded into one chamber of a custom PDMS mold consisting of two chambers separated by a metal plate, with a dry ice bath of absolute ethanol in the other half. The silk then froze in one direction, creating an

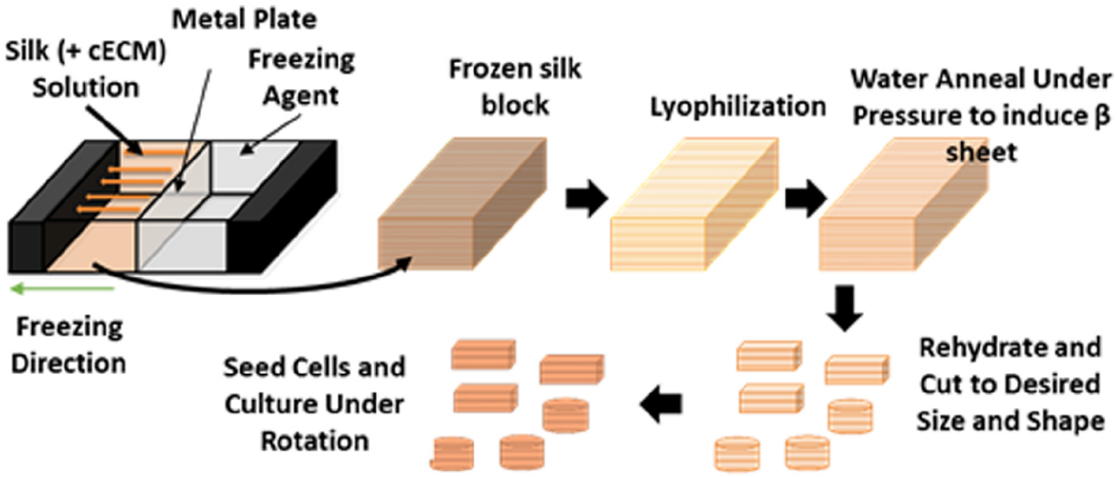

Figure 2. Schematic of freezing and preparing anisotropic silk scaffolds (6).

aligned, porous scaffold (6). The scaffold was then lyophilized and water annealed at room temperature to induce β-sheet formation, making it insoluble (7). It was then rehydrated in PBS with 1% penicillin/streptomycin overnight and cut with a biopsy punch into 10mm-diameter round scaffolds, which were then trimmed with a sterile razor blade to about 1mm thickness.

3.1.4 Preconditioning cells

Passage 4 c-kit+ CPCs isolated from infarcted rat hearts 1 week post-MI were thawed from liquid nitrogen storage and cultured for two passages in either low-oxygen conditions (5% O_2) or at normal cell culture oxygen tension (20% O_2). Cells were cultured in stem cell maintenance media (10% FBS, 1% P/S in F-12) supplemented with FGF-2 and leukemia inhibitory factor (LIF) (both 0.01%) to maintain stemness. The cells cultured at 20% O_2 were then divided into groups and supplemented with solubilized porcine left ventricle ECM (30 mg/mL), TNFαR antagonist antibody (10 µg/mL), or were untreated to serve as a control. All cell solutions were diluted to a concentration of $6.5x10^5$ cells/mL so that each construct would have a cell density of 650 cells/mm^3.

3.1.5 Cell seeding and culture

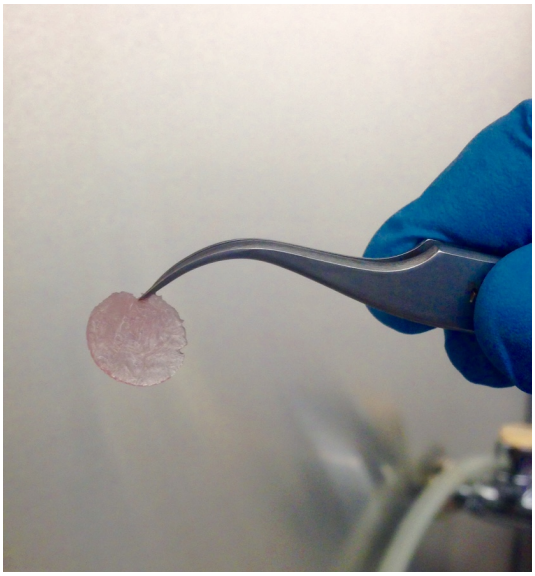

Figure 3. A seeded silk scaffold.

Silk scaffolds were seeded by using their absorption capacity (Fig. 3). To prevent cells from migrating to the plastic, 5% wt/vol Pluronic®F-127 was used to coat the wells of 12-well plates for at least two hours and then rinsed three times with sterile PBS and allowed to dry. The thickness of each wet scaffold was measured using calipers to calculate the volume of cell solution that could saturate them. Then each scaffold was dehydrated using a sterile glass pipette tip connected to an aspirator and moved to its own well of the 12-well plate. After seeding the constructs with the appropriate volume of cell solution, they were briefly stored in an incubator for 15-20 minutes before 3 mL of media was added to each well to prevent cells from immediately washing out of the scaffold. The constructs were cultured in serum-free media (50:50 F-12:DMEM, 0.5% ITS, 0.2% BSA, 1% P/S) for 5 days, 2 weeks, or 4 weeks, with media changes every four days. Constructs were cultured under various oxygen tensions, at 1% oxygen (mimicking the infarct environment), 5% oxygen (mimicking healthy physiological cardiovascular oxygen levels), or 20% oxygen (standard cell culture environment). Those in low- (5%) or very low- (1%) oxygen environments were supplemented with 0.01% TGF-β and 0.005% FGF-2 in the media.

3.1.6 Histology

Scaffolds were fixed in 4% paraformaldehyde (PFA) for 30 minutes, followed by three 10-minute rinses in PBS. Cellular actin was then stained with phalloidin (15 µL/mL) for at

least 1.5 hours, and then nuclei were stained with DAPI (16 μL/mL) for 15 minutes, followed by three 20-minute rinses in PBS. Scaffolds were then imaged at 40x magnification.

3.1.7 Isolation of protein, DNA, and RNA

RNA, DNA, and protein were isolated from the scaffolds at the 5-day and 4-week time points using a BioReagents™ SurePrep™ RNA/DNA/Protein Purification Kit (Fisher Scientific, Waltham, MA). First the scaffolds were flash frozen in liquid nitrogen, physically disrupted with a mortar and pestle and digested with lysis buffer, as recommended in the protocol for tissue samples. The samples were then drawn up and down in a 1 mL syringe before being transferred to a spin column in a collection tube. In some cases, samples were centrifuged for additional time to ensure the entire sample was processed through the spin columns. Protein was isolated from 200 μL of the lysate collected during RNA extraction.

