Summer 8-15-2013

Engineering Poly(ethylene glycol) Materials to Promote Cardiogenesis

Amanda Walker Smith
Washington University in St. Louis

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Engineering Poly(ethylene glycol) Materials to Promote Cardiogenesis
by
Amanda Walker Smith

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2013
St. Louis, Missouri
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>Am</td>
<td>amine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CM</td>
<td>cardiomyocyte</td>
</tr>
<tr>
<td>CSC</td>
<td>adult cardiac stem cell</td>
</tr>
<tr>
<td>CTE</td>
<td>cardiac tissue engineering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dubecco's modified eagle's medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>FB</td>
<td>fibroblast</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hESC</td>
<td>human embryonic stem cell</td>
</tr>
<tr>
<td>hMSC</td>
<td>human mesenchymal stem cell</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>lam</td>
<td>laminin</td>
</tr>
<tr>
<td>Mat</td>
<td>Matrigel</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>mESC</td>
<td>mouse embryonic stem cell</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>OSKM</td>
<td>Oct4, Sox2, Klf4, c-Myc (Yamanaka factors)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDMS</td>
<td>polydimethylsiloxane</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PSC</td>
<td>pluripotent stem cell</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TCPS</td>
<td>tissue culture polystyrene</td>
</tr>
<tr>
<td>TTF</td>
<td>tail tip fibroblast</td>
</tr>
<tr>
<td>VS</td>
<td>vinylsulfone</td>
</tr>
</tbody>
</table>
Acknowledgements

First of all, I would like to thank my advisor, Dr. Donald Elbert, for the excellent guidance I have received over the past 4 years. His positive outlook, enthusiasm for the project, and willingness to help troubleshoot technical issues has helped to make graduate school a fun and gratifying experience. Don has been a constant source of creative ideas, but has also given me the flexibility to work on projects that I find most interesting, for which I am truly grateful. I would also like to thank Dr. Igor Efimov for the continuous encouragement, insights, and support that he has provided to this project. Finally, I would like to thank Dr. Shelly Sakiyama-Elbert for providing mentorship that ranged from classes to research to careers to everyday life. Thank you all so very much.

Secondly, I’d like to thank the members of the Elbert lab (past and present), Casey Donahoe, Peter Nguyen, Megan Flake, Evan Scott, Claire Segar, Jake Hoyne, and Jacob Roam, for making our workplace a fun, easygoing, and collaborative environment. You have all been great colleagues in your willingness to evaluate new ideas and offer your advice. I would also like to thank the members (past and present) of the Sakiyama-Elbert and Efimov labs for their collaborative and helpful attitudes, particularly: Christina Ambrosi, Qing Lou, Philip Johnson, Matt Wood, Nithya Jesuraj, Di Lang, Deborah Janks, Jacob Laughner, Sarah Gutbrod, Dylan McCreedy, Laura Marquardt, Thomas Wilems, Hao Xu, Xi Lu, Jessie Butts, Chelsea Brown, and Nisha Iyer.

My committee has been very supportive and helpful, and I would like to thank Drs. Jeanne Nerbonne, Robert Mecham, and Guy Genin for agreeing to be on it and for offering great insights. I would also like to thank the American Heart Association for awarding me a predoctoral fellowship (11PRE7690043). Their support has been essential to this project and for that I am immensely grateful.

My family has made me who I am, and without their support I may not have had the confidence to go for a Ph.D. First, I want to thank my husband, Jody, for being supportive of everything I aspire to do, and for continuously looking for ways to help me get there. I am so lucky to have found you. I want to thank my dad, David Walker, who may be my biggest cheerleader… thank you for always saying how proud you are of me. I want to thank my sister, Melissa, for generally making everything about life more fun and exciting, especially when I’ve been working too much.

Finally, I want to thank my mom, Gail Walker, for setting high expectations because she knew that I could meet them, even when I doubted myself. Thank you for always believing in me. Thank you also for getting me a big screen microscope toy, for bringing hearts and brains to my middle school, and for convincing me that atoms were real.

Amanda Walker Smith

Washington University in Saint Louis
May 2013
ABSTRACT OF THE DISSERTATION

Engineering Poly(ethylene glycol) Materials to Promote Cardiogenesis

by

Amanda Walker Smith

Doctor of Philosophy in Biomedical Engineering

Washington University in St. Louis, 2013

Professor Donald L. Elbert, Committee Chair

Heart failure is one of the leading causes of death worldwide, and the current costs of treatment put a significant economic burden on our societies. After an infarction, fibrotic tissue begins to form as part of the heart failure cascade. Current options to slow this process include a wide range of pharmaceutical agents, and ultimately the patient may require a heart transplant. Innovative treatment approaches are needed to bring down costs and improve quality of life. The possibility of regenerating or replacing damaged tissue with healthy cardiomyocytes is generating considerable excitement, but there are still many obstacles to overcome. First, while cell injections into the myocardium have demonstrated slight improvements in cardiac function, the actual engraftment of transplanted cells is very low. It is anticipated that improving engraftment will boost outcomes. Second, cellular differentiation and reprogramming protocols have not yet produced cells that are identical to adult cardiomyocytes, and immunogenicity continues to be a problem despite the advent of autologously derived induced pluripotent stem cells. This dissertation will explore biomaterials approaches to addressing these two obstacles.
Tissue engineering scaffolds may improve cell engraftment by providing bioactive factors, preventing cell anoikis, and reducing cell washout by blood flow. Poly(ethylene glycol) (PEG) is often used as a coating to reduce implant rejection because it is highly resistant to protein adsorption. Because fibrosis of a material in contact with the myocardium could cause arrhythmias, PEG materials are highly relevant for cardiac tissue engineering applications. In Chapter 2, we describe a novel method for crosslinking PEG microspheres around cells to form a scaffold for tissue engineering. We then demonstrate that HL-1 cardiomyocyte viability and phenotype are retained throughout the fabrication process and during the first 7 weeks of culture.

In the third chapter of the dissertation, we demonstrate that the use of PEG cell culture substrates can improve efficiency of direct reprogramming from fibroblasts to cardiomyocytes for cell transplantation. Standard tissue culture plastic adsorbs proteins from the cell media, increasing experimental variability via non-specific signaling. Because of its protein resistant properties, PEG provides cells with highly specific signals. In addition to improving the efficiency, we found that presentation of RGD peptides stimulated proliferation during reprogramming. Combined, the improvements enabled us to approximately double the number of cardiomyocytes produced by the protocol.

In Chapter 4, we explore the effects of 3D culture on the direct reprogramming protocol described in Chapter 3. We demonstrate that the variables involved in 3D culture, including scaffold material, diffusion, cellular remodeling, and scaffold topography, have significant effects on reprogramming efficiency. This chapter provides the groundwork for future studies developing 3D microenvironments for efficient and scalable reprogramming to cardiomyocytes.
CHAPTER 1

Introduction

1.1 The role of biomaterials in cardiac tissue engineering

Nearly a hundred years ago, Ross Harrison, a Yale zoologist who is often considered to be the “father of tissue culture” (1), noticed that environmental stimuli were commonly reported to affect developing tissues, whether it be fish embryo chromatophores responding to circulating blood (2) or nerve guidance by chemotactic

Figure 1.1: Early examples of materials used in cell culture. A) cells migrating out from embryos onto spiderwebs, adapted from Harrison, 1914. B) Stretching fibrin clots was known to induce cell elongation (adapted from Weiss, 1952)
factors (3). In his own lab, he had noticed that cells embedded in firmly clotted lymph took on a more migratory phenotype than those suspended in unclotted lymph droplets. This made him curious about the effects of physical stimuli cell phenotype. In his 1914 paper, “The reaction of embryonic cells to solid structures” he describes diligently collecting Tegenaria spiders from “old tree stumps” to create webs upon which he could culture embryonic cell clusters. In perhaps the first description of cellular reactions to culture materials, Harrison reported that spider webs provided paths for “cell wandering”, or migration along the web (4) (Fig. 1a).