Protein was isolated after 2 weeks of culture by following a standard cell lysis protocol. Cells were trypsinized, centrifuged, and the cell pellet resuspended in 100 μL of lysis buffer (50% 2xNP40, 2.5% 40x sodium deoxycholate, 1% 100x sodium orthovanadate, 1% protease inhibitor cocktail [aprotinin, pepstatin, leupeptin], in diH_2O). After the lysis buffer was added, samples were sonicated on ice for 5 minutes and vortexed for 20 minutes before being centrifuged at 4°C at 13000 rpm for 15 minutes. The supernatant was transferred to a fresh tube and stored at -20°C.

Protein concentrations were quantified with a Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, MA). DNA purities and concentrations were measured using a NanoDrop (ThermoFisher Scientific, Waltham, MA).

3.1.8 Western blots

Western blots were used to quantify differences in protein expression across culture conditions. First equal amounts of protein (10-20 μg per lane) were loaded into precast 4-15% polyacrylamide gels (cat #456-1086, BioRad, Hercules, CA) and run before being transferred to a nitrocellulose membrane. Blots were blocked for at least an hour at room temperature in milk/TBST solution. Primary antibodies were used to evaluate the expression of proteins at the different time points for each condition. β-actin (1/1000) or GAPDH (1/1000) were used to normalize to total cell content. The blots were then stained with α-smooth muscle actin (1/400), von Willebrand factor (1/100), cardiac α-actin (1/200), GATA4 (1/200), and c-kit (1/200). Species-specific secondary antibodies conjugated to HRP were used (1/5000) for chemiluminescence. Blots were stripped in stripping buffer between stainings. Images were acquired on the G:Box Chemi XR5 (Syngene, Cambridge, United Kingdom). Expression intensities were analyzed using ImageJ (NIH, Bethesda, MD).

3.1.9 Statistics

Significance was determined with one- or two-way ANOVA's in SigmaPlot.

3.2 Ion Channel Characterization

3.2.1 Fetal cardiac ECM

Porcine fetal hearts were decellularized as described above for adult hearts, but with a 0.1% SDS solution. Complete decellularization occurred slightly faster than with adult hearts.

3.2.2 HRAA c-kit+ CPC culture

12- and 24-well plates were prepared for culture by coating with adult or fetal ECM, PLL, or nothing (plain tissue culture plastic (TCP)). Enough solubilized ECM was added to coat the bottom of each well at a concentration of 50 μg/cm^2 and allowed to dry overnight. The same amount of PLL was added and allowed to sit in the wells for 10 minutes before being aspirated off and rinsed twice in PBS. All wells were rinsed twice in PBS prior to seeding. c-kit+ CPCs previously isolated from the right atrial appendages (HRAAs) of five human patients were plated and grown up in normal culture conditions. At passage three, the cells were passaged and counted. Three samples of 1x10^5 trypsinized cells were lysed and the protein isolated for each line, to be used as the day 0 time-point. The remaining cells were seeded onto the coated plates at a concentration of 1x10^4 cells/cm^2. Plates coated with PLL, adult, and fetal ECM were cultured in serum-free B27 media with 0.01% FGF-2. The remaining TCP, adult, and fetal ECM-coated plates received HRAA media, with FBS and 0.01% FGF-2. The cells were cultured for two days before being lifted with tryple and lysed in lysis buffer as described above.

3.2.3 Western blots

Protein expression of all lines at Day 0, and for lines 2 and 3 after culture were analyzed using the BCA protein assays and western blotting procedures described above. β-actin (1/1000) was used to normalize to total cell content. The blots were also stained for Nav1.2 (1/230), HCN4 (1/430), c-kit (1/1000), Slo1BK (1/375) and RyR-2 (1/400). Species-specific secondary antibodies conjugated to HRP (1/5000) were used for chemiluminescence to measure and compare the expression of different ion channels between individuals and culture conditions.

3.2.4 Statistics

Significance was determined with one-way ANOVA's in SigmaPlot.

3.3 References

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4. Results

4.1 Cardiac Models

4.1.1 Cardiac α-actin increased with hypoxic preconditioning

Data was collected on the relative protein expressions in each sample using western blots. Proteins stained include cardiac markers: cardiac α-actin (mature CM marker) and GATA-4 (early CM marker); vascular markers: α-smooth muscle actin (early SMC marker) and von Willebrand factor (EC marker); and the stem cell marker, c-kit. Hypoxic preconditioning of

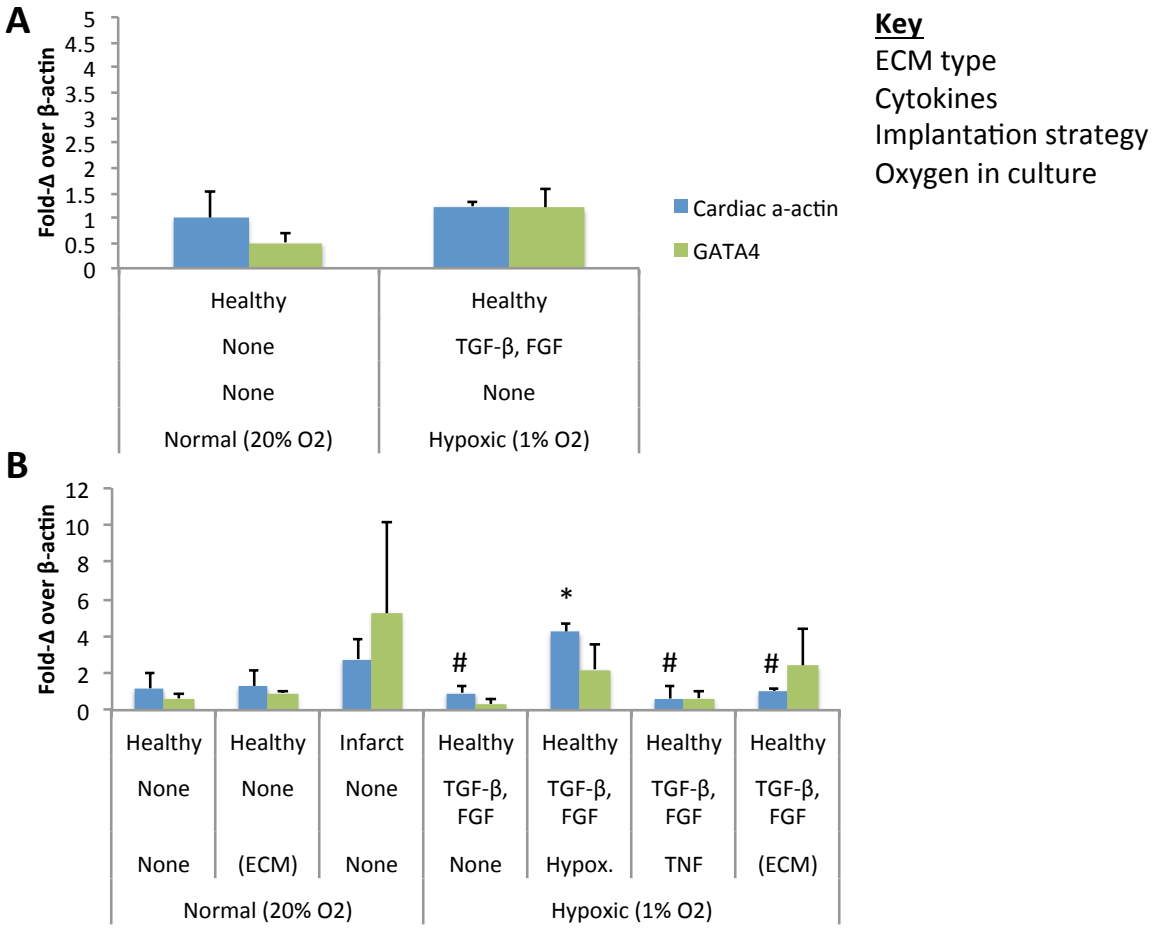


Figure 1. Expression of cardiac markers following culture in the infarct model after A) 5 days and B) 4 weeks.