Tissue culture was founded by growing three-dimensional (3D) explants or embryos in lymph or fibrin (5). However, 2D protocols were widely adopted by the field in the 1920s, after Warren and Margaret Lewis developed them in order to investigate the morphological details and compartments of single cells (6). These protocols allowed for easier visualization of cellular responses, and also gave scientists greater control over many variables (which will be discussed in Chapter 1.2 and Chapter 4). Recognizing the importance of cellular interactions with the extracellular environment, Paul Weiss re-introduced the fibrin clot culture protocols in the 1950s. In a particularly notable experiment, he described the tendency of cells to elongate in response to stretching the fibrin substrate (Fig. 1b) (7).

In the 1970s, multiple groups noticed that when dissociated cardiac harvests were reaggregated into 3D structures, they bore a closer resemblance to native tissue (8, 9). In 1977, Horres, Lieberman and Purdy engineered a 3D preparation with high mechanical integrity by culturing the cells onto nylon fibers (10). This work, which was inspired by
a need for easier “experimental manipulation” from 3D preparations, may mark the introduction of the “synthetic scaffold” to the field of cardiac tissue engineering (CTE).

Heart failure is one of the largest public health problems, and will ultimately affect 20% of Americans who are currently forty years old (11). Age-related fibrosis around the sinoatrial node is another major health concern (12-16). Pharmaceutical agents are the most common treatment, and in the worst case scenario a patient may be placed on a wait list for a heart transplant. However, the drugs may have serious side effects, and mortality rates on the transplant list hover around 10% per year (17). Over the past 30 years, research in the field of CTE has developed with the goal of fully repairing or replacing diseased myocardium. Research in this area usually takes one of four forms; 1) injection of cells expected to provide therapeutic benefit to the damaged area, 2) implantation of in vitro engineered cardiac tissue (often called a cardiac patch) to either replace the infarct zone or support it from the outer surface, 3) in vivo genetic alterations to return cells to cardiac phenotypes, or 4) introducing materials to induce resident stem cell homing. Materials engineering principles are being applied in all of these research areas. I will discuss the first and second in some detail. The third and fourth are beyond the scope of this dissertation, but the reader can find thorough reviews elsewhere (genetic manipulation: (18-20), stem cell homing: (21, 22)). For both cell injections and engineered tissue implants, a major decision is the type of cell to use.

1.2 Cardiac cell sourcing

1.2.1 Pluripotent stem cell derived cardiomyocytes
The ability to expand mouse embryonic stem cells (mESCs) *in vitro* (23, 24) was the 1981 milestone that ignited the field of regenerative medicine. It provided a platform for researchers to unravel embryonic development mechanisms and a hope of ultimately engineering tissue replacements. Within 4 years, Doetschman *et al.* described the differentiation of mESCs to cardiomyocytes (mESC-CMs) (25). However, it would be nearly 20 years before human ESCs could be expanded in culture (26) and differentiated to cardiomyocytes (27), highlighting major differences between the species. Nonetheless, murine stem cell studies continue to provide preliminary research that jump-starts human cell research.

In addition to ethical concerns, a major obstacle for ESC-derived transplants is the potential for immune rejection due to their allogenic origin. In 2006 and 2007, Yamanaka and colleagues introduced the induced pluripotent stem cell (iPSC), providing a less contentious, autologous, and (presumably) safer alternative. Human and mouse iPSCs are generated by activating a combination of four transcription factors (collectively called the Yamanaka factors) (28, 29), which induce epigenetic changes to reset the genetic expression profile of the cell (30). Using the knowledge gained from ESC studies, several groups quickly demonstrated that mouse and human iPSCs could be differentiated to cardiomyocytes by similar protocols (31-34). In 2007, LaFlamme *et al.* described a growth factor based protocol that drastically increased efficiency of cardiomyocyte derivation to ~30% (35). More recently, small molecule differentiation has been shown to promote cardiogenesis with 98% efficiency (36), providing a cheaper, more efficient, and more stable alternative to growth factor based protocols.
While iPSCs initially seemed like the perfect alternative to ESCs, recent studies have found that the two may not be as similar as originally thought. Derivatives of both cell types carry carcinogenic risk unless they are immaculately purified (37, 38), and at least one group has reported that iPSC cells develop into more aggressive tumors than ESCs (39). Unlike ESCs, iPSCs are sourced from adult somatic cells, which may have accumulated mutations over time. The reprogramming process also introduces new opportunities for genetic errors. Mutations resulting in uncontrolled proliferation and resistance to apoptosis (tumorogenic qualities) are evolutionarily favorable traits within the reprogramming cell population (40, 41), which may explain why mutations at known oncogenic genes are more common in iPSCs (42). These errors are in addition to genetic aberrations that appear with repeat passaging, as is seen with hESCs (43-46). Errors that occur during reprogramming may help to explain a surprising recent study that found iPSCs to be immunogenic in syngenic hosts (47). Another problem with iPSCs is “epigenetic memory”, or remnants of epigenetic modifications from the original cell (48-50). Cells with epigenetic memory show differentiation biases toward the germ layer of the cell source (51).

A recent study by Newman and Cooper (52) reanalyzed gene expression data from seven different laboratories that had previously compared iPSCs to ESCs. Many of these groups had reported distinct differences between the two cell types, but the differences found were not consistent between labs. Newman et al. performed a computational meta-analysis of the data. They determined that gene expression signatures were strongly correlated with the laboratory of origin, calling the previous conclusions into question. They wrap up the paper with a call to consider the effects of
the microenvironment on reprogramming processes. In chapter 1.2, I will discuss how materials can be used to control microenvironmental variables.

Despite the obstacles, *in vivo* studies in small mammals have demonstrated modest improvements after injection of pluripotent-cell derived cardiomyocytes (35, 53, 54) or even undifferentiated pluripotent cells (55, 56). Actual cell engraftment is quite low, suggesting that the benefits are likely related to paracrine effects (35, 57). Due to the hurdles outlined above, the progress of large animal *in vivo* studies has been slow, especially when considering that human trials of adult stem cells injections are well under way (58). However, the potential for unlimited expansion of pluripotent stem cells necessitates that we continue to explore this area of research, as the sheer number of cells needed for these types of therapies is quite large (59).

### 1.2.2 Adult stem cell derived cardiomyocytes

While embryonic and induced pluripotent stem cells have the capacity for greater scalability, many groups are studying adult stem cell techniques due to the concerns outlined above. This area of research is quite broad, and generally beyond the scope of this dissertation. However, the field merits some discussion since several human trials are already underway, many showing modest improvements in cardiac function (34, 58, 60). The majority of groups working with adult derived stem cells are using bone marrow derived mesenchymal stem cells (MSCs). When Orlic *et al.* (61) and Strauer *et al.* (62) first provided evidence that MSCs induced myocardial regeneration and functional improvement in 2001, it was unclear if the benefits were related to true differentiation of the MSCs to cardiomyocytes, or to some other effects (63, 64). This
controversy has still not been fully settled \((58, 65)\). However, since actual stem cell engraftment is low (~5%) \((66, 67)\), most groups now agree that increased vascularization \((68)\) and paracrine signals from the transplanted MSCs play a much larger role \((58, 69)\) than any differentiation. One such paracrine mechanism may be the activation of resident adult cardiac stem cells (CSCs), which normally play only a minor role in cardiac regeneration \((70)\). Interestingly, CSCs themselves can be harvested, expanded \textit{in vitro} \((71, 72)\), and injected back into damaged myocardium. Cardiac ejection fraction (a common and useful functional endpoint, meaning the volumetric fraction that the heart pumps out during contraction) has been improved in a number of studies in different animal models. As with MSCs, these improvements appear to be more closely related to paracrine signaling since CSC engraftment is low \((73)\). While studies are promising, the clinical adoption of this cell source will likely require functional improvements of a much higher magnitude \((58)\).

\subsection{1.2.3 Direct reprogramming}

Direct reprogramming protocols are a recent addition to the list of potential cardiomyocyte sources, and have generated much excitement over the past few years. Direct reprogramming (also referred to as direct conversion or transdifferentiation) refers to a phenotypical switch that does not involve going through the pluripotent state, and has been reported for multiple different lineages (recently reviewed by Morris \textit{et al.} \((74)\)). In 2010, Ieda \textit{et al.} first reported direct reprogramming of mouse fibroblasts to cardiomyocytes using a combination of transcription factors (Gata4, Mef2c, and Tbx5) crucial to cardiac development \((75)\), and several variations of that protocol have since
been published (76, 77). The conversion of fibroblasts to iPSC is usually on the range of 1% (78), and cardiac differentiation efficiency from there is as high as 98% (36), giving an overall fibroblast to cardiomyocyte efficiency of about 1%. Ieda et al. reported higher efficiency conversions (~5% troponin-T positive) of fibroblasts directly to cardiomyocytes by 4-5 weeks (75), as opposed to ~6-7 weeks for fibroblast to iPSC to cardiomyocyte derivations (36, 79).

Potentially the most exciting application of this research is the potential to reprogram scar tissue fibroblasts to cardiomyocytes in vivo, potentially negating the need for cell transplants. This has been demonstrated in small mammals by multiple groups (77, 80-82), one that used micro-RNAs (1, 133, 208, and 499) instead of the transcription factors (82). Up until recently, all research has been performed using mouse somatic cells. In March of 2013, the first article reporting direct reprogramming of adult human fibroblasts to cardiomyocytes was published online (83), confirming the potential for clinical use. This protocol used a combination of transcription factors (Gata4, Hand2, Tbx5 and Myocardin) in addition to miRNAs-1 and -133.

Less than a year after the first report of direct reprogramming to cardiomyocytes, the Deng group demonstrated that similar results could be achieved using a fundamentally different method. Efe et al. briefly activated the Yamanaka factors (normally used to generate iPSCs) to push cells into an epigenetically unstable state, while simultaneously inhibiting pluripotentcy pathways (84). After 9 days, they introduced BMP4 to stimulate cardiogenesis. By day 18, they reported widespread beating and ~40% troponin-T positive cells. During the process, they did note a small population of pluripotent cells, but demonstrated that these cells were not the source of
the cardiomyocytes. Instead, the pathway led through a proliferative progenitor state that is known to also produce cardiac vasculature (84, 85).

The protocol described by Efe et al. is faster than the one by Ieda et al., which may be clinically important as the cardiac remodeling cascade starts immediately following infarction (86, 87). The possibility of forming the other myocardial cell types is a huge advantage, as revascularization will be important to myocardial scar healing. Several groups have noted the improvements in cardiomyocyte organization in vitro (88) and cardiac function in vivo when a vascular system is also provided within the construct at implantation (“prevascularization”) (89, 90). Further characterization of this protocol is needed to quantify endothelial and smooth muscle cells differentiation efficiency.

Despite the advantages listed above, excitement for the Efe et al. protocol is tempered by the formation of pluripotent byproducts (Nanog levels were low but detectable by day 18 (84)). This teratoma risk will have to be eliminated before any human trials could take place. Unlike other protocols, it has not yet been replicated with human cells, or shown to work in vivo. Furthermore, all direct reprogramming protocols may encounter the same “epigenetic memory” problems that have plagued iPSC research. Nonetheless, this area of research is growing and advancing quickly (91), and these hurdles may soon be irrelevant. Further detail and discussion of direct reprogramming can be found in chapter 3 of this dissertation.

1.3 Materials for stem cell microenvironment engineering
The meta-analysis that found gene expression of reprogrammed cells to correlate strongly with the lab of origin (52) elucidated the effects of the microenvironment on stem cell research. The “microenvironment” can encompass a wide range of variables, including oxygen levels, temperature, pH, and media components. However, one that often goes unappreciated in stem cell research is the culture surface, which in reality should be broken down into several additional variables. Cell adhesion to the extracellular matrix (ECM) is accomplished through cell-surface integrins that link to the cytoskeleton (92, 93). Cytoskeleton-mediated intracellular pathways may then affect cell phenotype, making ECM-integrin interactions important to differentiation and reprogramming (94-99). Cellular phenotype can be affected by ECM protein types (100), spacing (7, 101) and concentrations (102), as well as substrate topography (103-107) mechanical properties (108, 109), and cell shape (110, 111).

While these factors are known to have profound effects on stem cells (100, 112), the majority of differentiation and reprogramming studies still use tissue culture polystyrene (TCPS) as the cell culture substrate. The topographical and mechanical features of TCPS (flat and stiff) may be sub-optimal for differentiation to many cell types. For example, in Engler et al., mesenchymal stem cells cultured on very hard substrates preferred osteogenic differentiation, whereas those cultured on softer substrates preferred myogenic or neurogenic pathways (112). To provide a closer mimic the natural environment, ECM proteins are often electrostatically adsorbed to the TCPS prior to cell seeding. However, proteins from serum in the media may also adsorb, and these may activate non-specific intracellular pathways that vary by serum batch. While the breakneck speed of stem cell advances makes it inconvenient to question long-established
methods, true therapeutic value has not yet been realized. Innovative approaches are needed to increase the value of stem cell research.

Multiple examples exist in the literature where engineering the stem cell microenvironment has changed differentiation patterns. In 2004, McBeath et al. demonstrated that the size of “cell islands” (micropatterned ECM) affected the extent of hMSC spreading, which in turn dictated their differentiation to either adipocytes (small islands) or osteoblasts (large islands). They determined that the cytoskeletal shape affected the RhoA pathway, which in turn affected the differentiation (110). Later, Kilian et al. demonstrated that cell shape still affected hMSC differentiation even if the patterned area (and therefore, number of cell-to-ECM contacts) was held constant (111). Nanotopography has shown to dictate differentiation outcomes to neural (104) or osteogenic (105) phenotypes, likely by changes in cytoskeletal organization (101, 113). In addition to the role of substrate stiffness on hMSC differentiations (112), mESCs may also be affected by substrate stiffness, with softer substrates being better suited for the maintenance of pluripotency (114). Studies with hESCs have recently shown that culture under biaxial strain helps to maintain pluripotency via the TGFβ-Activin-Nodal pathway (99, 115).

In addition to the effects of ECM composition, stiffness, topography and other mechanical factors, natural stem cell niches are 3D environments. Culture in 2D forces cell polarity, changes the membrane-to-cytoplasm ratio, and alters the cell shape (116), all of which have physiological repercussions (110, 111, 117, 118). This might help to explain drastic phenotypical differences that are often seen between 2D and 3D cultured cells (119-123). Schenke-Layland et al. recently demonstrated that designing 3D stem
cell culture to mimic *in vivo* CSC niches improved differentiation from mESCs to cardiac progenitors (124). However, variations cannot be attributed solely to differences in dimension, since 3D environments incorporate all of the variables mentioned above, as well as diffusion limitations not present in 2D culture. Nonetheless, 3D environments more closely mimic natural environments, while simultaneously addressing scalability problems encountered with 2D culture (59). Further discussion on 3D stem cell culture, as well as additional examples from the literature, can be found in Chapter 4 of this dissertation.

### 1.4 Using materials to improve cell engraftment *in vivo*

As mentioned previously, clinical trials of MSC injections have yielded only modest improvements (0-5% increase in ejection fraction) (125-128), and only ~5% of cells are actually engrafting into the myocardium (66, 67). Anoikis, or apoptosis resulting from lack of adhesion, is a likely reason for this problem (129). Laflamme *et al.* found that including Matrigel during injection to prevent anoikis of hESC-CMs helps to improve engraftment and functional outcomes (35). Similarly, Kutschka *et al.* found that delivery of cardiomyocytes to rat infarcts using collagen-Matrigel composites improved cell engraftment and ejection fraction over injections alone (130). Zimmermann *et al.* demonstrated similar effects when suturing a cardiomyocyte-loaded collagen gel on the surface of infarcted hearts (131).

In addition to anoikis, other reasons that transplanted cells are lost are 1) they are killed going through the needle, 2) they cannot survive the ischemic environment, 3) they are killed by reactive oxygen species (ROS) secreted by inflammatory cells that are
responding to the infarction, or 4) they are washed out by circulation. Materials are being developed to address these issues, as well. For example, Aguado et al. demonstrated that cells encapsulated within a highly compliant alginate hydrogel during injection had a higher rate of survival (132). To combat ischemia, many groups have focused on “prevascularizing” the graft, or including a ready-made capillary network. In one example, cardiomyocyte sheets were fabricated with or without endothelial cells and fibroblasts. The inclusion of endothelial cells enabled perfusion by the host vasculature and improved graft survival (89). Simultaneous delivery of superoxide dismutase, a superoxide scavenger, can reduce cell death due to ROS (133). Delivery of this unstable enzyme can be improved by encapsulating it in polyketal microspheres (134).