cells significantly increased the expression of cardiac α-actin over other implantation strategies when cultured in a very low oxygen environment for 4 weeks (Fig. 1).

4.1.2 Preconditioning and oxygen tension do not affect the expression of vascular proteins

None of the parameters tested in our model had a significant influence on the expression of vascular proteins αSMA or vWF (Fig. 2).

4.1.3 c-kit expression did not change with implantation strategies or oxygen tension

The expression of c-kit in cells that were preconditioned or cultured under hypoxia did not change significantly over controls (Fig. 3).

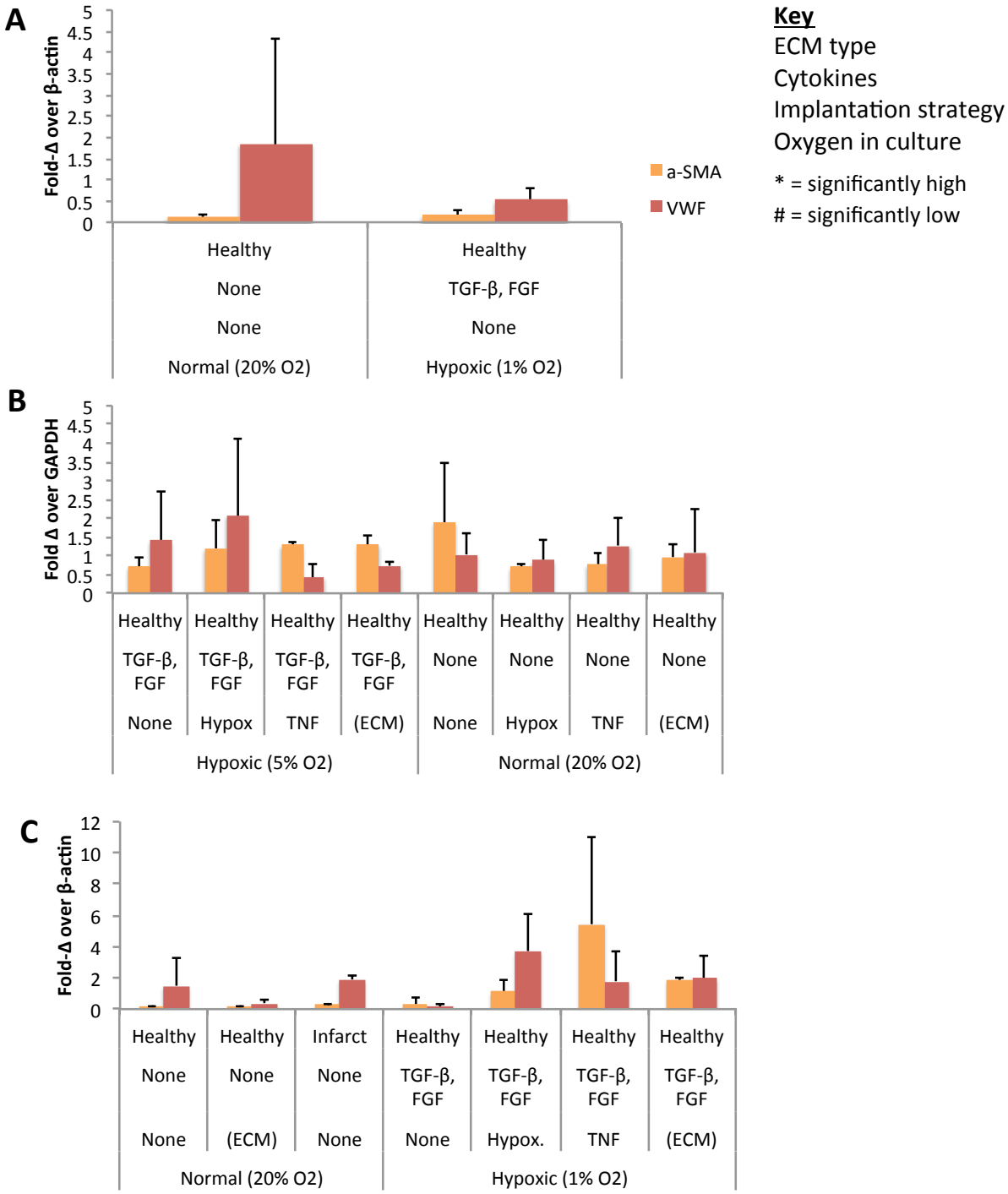


Figure 2. Expression of vascular markers following culture in the infarct model after A) 5 days, B) 2 weeks and C) 4 weeks.