Injection of in situ polymerizing hydrogels alone (without cells) may also improve functional outcomes (135-137), possibly by providing structural support, releasing bioactive factors, or recruiting endogenous CSCs (138). Because cells are not involved, human clinical trials are farther along (139). However, the addition of cells within materials remains a promising area of research, since they provide functional improvements over materials alone in small animal models (130, 137, 140). The major challenges are 1) optimizing cell sourcing, 2) ensuring electrical and mechanical coupling with the myocardium, and 3) vascularizing constructs to improve graft viability (141).

Another hurdle to this area of research is difficulty in comparing results between studies. Because the field has developed so recently, preparation protocols, delivery methods, animal models, and functional endpoints are still highly variable between research groups (141). Also, studies often focus on one specific material instead of
comparing several (138), making it difficult to determine which will ultimately perform best.

### 1.5 Poly(ethlyene glycol) biomaterials for cardiac tissue engineering

Poly(ethylene glycol) (PEG) has been described as a “stealth material” and has long been approved by the FDA for clinical use (142). Because of its resistance to protein adsorption and cell adhesion, PEG is a highly favored material for many therapeutic applications. Here, I will discuss its potential in stem cell niche engineering and myocardial cell transplantations.

#### 1.5.1 History of PEG materials

Over long periods of time, most materials adsorb proteins from the blood, triggering the foreign body response that walls off the implant in a fibrotic capsule (143). PEG was first reported to prevent cell adhesion during coagulation studies in the early 1970s (144). Furasawa et al. later demonstrated that including PEG in copolymers decreased protein adsorption (145), which was confirmed by Brash, Whicher, and Uniyal (146, 147). A few years later, it was found that platelet adhesion was lower on PEG surfaces than on PVP surfaces (148). Considering results from his own lab (149-151) in conjunction with various results from other labs, Merrill composed a convincing argument that PEG may be one of the least thrombogenic materials available (152).
These findings supporting PEG as a nonthrombogenic material set off a wave of research into “PEGylation” of previously studied materials, as well as investigation into PEG-copolymers. In 1994, Hill-West et al. demonstrated that balloon angioplasty-induced restenosis could be nearly eliminated by coating the surface of the treated artery with PEG-diacylate (153). In 1993, Hubbell and colleagues developed a PEG-polylactide surgical sealant to use as a preventative measure against adhesions that frequently occur as a result of certain surgical procedures (154). While these performed relatively well in small animals, results in humans were more difficult to assess. However, the sealant has been adapted for use in lung (155) and brain (156) surgeries.

1.5.2 PEG for engineering the stem cell niche

As mentioned previously, the majority of stem cell research studies have utilized tissue culture polystyrene (TCPS) as the culture substrate. In addition to being a poor reproduction of the in vivo environment, TCPS adsorbs proteins from the serum, presenting cells with non-specific signals that may vary by serum batch. Because of its protein-resistant properties, PEG is an excellent candidate substrate for stem cell research studies (157) because it provides a silent “background” for probing the effects of other molecules (158). In addition, PEG can be tuned to adjust the matrix stiffness (159), incorporate bioactive moieties (160-162), and degrade at a controlled pace (163), all of which can affect stem cell differentiation outcomes (112, 164). Recently, Lutolf and colleagues have developed a high throughput method to investigate several of these variables simultaneously in 2D using a PEG microwell array (165). The Khademhosseini and Hubbell groups have recently published interesting studies of cardiac differentiation
from embryoid bodies embedded in PEG gels (159, 166). Further discussion on using PEG materials to engineer the stem cell niche can be found in chapter 3 of this dissertation.

1.5.3 PEG for myocardial cell transplantation

Of the synthetic materials available, PEG is one of the least likely to cause protein adsorption and fibrosis. For cardiac tissue engineering, this is highly important as fibrosis could lead to arrhythmias and death. Most materials used so far in the cardiac setting are degradable to ensure a limited foreign body response. The Suggs group has developed an injectable PEGylated fibrin material for use in cardiac repair (167). This hybrid material provides dual benefits of natural and synthetic materials: the fibrin provides topographical cues, degradation, and adhesion sites, while the PEG acts as a spacer molecule to enhance cell viability and also provides an inert vehicle for delivering growth factors (167). When delivered via injection, the MSC-loaded PEGylated fibrin group had the highest ejection fraction after 4 weeks (as opposed to PEGylated fibrin without cells or cells injected alone) (140).

The Hubbell group has developed injectable matrix metalloproteinase sensitive PEG hydrogels for myocardial repair. In a recent study, these were loaded with hESC-derived endothelial and smooth muscle cells to provide paracrine benefits and vascularization potential, without the complications of electrically and mechanically integrating cardiomyocytes (137). They saw improvements in ejection fraction at 6 weeks in a rat myocardial infarction model. The highest performing group had been
treated with a cell-loaded gel that also included thymosin β4, a pro-angiogenic survival factor. This was significantly higher than improvements made by the gel alone (168).

A recent study by Wu et al. reports low cytotoxicity of a supramolecular PEG-based hydrogel that incorporates poly(caprolactone) for degradation and α-cyclodextrin for enhanced drug delivery (169). Four weeks after injection into a rabbit myocardial infarction, control groups (injection of DMEM alone) demonstrated a 30.3% decrease in ejection fraction. MSC injection alone demonstrated a significant improvement over the control (17.5% decrease, up 12.8% from control), but injection of MSC loaded gels performed the best (6.8% decrease, up 23.5% from control) (170). The delivery of erythropoietin (EPO) from the hydrogels improved outcomes over EPO injections alone and hydrogel injections alone (171).

Several other groups have also recently published in vivo studies analyzing the protective effects of PEG hydrogels after myocardial infarction (172-174), and at this point the number of potentially useful strategies (biocompatible material X cell source X drug to be delivered) seems limitless. A common theme is that the most improvement comes when cells are delivered within a hydrogel. PEG gels alone do not present the same level of improvements in the studies described above or in recent publications by Rane et al. (173) and Dobner et al. (174). The next decade of research in CTE involving cell transplantation will need to focus on starting human trials to test these strategies.
CHAPTER 2*

Long term culture of HL-1 cardiomyocytes in modular poly(ethylene glycol) microsphere-based scaffolds crosslinked in the phase separated state

2.1 Abstract

Poly(ethylene glycol) (PEG) microspheres were assembled around HL-1 cardiomyocytes to produce highly porous modular scaffolds. In this study, we took advantage of the immiscibility of PEG and dextran to improve upon our previously described modular scaffold fabrication methods. Phase separating the PEG microspheres in dextran solutions caused them to deswell and crosslink together rapidly, eliminating the need for serum protein-based crosslinking. This also led to a dramatic increase in the stiffness of the scaffolds and greatly improved the handling characteristics. HL-1 cardiomyocytes were present during the microsphere crosslinking in the cytocompatible dextran solution, exhibiting high cell viability following scaffold formation. Over the course of 2 weeks, a 9-fold expansion in cell number was observed. The cardiac functional markers sarcomeric α-actinin and connexin 43 were expressed at 13 and 24 days after scaffold formation. HL-1 cells were spontaneously depolarizing 38 days after scaffold formation, which was visualized by confocal microscopy using a calcium-sensitive dye. Electrical stimulation resulted in synchronization of activation peaks.

* Chapter 2 has been adapted from the following manuscript: Smith AW, Segar CE, Nguyen PK, MacEwan MR, Efimov IR, Elbert DL. Long-term culture of HL-1 cardiomyocytes in modular poly(ethylene glycol) microsphere-based scaffolds crosslinked in the phase-separated state. *Acta Biomaterialia*. Jan 2012. 8(1):31-40
throughout the scaffolds. These findings demonstrate that PEG microsphere scaffolds fabricated in the presence of dextran can support the long-term three-dimensional culture of cells, suggesting applications in cardiovascular tissue engineering.