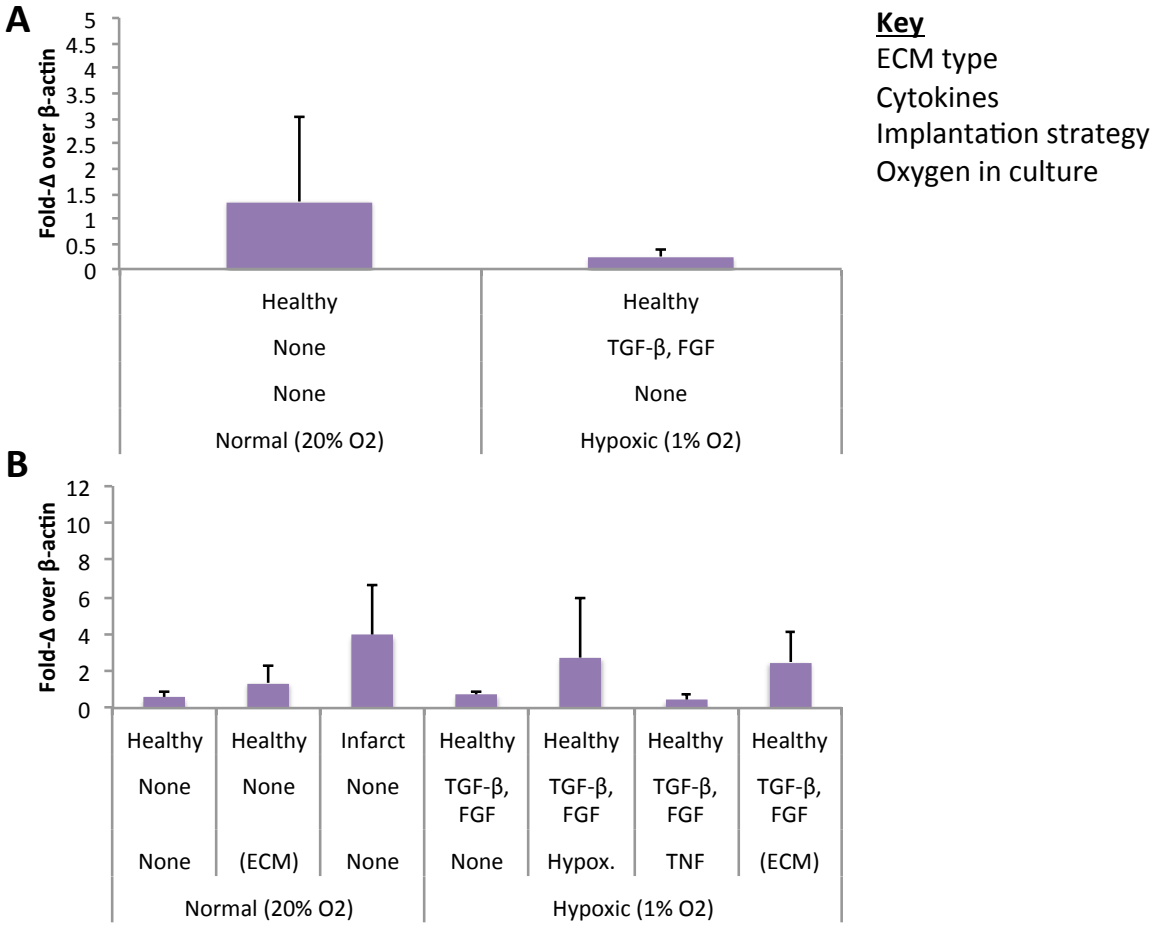


Figure 3. Expression of c-kit following culture in the infarct model after A) 5 day and B) 4 weeks.

4.1.4 Proliferation does not change over time

DNA content of samples was quantified to observe cellular adhesion after 5 days and cell density after 4 weeks. There were no significant changes in DNA content over time or between samples (Fig. 4).

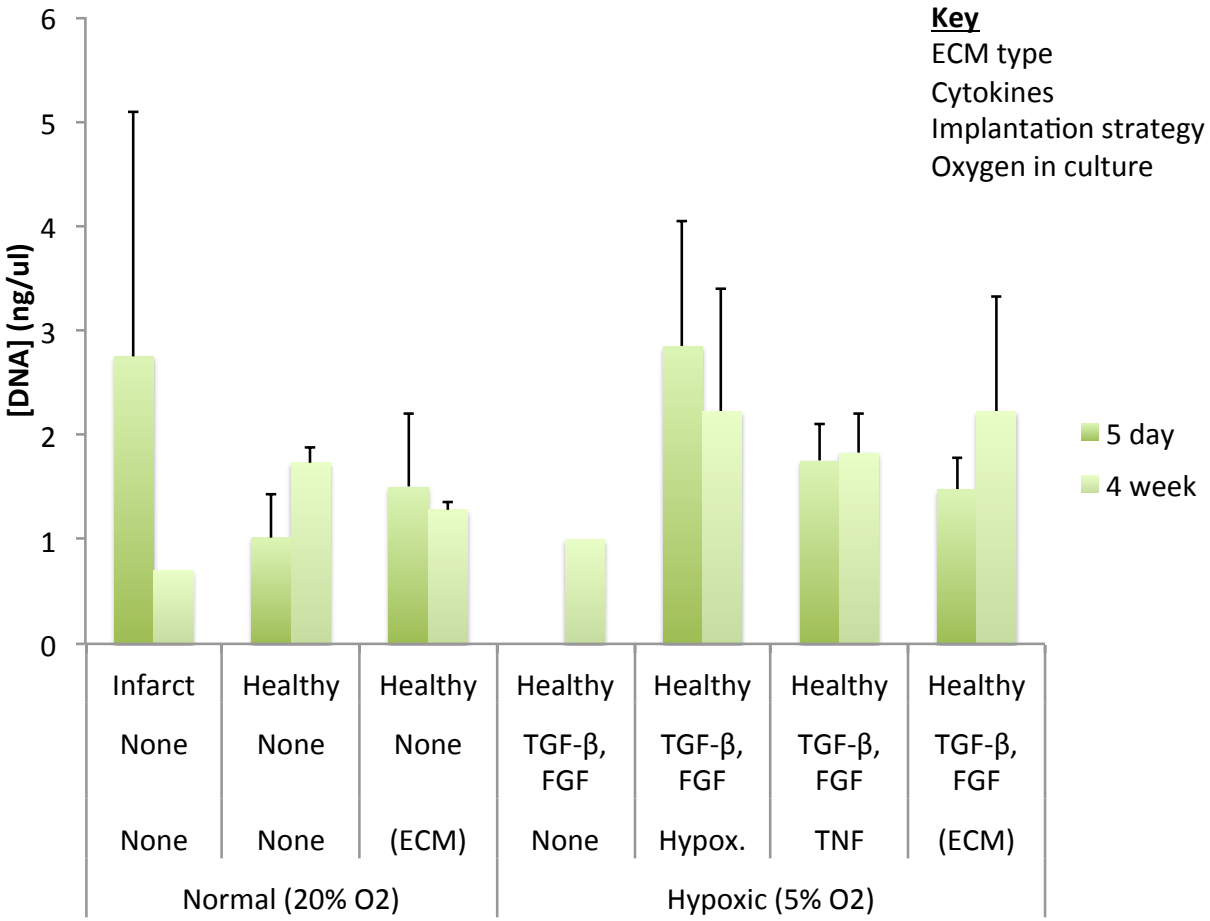


Figure 4. DNA content in cardiac models over time under different culture conditions.

4.1.5 Implantation strategies affect cell adhesion and proliferation

When preconditioned cells were cultured on tissue culture plastic (TCP) for 2 weeks under normal oxygen tension, qualitatively, there appeared to be a difference in the adhesion and proliferation of cells. Those expanded under hypoxia saw the greatest proliferation, while

delivery with solubilized ECM seemed to inhibit adhesion and expansion (Fig. 5). Delivery with TNF-αR antagonist appeared to increase cell density somewhat over no preconditioning. These trends seemed to hold when the cells were introduced to the 3D cardiac models (Fig. 6A, B). The control models did not display much proliferation or spreading.

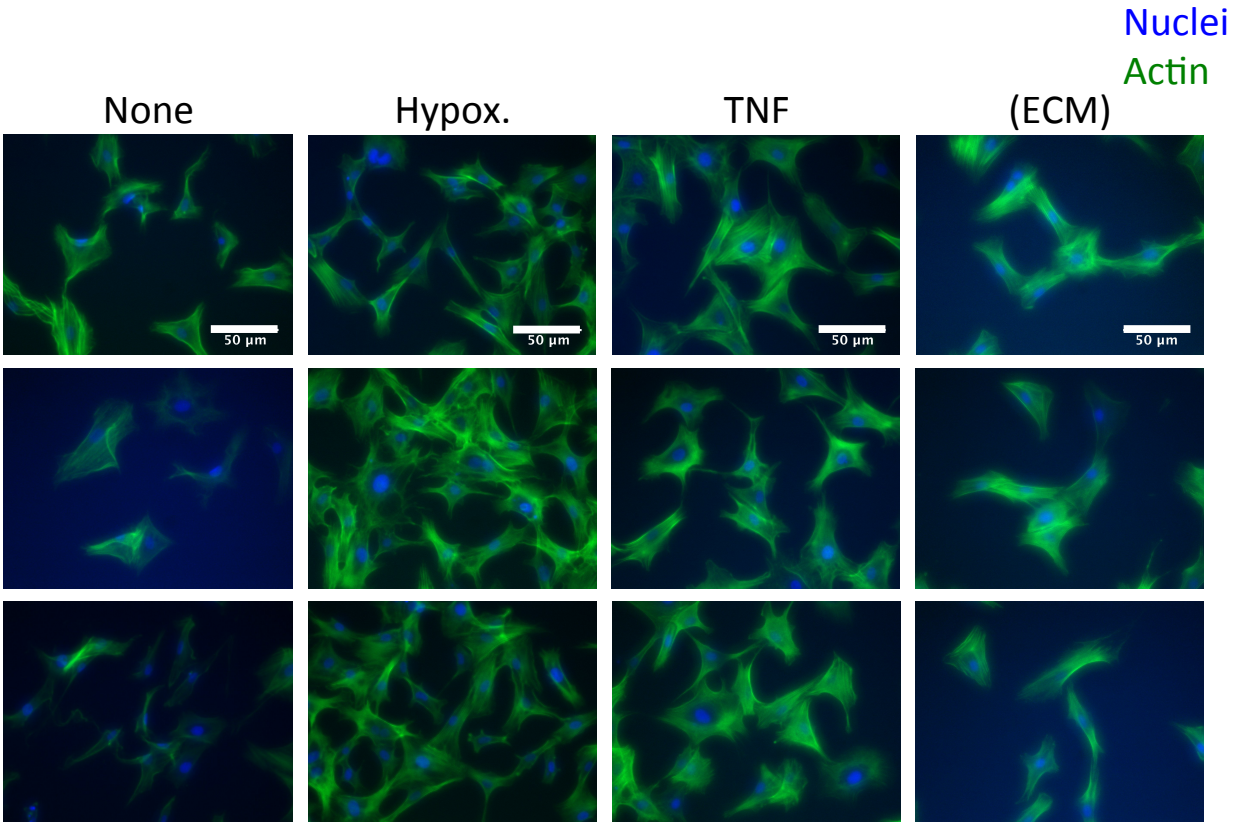
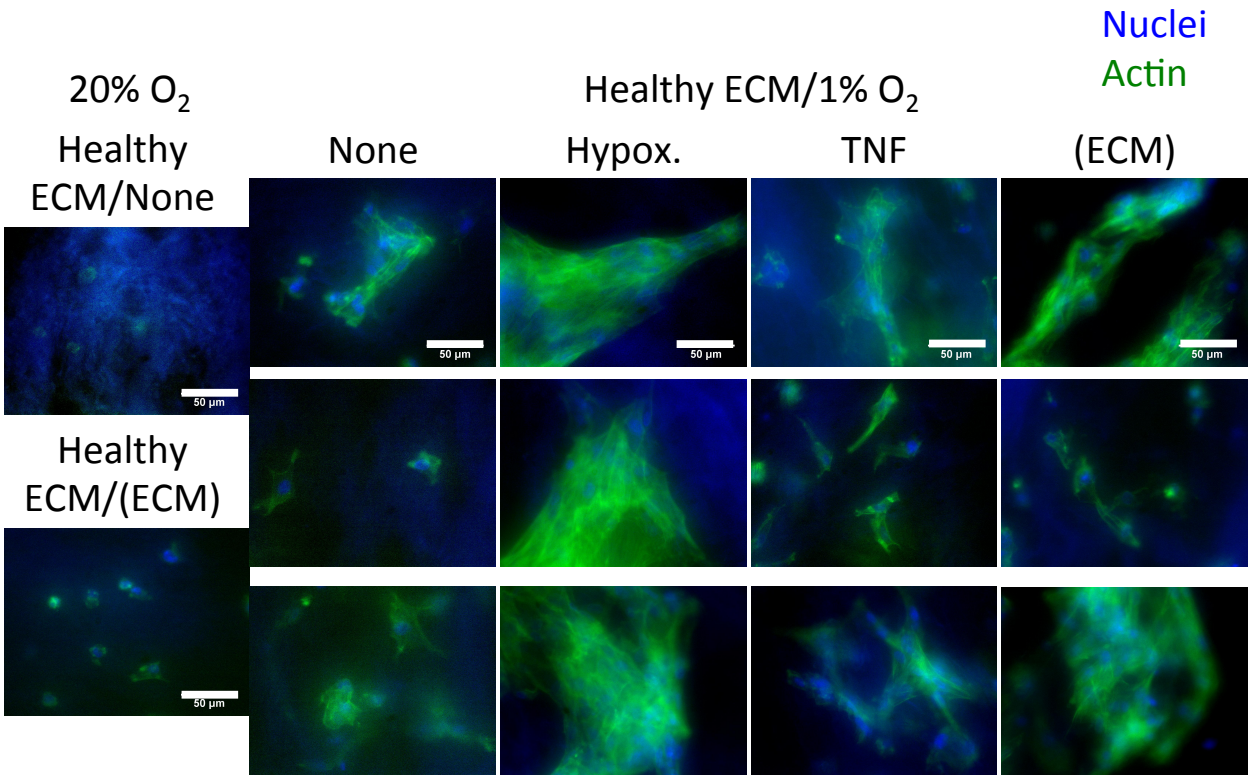