2.2 Introduction

Advancements in cardiac tissue engineering (CTE) are currently limited by a number of engineering challenges. Engineered myocardium must contain a dense population of properly aligned and electrically connected cardiomyocytes. Scaffold materials must be extremely resistant to foreign body reaction because fibrous encapsulation might electrically isolate the engineered tissue and cause arrhythmias. Cardiomyocytes are highly metabolically active, form a well-coupled electromechanical syncytium, and must be in intimate contact with the vascular system (175, 176).

Scaffolds may also need to be highly porous to allow passage of nutrients so that cells survive even at the center of a large construct (177-179).

Most methods for producing porous scaffolds utilize processing conditions that are not compatible with cell survival. Consequently, cells must be seeded onto the exterior of a pre-formed scaffold. However, this may result in uneven cell distribution unless steps are taken to promote deeper cell penetration (180-183). To achieve well distributed cell density, cytocompatible crosslinking and pore formation techniques may be needed. Bulk poly(ethylene glycol) (PEG) hydrogels are easily formed in the presence of cells with high cell viability (184-186). However, the porosity of these scaffolds is at the nanoscale and methods to introduce pores are usually not cytocompatible (187-189).

A different approach is the formation of cell scaffolds by crosslinking hydrogel
microparticles in the presence of cells. This technique introduces pores between the microparticles, which may provide greater nutrient diffusion and pathways for cells to migrate and proliferate. Sefton and colleagues have encapsulated cardiomyocytes and other cells in collagen-based modules, which were then coated with endothelial cells. The modules were stacked together, forming constructs with evenly distributed cells and endothelial cell-lined channels (190-192). Additionally, Khademhosseini and colleagues have extensively engineered the self-assembly of cell-laden microgels (166, 193-197). Other modular approaches have been recently reviewed (198).

Modularity is desirable because different microparticles may perform different functions in the scaffold. We have recently developed scalable, aqueous methods for the fabrication of stable microspheres from eight arm PEG-amine (PEG₈-Am) and PEG₈-vinylsulfone (PEG₈-VS), as well as hydrolytically degradable microspheres from PEG₈-Am and PEG₈-acrylate (199, 200). The microspheres were modified with arginine-glycine-aspartic acid (RGD) peptide to enable cell adhesion (201). Microspheres had unreacted VS groups and aggregated by reaction with free amines in serum proteins, enabling assembly of microsphere-based scaffolds. By including both degradable and non-degradable microspheres, scaffolds with porosities on multiple length scales were fabricated around HepG2 liver cells. Some microspheres released sphingosine-1-phosphate (S1P), which promoted endothelial cell migration into the pores of the scaffold (200). We have also utilized microsphere density differences to incorporate growth factor gradients into microsphere-based scaffolds (202).

We continued to engineer these scaffolds with the goal of developing functional, implantable cardiac tissues. Crosslinking of microspheres via serum proteins is
undesirable because it may cause an antigenic response. Furthermore, the mechanical strength of scaffolds crosslinked by the previous method was limited. We hypothesized that by performing microsphere crosslinking in a cytocompatible dextran solution, phase separation between dextran in solution and PEG in the microspheres would result in microsphere aggregation and more rapid reaction kinetics. This may enhance the strength of the scaffolds and eliminate the need for serum proteins to crosslink the microspheres together. PEG/dextran aqueous two-phase systems have been widely used for cellular and molecular partitioning (203-207). Two-phase dextran/PEG solutions have also been used to produce microspheres and alter the porosity of hydrogels (208-212). Dextran solutions were therefore seen as an attractive choice for enhancing the crosslinking of PEG microspheres in the presence of HL-1 cells (a cardiomyocyte cell line), allowing in vitro expansion and long-term culture in the resulting porous 3D scaffolds.

2.3 Materials and Methods

2.3.1 Synthesis of PEG derivatives

Eight arm PEG-vinyl sulfone (PEG₈-VS) and eight arm PEG-amine (PEG₈-Am) were synthesized from eight arm PEG (Creative PEGworks) as previously described (213, 214). Stock solutions of polymers (20%) in phosphate buffered saline (PBS) were sterilized by filtration (0.22 µm, Millipore). For fluorescent labeling, 20% PEG₈-Am and Dylight-633 NHS ester (Pierce) were reacted overnight at 25°C at a 100:1 molar ratio of PEG:dye.
2.3.2 Microsphere formation

PEG₈-VS and PEG₈-Am stock solutions (20%) were mixed at a 2:1 volume ratio and pre-reacted at 37°C for 70% of the time needed to reach bulk gelation (5.25 h). The pre-reacted solutions were aliquoted and stored at -80°C. RGD cell-adhesion peptide (1 mM, sequence: GCGYGRGDSPG, Genscript) was added to the pre-reacted solution and reacted at 37°C for 30 min. For microsphere formation, PBS and 1.5 M Na₂SO₄ were pre-warmed to 37°C. Pre-reacted solution (50 µL, room temperature) was then diluted with pre-warmed solutions in an Eppendorf tube to a final concentration of 2% PEG and 0.6 M Na₂SO₄ (500 µL total). Phase separation occurred upon addition of Na₂SO₄. The mixture was trituration once and quickly transferred to a 37°C heating block. Reaction above the cloud point proceeded for 45 min. Microsphere suspensions were then diluted 3:1 in PBS, centrifuged at 14,100 g for 2 min, and the supernatant was replaced with an equivalent volume of PBS. This was repeated 2x to ensure all Na₂SO₄ was removed. After the final centrifugation, the pellet was resuspended in 500 µL of PBS. The post-swelling microsphere volume was determined by measuring the total volume of solution after the addition of 500 µL of PBS. Microspheres were stored in PBS at 4°C and were stable for at least 6 months.

2.3.3 Microsphere sizing, swelling and aggregation

For microsphere sizing, aggregation was minimized by incubating microspheres in a 350 µM cysteine solution for 2 hr at 25°C to cap free VS groups. Fluorescent microspheres (5 µL) were suspended in 45 µL of PBS, then 350 µL of dextran in PBS was added just prior to imaging to attain the desired dextran concentration. Suspensions
were vortexed for 30 sec, then samples (10 µL) were imaged on a glass slide by confocal microscopy. Microsphere diameters (n > 40 microspheres per group) were measured using ImageJ software (National Institutes of Health) and volumes were calculated accordingly. Reported values are averages from 3 separately made microsphere batches, with error bars representing the 95% confidence interval.

To investigate dextran-induced aggregation, fluorescent microspheres were resuspended at a 1:1 volume ratio with 2%(v/v) fetal bovine serum (FBS, Sigma-Aldrich) or 20% or 40% dextran (M_r = 100,000, Sigma Aldrich) in PBS. Suspensions were vortexed and incubated at room temperature for 15 min. Aggregation of microspheres was assessed by fluorescence microscopy. Dextran concentrations are reported as %%(w/v).

2.3.4 HL-1 culture

HL-1 cells, a gift from Dr. William Claycomb, are a continuously proliferating cardiomyocyte cell line derived from mouse atrial tumors (215, 216). Cells were maintained according to the protocol supplied by the Claycomb lab (217). Briefly, Claycomb Medium (Sigma) was supplemented with 10% FBS, 100 µg/mL penicillin/streptomycin, 0.1 mM norepinephrine, and 2 mM L-glutamine. Supplementation with norepinephrine is key to maintenance of the contracting cardiac phenotype (215-217). Cells from passages 50-70 were seeded at a density of 10^5/cm^2 onto gelatin/fibronectin coated plates, supplied daily with 1 mL medium per 5 cm² culture area, and split 1:3 every 3.5 days. HL-1 monolayers were contractile by day 3 after passage.
2.3.5 Scaffold formation

Scaffolds crosslinked via serum proteins were fabricated as previously described (200). For scaffolds crosslinked without serum proteins, dextran (20% unless otherwise noted) was dissolved in serum-free DMEM if cells were present or PBS otherwise. Scaffolds were made using 50 µL of RGD-containing microspheres. HL-1 cells were removed from flasks by trypsin dissociation, then rotated in 1.65 mL serum-free DMEM in an Eppendorf tube with the RGD-containing microspheres (10⁷ HL-1s/mL microspheres) for 1 h at 37°C to promote cell adhesion (the “adhesion step”). The cells and microspheres were centrifuged at 500 g for 3 min and the supernatant was removed. Dextran-containing serum-free DMEM was added at a 1:1 volume ratio with the microspheres to resuspend the pellet. The suspension was centrifuged at 500 g for 3 min, then transferred to 37 °C/5% CO₂ for 1 h. Scaffolds were transferred to 6-well plates (BD Falcon) containing either Claycomb medium if cells were present, or PBS otherwise. Claycomb medium (4 mL) was replaced daily. Post-swelling dimensions were approximately 8 mm in diameter and 1 mm in thickness.