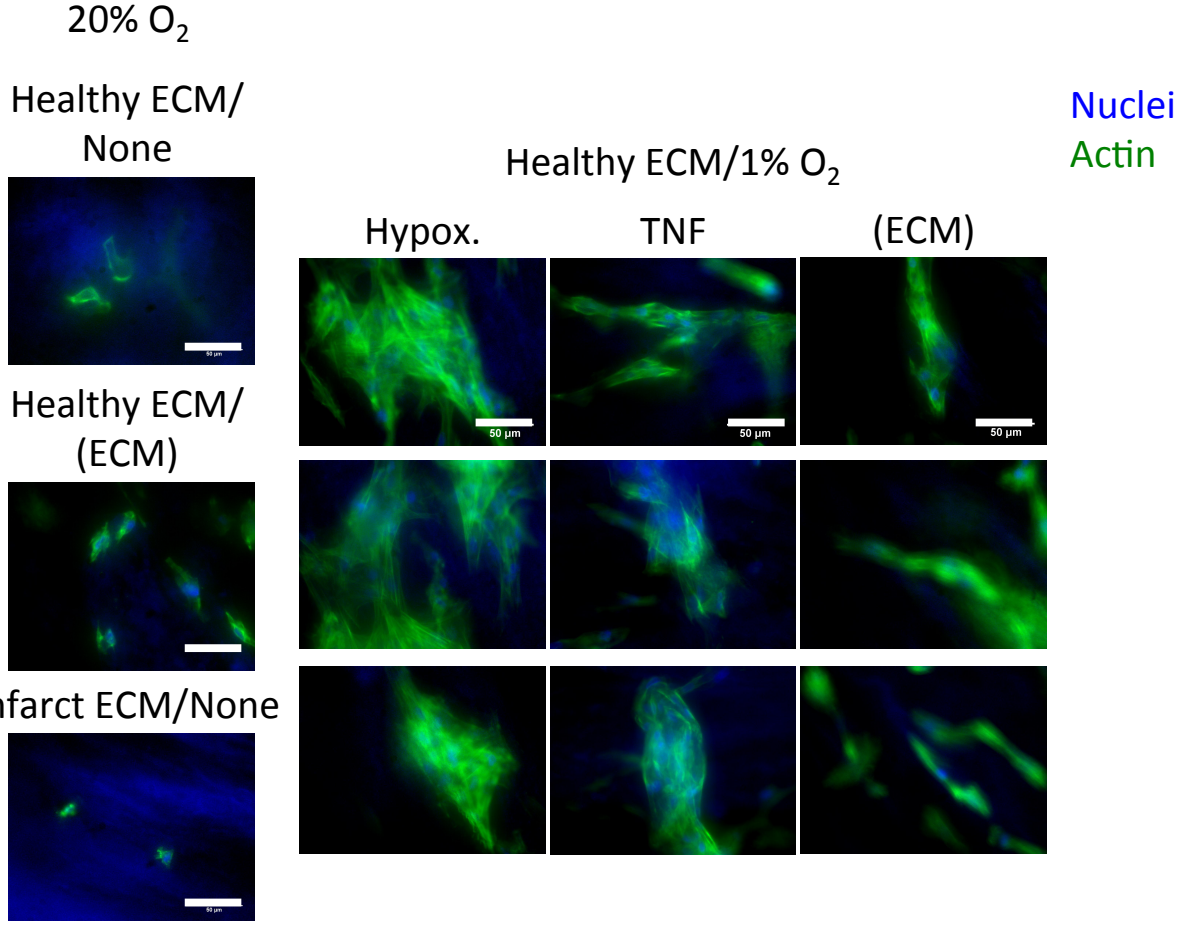
Figure 5. Preconditioned cells seeded on TCP and cultured for 2 weeks under normal oxygen tension (20%). 3 representative images of each condition. All scale bars are 50 μ m. Actin in cells is stained with phalloidin (green) and nuclei are stained with DAPI (blue). (ECM) represents delivery with solubilized ECM.

(Following page) Figure 6. Preconditioned cells were seeded in the cardiac models and imaged after 5 days (A) and 4 weeks (B) of culture. All scale bars are 50 μ m. Actin in cells is stained with phalloidin (green) and nuclei are stained with DAPI (blue). Three representative images are displayed for all preconditioned cells. (ECM) represents delivery with solubilized ECM.

A


Nuclei
Actin
20% O_2
Healthy ECM/1% O_2
Healthy ECM/None
None
Hypox.
TNF
(ECM)
Healthy ECM/(ECM)
50 µm

B


20% O_2
Nuclei
Actin
Healthy ECM/ None
Healthy ECM/1% O_2
Hypox.
TNF
(ECM)
Healthy ECM/ (ECM)
Infarct ECM/None
50 µm

4.3 Ion Channel Characterization

4.3.1 Initial trends between lines

Channels tested included Nav1.2 (sodium channel), HCN4 (potassium/sodium channel), Slo1BK (potassium channel), and RyR-2 (calcium channel). c-kit was also measured to monitor changes in levels of stemness. Each line began with a unique level of expression of all ion channels (Fig. 7). In some cases, the initial relative expression of these channels was significant between lines. Nav1.2 expressed in line 1 at day 0 was significantly greater than that expressed in lines 2 and 3. The level of c-kit expressed in line 4 was significantly

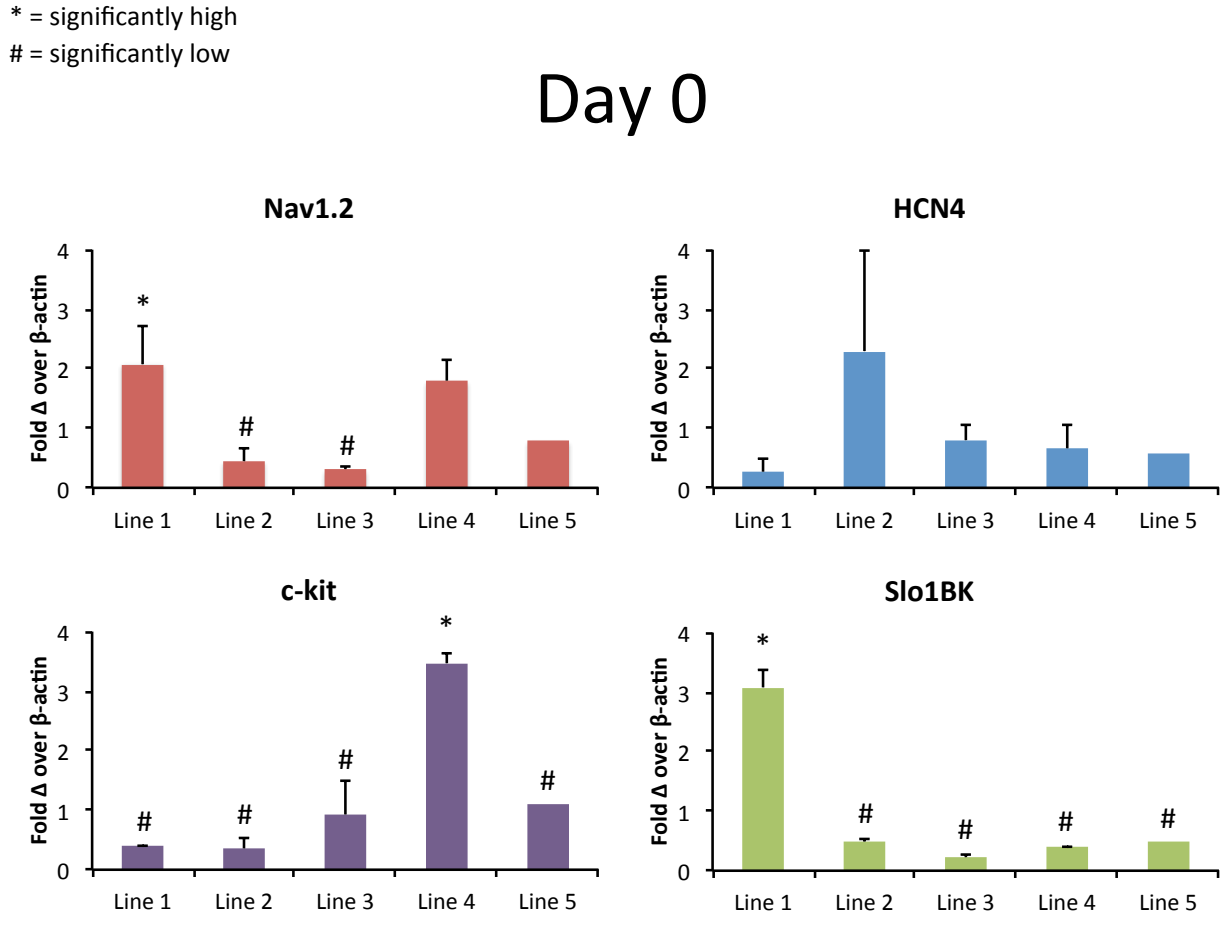


Figure 7. Ion channel expression of human c-kit+ CPCs before culture. Significance was determined with one-way ANOVA's.

greater than that expressed in all the other lines. The amount of Slo1BK channels expressed in line 1 was also greater than in any other lines.

4.3.2 Post-culture trends

The expression of ion channels changed when cultured on various substrates in different culture media for two days. Not enough cellular protein was isolated from the PLL cultures to accurately compare ion channel expression in those samples. In line 2, Nav1.2 was upregulated significantly on fetal ECM in media with serum over all other conditions and on fetal ECM in serum-free media over adult ECM and TCP in media with serum (Fig. 8A). HCN4 was also significantly increased on fetal ECM in media with serum over all other conditions. c-kit expression did not change significantly in line 2. Slo1BK was significantly upregulated on fetal ECM in media with serum over adult ECM in serum-free media.

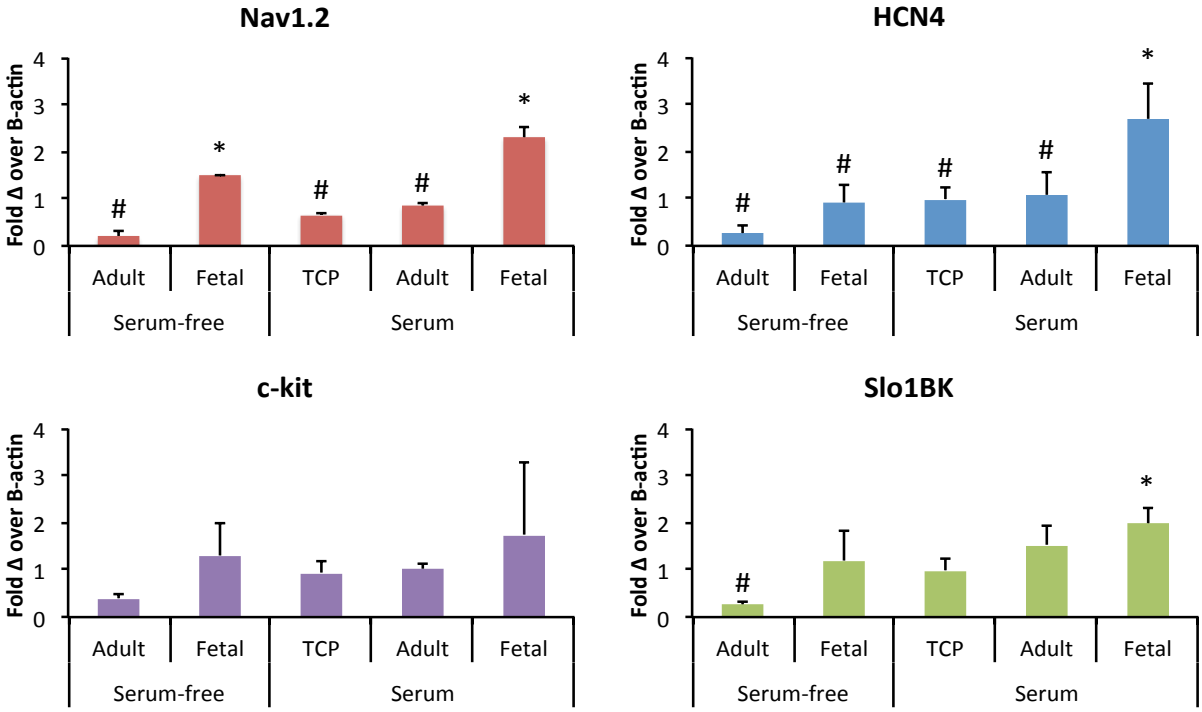
In line 3, there were no significant relative changes in the expression of the identified ion channels between culture conditions (Fig. 8B).

(Following page) Figure 8. Ion channel expression in Line 2 (A) and Line 3 (B) after two days of culture on different substrates (first line of horizontal labels) in different media formulations (second line of horizontal labels). Significance was determined with one-way ANOVA's.