2.3.6 Microstructural and mechanical analyses of scaffolds

Confocal microscopy was used to enable measurement of scaffold porosity in the hydrated state. At least 8 confocal images per scaffold (n=3) were taken at randomly selected depths and analyzed in Matlab (MathWorks Inc.) to calculate percent porosity (area without microspheres/total area). Scaffolds for oscillatory shear rheometry (RFS3, Rheometric Scientific) were made using 100 µL microspheres and were crosslinked in
Eppendorf tubes (PDMS was used to fill the conical portion of the tubes such that crosslinking occurred in the top of the tube). PBS-hydrated scaffolds (1 mm thick) were die cut to 8 mm and characterized using 8 mm parallel plates, with a normal force of 40 mN to prevent slipping. An amplitude sweep (G’ as a function of strain) was performed to confirm that measurements were in the linear viscoelastic regime. Frequency sweeps (1-100 radians/second) were then performed at 5% strain.

2.3.7 Characterization of HL-1 response to scaffolds crosslinked in 20% dextran

For cell-viability analysis, scaffolds were incubated in serum-free DMEM containing calcein AM (2 µM) and ethidium bromide (4 µM) (Invitrogen) for 30 min. Ten images per scaffold (n=3) were taken at randomly selected depths and analyzed in Matlab to calculate percent viability. For measurement of scaffold cell densities, scaffold volume was measured prior to storage at -80 ºC. At the time of the assay, scaffolds were thawed, mechanically dissociated by pipetting, and suspended to a final volume of 1 mL in dye/cell lysis buffer from a cell quantification kit (CyQUANT®, Invitrogen, excitation/emission: 480/520 nm). Suspensions were vortexed for 2 min, and 5 µL samples were diluted in an additional 195 µL of dye/lysis buffer on a 96-well plate. Spectrofluorometric measurements were compared to a standard curve of known HL-1 densities.

To ensure that cells were well distributed throughout the depth of the scaffold, a thicker (~1.65 mm), fluorescently labeled scaffold was fixed (24 hr after scaffold crosslinking) in 3.7% formaldehyde for 45 min at room temperature then treated with
30% sucrose solution for 3.5 h at room temperature for cryoprotection. Scaffolds were frozen in OCT (Tissue-Tek®, Sakura) at -80°C for 30 min then cryostat sectioned with careful attention to depth of sectioning. Nine evenly spaced serial sections (16 μm thickness) were treated with a nuclear stain (Hoescht 33258, Molecular Probes, 1:1000 in PBS) for 15 min at room temperature prior to imaging by fluorescence microscopy. The first 3 sections were designated as the top region of the scaffold, the middle three as the center, etc., with each region being ~550 μm thick. Fifteen cell counts were made per region, averaged and divided by the average area of microspheres for that region.

For immunohistochemistry (IHC), non-fluorescent scaffolds were prepared for cryostat sectioning as described above. After sectioning, slides were blocked/permeabilized in 10% normal donkey serum/0.1% Triton X-100 in PBS. Primary antibodies were goat anti-connexin 43 (cx43, 1:100 dilution, Santa Cruz Biotechnologies, SC6560) and rabbit anti α-actinin (1:500, Sigma Aldrich, A2543). Alexa Fluor 488-labeled donkey anti-goat (1:1000, Invitrogen, A11055) and Cy5-labeled donkey anti-rabbit (1:100, Chemicon, AP1825) secondary antibodies were used to visualize the staining. Coverslips were added with Vectashield® mounting medium containing DAPI (Vector Labs) for nuclear detection.

2.3.8 Microscopy

For scanning confocal microscopy (Eclipse C1/80i, Nikon), a hydrated scaffold was placed on a glass slide and covered with a circular glass coverslip (150 μM thick). PBS was pipetted under the coverslip to prevent dehydration. For scanning electron microscopy (SEM, NanoSEM 2300, FEI Nova), hydrated scaffolds were fixed in 3.7% formaldehyde for 30 min at room temperature, suspended in diH2O, frozen in liquid
nitrogen, and freeze-dried overnight. Scaffolds were gold-coated (Cressington 108) and imaged at 15 kV. Images of fluorescent microspheres and immunohistochemistry (IHC) were acquired by fluorescence microscopy (IX70, Olympus) using a Magnafire camera (Optronics).

2.3.9 Detection of HL-1 electrical activity

Calcium sensitive Fluo-4 AM (10 mM in DMSO, excitation/emission: 494/516 nm, Invitrogen) was mixed (1:1 volume ratio) with 20% (w/v) Pluronic® F-127 (Invitrogen), diluted to 10 µM in DMEM, and applied to scaffolds for 30 min at 37°C/5% CO₂. Scaffolds were washed 3x in PBS and incubated 15 min in DMEM + 0.1 mM norepinephrine to allow for complete de-esterification of the Fluo-4. Scaffolds were rinsed once in Tyrode’s solution (1.33 mM CaCl₂, 1 mM MgCl₂, 5.4 mM KCl, 135 mM NaCl, 0.33 mM NaH₂PO₄) containing 0.1 mM norepinephrine, 5 mM glucose, and 5 mM HEPES, and this solution was used to keep scaffolds hydrated during imaging.

Norepinephrine is required in the culture media to maintain the beating phenotype over long culture periods, and was similarly added to the Tyrode’s solution (215-217).

Electrical stimuli were delivered to tissue constructs via a bipolar electrode made of two silver microwires (8 mil, A-M systems Inc.) inserted under the glass cover slips and into the Tyrode’s solution surrounding the scaffolds. Sequences of images (sampling rate = 1 - 2 Hz) were acquired by confocal microscopy at a depth of 100 µm into the scaffold while monophasic electrical impulses were applied to the scaffolds (amp = 10 V, duration = 200 msec, frequency= 1 Hz, burst width = 10 sec.) using an isolated pulse stimulator (Model 2100, A-M systems Inc.). Resulting images were analyzed using Matlab.
software for temporal patterns of fluorescence. Average fluorescence intensity was calculated over time for 8x8 pixel regions within the field of view, which were normalized to location-specific background intensity values to remove biases associated with the variability in cell density across the scaffold.

2.3.10 Statistics

Comparisons between 2 means were by Student’s T-Test with p < 0.05 considered significant. Three or more means were compared by ANOVA with post hoc Scheffé test in Matlab with p < 0.05 considered significant. Data are mean ± standard deviation or 95% confidence interval, as specified in the text.