A

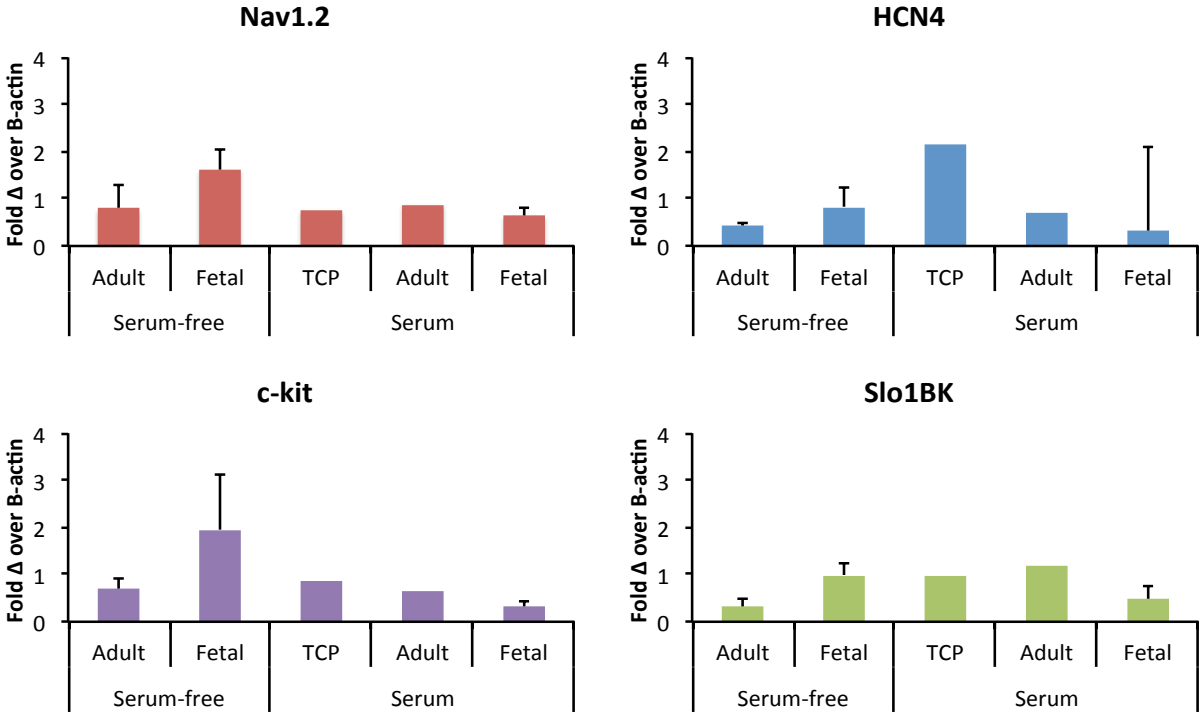
* = significantly high
# = significantly low

Line 2



B

Line 3



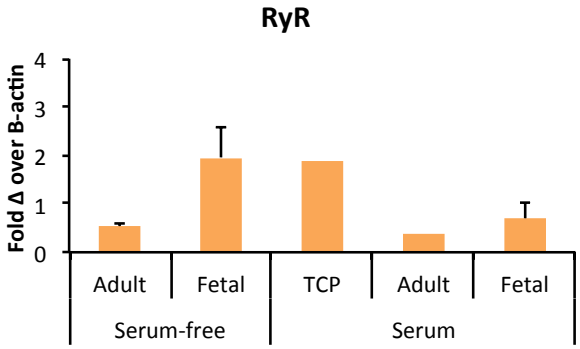


Figure 9. RyR-2 expression in HRAA Line 3.

Line 3 saw expression of RyR-2 when Line 2 and Day 0 did not (Fig. 9). Relative levels of expression were not determined to be significant.

5. Discussion

5.1 Preconditioning strategies affect cell fate

One major environmental change that cells encounter when introduced to the infarct region is reduced oxygen content (1). While this can be harmful to cells, controlled hypoxic preconditioning has been shown to induce protective responses that can prepare them to thrive in the hostile infarct environment, which would be useful in cell therapy applications to improve cell uptake (2). While enhanced proliferation has been reported for a few types of stem cells under hypoxic conditions (3-7), it had yet to be investigated for c-kit+ CPCs before this study.

Preconditioning CPCs under hypoxic conditions encouraged their differentiation and improved their performance in the *in vitro* model. Particularly, hypoxic preconditioning of CPCs encouraged differentiation toward CM's, adhesion, and proliferation. This means that preconditioning cells in lower levels of oxygen tension, more like that they would see in native or infarct myocardium after isolation from the patient, could better prepare them for implantation and enhance their performance in the infarct environment. This type of treatment could predispose the CPCs toward contractile CMs, which is a critical step towards restoring cardiac function post-MI. Enhancing their adhesion and proliferation would also be a step towards solving the problem of low cell retention in cell therapies.

The other implantation strategies studied did not end up having as significant effects as hypoxia. Delivering cells with solubilized ECM possibly could have been too much with the model system and prevented cells from adhering properly. Perhaps the TNFαR antagonist was not delivered in a high enough quantity to significantly enhance cell viability or it also

did not perform well in the model system. Whatever the case, hypoxic preconditioning holds the most promise for enhancing c-kit+ CPCs regenerative potential in this *in vitro* model.

5.2 Ion Channel Characterization

If a small-molecule treatment for c-kit+ CPCs were developed to hyperpolarize certain ion channels and encourage cell differentiation, any variation in the expression of ion channels between individuals could alter the efficacy of the treatment across the population of patients (8-10). Therefore, it is important to understand this variation before creating the treatment to target ion channels that are largely conserved between cell populations. By characterizing five human cell lines of c-kit+ CPCs, it was determined how channel expression varies both before and after culture. It is important to observe how changing culture conditions further affects the variation to determine which factors have a significant influence on channel expression.

The ion channels characterized in this study included those that play various important roles in the electrical activity of CMs and are present in both c-kits and CMs. Each cell line had unique ion channel expression both before and after culture. Overall, culture conditions appeared to affect cell lines differently, with line 2 experiencing significant channel upregulation on fetal ECM, while line 3 saw no significant changes in channel expression post-culture. These changes in channel expression need to be considered when designing a differentiation protocol using hyperpolarization for cells cultured on different substrates, because it could change the treatment.

The expression of RyR-2 in line 3, and not in line 2 or day 0, suggests that line 3 could have inherently possessed greater differentiation capabilities, as RyR-2 is present in differentiated CMs (11). Variation in differentiability would also change treatments for cells, in that some cell lines may need longer treatment or more hyperpolarizing molecule to achieve the same levels of differentiation as other lines. While some work has been done to investigate line variation in differentiation in some types of cells (12), this would be very important to investigate further in c-kit+ CPCs before relying on them as a source for clinical treatments.

5.3 References

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6. Conclusion

Hypoxic preconditioning of c-kit+ CPCs increased CM differentiation, adhesion, and proliferation in these cardiac models. This type of strategy could be used to enhance cell uptake and performance in cell therapy treatments. None of the other implantation strategies or culture conditions had a significant effect on these parameters. The HRAA cell line ion channel expressions varied significantly initially. After culture, there were significant changes based on culture conditions in line 2, but not line 3. In line 2, channel expression was generally upregulated by fetal ECM. Line 3 displayed greater differentiation potential than line 2 in its expression of RyR-2. These types of differences between lines could affect small-molecule treatments designed to hyperpolarize and differentiate cells before use in cell therapy.

All of these findings could contribute to the development of pretreatment strategies to enhance the reparative capabilities of CPCs introduced to the infarct environment. Increasing the sample size in the study of implantation strategies with a focus on hypoxic preconditioning, possibly at different levels of oxygen, could yield more significant conclusions on ways to enhance its efficacy. The cardiac infarct model used in these studies could be further refined to be used to study different implantation techniques or culture parameters. Understanding the variation in the expression of ion channels between patients could aid in the development of a small molecule treatment to hyperpolarize particular channels and encourage the differentiation of CPCs. The possible differences in differentiation abilities between cell lines could also be investigated in CPCs to aid in the development of cell therapy treatments. Overall, this research contributes to the

optimization of cell therapy techniques that will hopefully reach clinical stages to heal damaged myocardium in the future.