2.4 Results

2.4.1 Effect of dextran on PEG microsphere swelling and aggregation

Individual microsphere diameters were used to calculate the average individual microsphere volume across a range of dextran concentrations. These were normalized to average volume of microspheres in PBS (diameter: 20.1 ± 5.3 µm, volume: 2109.8 ± 1066.1 µm³). Microsphere volume did not significantly change in the presence of 2% serum (1.0 with a 95% CI of 0.8-1.3 as normalized to PBS alone), but decreased upon the addition of dextran, becoming more compact as dextran concentration increases (Fig. 2.1 A). The wide variation in volume between microsphere batches at 5% dextran was because this was close to the critical concentration for phase separation. PEG concentration increases upon deswelling, enabling increased crosslinking between free
VS and Am groups. Phase separation also causes microspheres in dextran solutions to aggregate, with increased packing at higher dextran concentrations (Fig. 2.1 B; note that the dextran concentration prior to dilution 1:1 (v/v) with PEG microspheres is reported). This compaction should increase the local concentration of PEG for microsphere-to-

![Graph A](#)  

**Figure 2.1** Phase separation in dextran increases localized PEG concentration through microsphere deswelling and aggregation. (A) The effect of dextran concentration on microsphere size was studied. Fluorescent microspheres were suspended in dextran solutions of varying concentrations. Care was taken to diminish microsphere aggregation prior to confocal imaging. Microsphere volumes (●) were determined by image analysis (n=3 microsphere batches, >40 microspheres per batch) and plotted alongside corresponding microsphere PEG concentrations (●) (both normalized to PBS alone =1). Microspheres showed a decreasing size trend with increasing dextran concentrations. Error bars correspond to the 95% confidence intervals at each point. (B) Pelleted fluorescent microspheres were resuspended at a 1:1 volume ratio in 2% fetal bovine serum, 20% dextran or 40% dextran, vortexed for 1 minute, allowed to aggregate at 25°C for 15 min, and imaged by fluorescence microscopy. Magnified insets highlight the packing of multiple microspheres to form large aggregates at higher dextran concentrations.
Microsphere crosslinking.

2.4.2 Fabrication and swelling of scaffolds crosslinked in 20% dextran

Due to the higher density of the dextran solutions, centrifugation resulted in microspheres rising to the top of the solution (Fig. 2.2 A). Scaffolds made by our previous method (via serum proteins) required overnight immobilization for crosslinking into scaffolds that were strong enough to support their weight in solution, but could not be removed from the centrifugation container. Scaffolds fabricated in 20% dextran could

![Figure 2.2 Microsphere scaffold formation and swelling. (A) Microspheres were suspended in medium containing either 2% serum or 20% dextran, centrifuged, then crosslinked to form scaffolds. Phase separation in dextran allowed microsphere crosslinking in the absence of serum proteins. Scaffolds formed in dextran were much stronger and could be transferred to cell culture plates and cell-specific medium after only 1 h at 37°C. Scaffolds crosslinked in 2% serum had to be kept in the same tubes in which they were formed to avoid breakage. (B) To demonstrate microstructural changes caused by swelling upon dextran removal (after crosslinking step), a scaffold crosslinked in 40% dextran was imaged by confocal microscopy before and after buffer exchange into PBS.](image-url)
be removed from their centrifugation containers after just one hour. The microsphere swelling data (Fig. 2.1 A) helps to explain this phenomenon. Being a second order reaction, the 4-fold increase in PEG concentration in 20% dextran should increase the rate of the crosslinking reaction $4^2=16$ fold. Sixteen hours approximates the previously used overnight crosslinking step. Scaffolds that were crosslinked in dextran demonstrated substantial re-swelling upon transfer to physiological buffer (Fig. 2.2 B).

### 2.4.3 Scaffold microstructure and mechanical properties

We noted that scaffolds crosslinked in dextran better withstood routine handling, 

![Figure 2.3](image.png)

**Figure 2.3** Scaffolds crosslinked in the presence of 20% dextran are stiffer. Storage moduli ($G'$) of microsphere scaffolds crosslinked in either 20% dextran ($n=3$) or 2% serum ($n=2$) in PBS, 1 d after scaffold formation. Error bars = std. dev. *p < 0.05 for all frequencies
possibly due to denser microsphere-to-microsphere crosslinking afforded by the increases in local PEG concentration. Parallel plate rheometry was used to quantify differences in stiffness. The storage moduli of scaffolds crosslinked in 20% dextran was approximately 3 times higher than the previously published scaffolds crosslinked in 2% serum (Fig. 2.3) (200). However, the nonlinearity of the storage modulus curve is unusual, and further characterization is needed to confirm the value.

Interestingly, confocal microscopy demonstrated larger pores in scaffolds crosslinked in dextran compared to scaffolds formed by centrifugation in 2% serum without dextran (Fig. 2.4). Scaffolds crosslinked in 2% serum without dextran were not

![Figure 2.4](image)

**Figure 2.4** Scaffolds crosslinked in the presence of 20% dextran have increased porosity. Hydrated scaffolds containing fluorescently labeled PEG8-Am were imaged by scanning confocal microscopy (top row). Fixed and dehydrated scaffolds were also analyzed by scanning electron microscopy (bottom row). Microsphere-based scaffolds were compared to bulk PEG hydrogels made from the same precursors by Michael-type reaction (2:1 PEG8- VS:PEG8-Am). Scaffolds formed in dextran were porous without the addition of porogens. Scaffolds formed in 2% serum appeared to be porous, but these are actually large fractures, and the microspheres are otherwise tightly packed.
highly porous, thus requiring porogens to enhance porosity (200). These scaffolds also fractured easily, making it impossible to quantify their porosity by image analysis.

Scaffolds crosslinked in the presence of 20% dextran were porous (42.9 ± 1.2% porosity, n=3) even without addition of a porogen. We suspected that this was due to pre-crosslinking of the microspheres upon addition of dextran, perhaps around spherical dextran-rich droplets, leading to inefficient packing upon centrifugation. SEM images of these scaffolds are in general agreement with the scanning confocal images. However, it is clear that SEM drying artifacts make interpretation of scaffold porosity much more tenuous, and that scanning confocal microscopy is clearly superior for characterizing porosity (218).

![Figure 2.5](image)

**Figure 2.5** HL-1 cells retain high cell viability in scaffolds assembled in the presence of 20% dextran. (A) Scaffolds (blue) were formed from microspheres with and without RGD peptide. HL-1 cells were mixed with the microspheres and rotated for 1 h at 37°C to promote attachment of the cells to the microspheres prior to scaffold formation in 20% dextran. One day after scaffold formation, HL-1 cells were labeled with calcein AM (green) and imaged by scanning confocal microscopy. Cells were more spread in scaffolds with RGD peptide. (B) Live/dead assay of HL-1 cells in a scaffold 1 d after cross-linking, as imaged by confocal microscopy. Cell viability was 81.2 ± 4.7% (n = 3).
2.4.4 HL-1 incorporation, viability, and proliferation in microsphere scaffolds crosslinked in the presence of 20% dextran

Scaffold fabrication in dextran solutions was also possible in the presence of living cells, giving well distributed cell density throughout the scaffold’s thickness (Supplementary Fig. S2.1). To enable cell adhesion to the microspheres, RGD peptides were reacted with PEG₈-VS during the pre-reaction stage, as previously described (200). Cells in RGD-containing scaffolds had a more spread morphology, as expected (Fig. 2.5 A). Cell viability 24 h after scaffold fabrication was 81.2 ± 4.7% (Fig. 2.5 B, n=3). The cell density with 1 mM RGD-containing microspheres, measured 2 h after transfer of the

![Figure 2.6 HL-1 cell proliferation in scaffolds. (A) HL-1 cell density immediately following scaffold formation (day 0) and after 6 and 13 days. Cell density increased nine-fold by day 13. *p<0.05 versus day 0 and day 6. (B) HL-1 cells labeled at day 0 with Cell-Tracker dye (green) then incorporated into scaffolds (blue) to follow the increase in cell density over time. Scanning confocal images are from the interior of each scaffold.](image-url)
scaffold to cell-specific medium, was $2.2 \pm 0.3$ million cells/mL scaffold ($n=3$), and only slightly higher by day 6 (Fig. 2.6 A). However, by day 13, HL-1 cells had proliferated to a density of $18.3 \pm 1.0 \times 10^6$ cells/mL scaffold (Fig. 2.6 A, $n=3$), and much of the porous space in the center of the scaffold was filled with HL-1 cells (Fig. 2.6 B). Proliferation of cells resulted in the formation of large cell clusters in the pores of the scaffold that changed the macroscopic appearance of the scaffold (Supplementary Fig. S2.2).

The experimental design of the proliferation assay originally contained control scaffolds that were crosslinked via serum proteins. However, they broke if removed from their centrifugation containers. In our previous study (200), scaffolds were maintained in the centrifugation containers and the latest time point was 108 hr (meaning 2-3 media changes). It is difficult to maintain serum protein-based scaffolds in centrifugation

![Figure 2.7](image)

**Figure 2.7** HL-1 cells express cardiac functional markers after long term culture in microsphere-based scaffolds crosslinked in 20% dextran. Immunohistochemical staining for connexin 43 (green), sarcomeric α-actinin (red), and DAPI (blue) at: (A-C) day 13 and (D-F) day 24 after scaffold formation. (A and D) Low magnification view of both sarcomeric α-actinin and connexin 43. (B and E) Magnified staining for connexin 43/DAPI alone and (C and F) sarcomeric α-actinin/DAPI alone. Connexin 43 staining was punctuated suggesting substantial cell-cell contacts, while sarcomeric α-actinin expression indicated that cells had retained the cardiomyocyte phenotype. Section slices were 10 µm thick for all images. Images were acquired by fluorescence microscopy.
containers much longer than that because frequent media changes cause scaffold breakage.

2.4.5 HL-1 expression of cardiac functional markers in microsphere scaffolds crosslinked in the presence of 20% dextran

When cultured on two-dimensional substrates, HL-1 cells are known to express both connexin 43 and sarcomeric α-actinin, which are key proteins in excitation and contraction of myocytes, respectively (215, 216). Punctuated expression of the gap junction connexin 43 indicates connexon/gap junction assembly and suggests intercellular electrical connectivity. These markers were probed by immunohistochemistry at days 13 (Fig. 2.7 A-C) and 24 (Fig. 2.7 D-F) after crosslinking. Extensive proliferation (Fig. 2.7 E-F) and cell clustering (Fig. 2.7 D) was further confirmed by nuclear staining at day 24. Both connexin 43 (Fig. 2.7 B and 2.7 E) and sarcomeric α-actinin (Fig. 2.7 C and 2.7 F) were expressed at day 13 and continued to be expressed through day 24 (Fig. 2.7). The continued expression of these proteins suggested that the cells retained their cardiomyocyte phenotype during culture in the microsphere scaffold and were capable of electrical communication via gap junctions.

2.4.6 Detection of HL-1 electrical excitation in microsphere scaffolds crosslinked in the presence of 20% dextran

As a final test of maintenance of cardiac phenotype, we looked at HL-1 excitation after over 5 weeks in culture. Activation of HL-1 cells in day 38 scaffolds was detected by confocal microscopy of Fluo-4 stained cells (Fig. 2.8). Fluorescence intensity traces
were collected for multiple pixels in different regions of interest (ROI). Pixel traces shown in a single ROI graph are spaced by a distance of at least 16 µm, such that some of the traces were likely from adjacent cells. Spontaneous excitation was observed throughout the scaffolds (Fig. 2.8 A), with pixels from various areas of a single ROI peaking simultaneously. This suggests some degree of electrical connectivity between cells of a single cluster. The duration of the spontaneous transients varied between ROIs, possibly due to variation in the activity of Ca\(^{2+}\)-ATPase on the surface of the sarcoplasmic reticulum. In paced scaffolds, cells in various ROIs were excited at the same frequency (0.5 Hz) for the 10 s duration of the stimulus (Fig. 2.8 B). Surprisingly the excitation frequency of these clusters was only half the stimulation frequency (i.e. a 2:1 block), suggesting that the cells have a long refractory period (normal murine refractory periods are on the range of 50 ms (219)).

Contraction of cell clusters was not visualized at the microscopic level, but the use of an excitation-contraction uncoupler (e.g. blebbistatin) would be necessary to absolutely verify that transients were not influenced by contraction-induced motion. Although the traces do not appear to represent pure action potential, the presence of calcium transients and/or motion indicate that the cells are still electrically active after 5 weeks in the 3D scaffolds. This suggests a promising outlook for future work investigating HL-1s or other types of cardiomyocytes. Future studies will focus on optimization of materials to support a contractile phenotype.
Figure 2.8 HL-1 cells retain electrical activity after 38 days of culture within microsphere-based scaffolds crosslinked in 20% dextran. Day 38 scaffolds containing HL-1 cells were incubated with calcium-sensitive Fluo-4 AM to monitor changes in intracellular calcium concentrations. Activity was imaged by scanning confocal microscopy at a depth of 100 µm into the scaffolds. (A) Spontaneous transients were seen in various regions of interest (ROI) throughout the scaffold. (B) Transients from various ROI in response to stimulation by monophasic electrical impulses (amplitude = 10 V, duration = 200 msec, frequency= 1 Hz, burst width = 10 sec). Traces of transients from different ROI or random pixels are shown. The multiple traces from within each ROI were from pixels spaced at least 16 microns apart.
2.5 Discussion

Phase separations have been utilized to produce porous hydrogel materials for at least five decades (220-222). Crosslinking of a polymer above its LCST results in the formation of sponge-like morphologies that sometimes appear to be aggregates of microspheres (218, 223-225). The current method differs from traditional phase separation methods because scaffold formation is divided into a microsphere formation step and a microsphere assembly step. Like our previously reported methods (199, 200), the microsphere formation step is performed in sodium sulfate solutions rather than dextran because the microsphere yield is much higher. The scaffold formation step is then performed in cytocompatible dextran solution, which provides a stiffness increase that enables more extensive biological characterization than was possible using the serum protein-based protocol. The two-step method allows the fabrication of large quantities of high quality microspheres that may be stored for longer than six months prior to scaffold formation. It is our experience that the complexity of processing materials in the phase-separated state is mostly captured in the first step. Thus, if the first step yields high quality microspheres, the same microsphere batch may be used over the course of many experiments.

Materials used for cardiac tissue engineering are typically: 1) macroporous scaffolds formed apart from cells, or, 2) non-macroporous hydrogels crosslinked around cells. As an example of pre-formed scaffolds, Langer and colleagues produced highly porous poly(glycerol sebacate) materials that had mechanical properties similar to myocardium (226). The scaffolds were engineered with intricate, biomimetic pore structures that promoted the alignment of cardiomyocytes (227). As an example of a
non-macroporous hydrogel, collagen gels have been extensively characterized by Eschenhagen, Zimmermann, Elson and colleagues (131, 179, 228-232). Primary heart cells were added to collagen solutions prior to gelation. Cardiac fibroblasts contracted the gels, which resulted in highly aligned, electrically active tissues (131, 179, 228-232). While the lack of porosity may place size limitations on the tissues, researchers have circumvented this problem by fusing multiple small collagen gels to create larger cardiac patches (131, 231, 232).

Materials for cardiac tissue engineering must be especially resistant to the formation of a fibrous capsule, which may result in electrical isolation of the transplanted tissue. Because of its low protein adsorption properties, PEG is an excellent candidate for these types of applications (142, 153, 233). The PEG-based scaffolds described here are macroporous when assembled in the presence of living cells. Similar to traditional macroporous scaffolds, the cells have space within the pores to migrate and proliferate. Similar to a collagen gel, the cells are evenly distributed regardless of scaffold thickness from the time of scaffold formation. The storage modulus is also higher than reported values for collagen gels (10-100 Pa) (234-237).

Collagen gels are typically remodeled by cardiac fibroblasts that are present in the primary heart cell harvests. Unfortunately, proliferating fibroblasts may quickly overtake the cardiomyocytes, limiting the window of study to a few days and making it difficult to analyze cardiomyocyte-specific responses (231, 238). While patient-derived stem cells may be the optimal cell source for cardiac tissue engineering, HL-1 cells are a useful model for studying cell responses to biomaterials because they maintain many of the characteristics of cardiomyocytes. HL-1 monolayer cultures have previously been used
to probe cardiomyocyte responses to various drugs and other treatments \((216, 239-241)\).

Several recent studies have used HL-1 cells to study cardiomyocyte responses to biomaterials \((242-245)\). For example, Stupp and colleagues demonstrated HL-1 alignment and electrical activity at day 10 in nanofibrous amphiphilic peptide gels \((242)\), but viability and retention of cardiac phenotype in longer-term cultures were not investigated. Jongpaiboonkit et al. investigated viability over time for HL-1s encapsulated in bulk PEG-diacrylate hydrogels, which have negligible porosity \((245)\). Viability in non-degrading bulk gels at day 1 was slightly higher than what was seen in the PEG microsphere scaffolds (~90% versus ~81%, respectively). This slight viability difference may be a product of the length of the adhesion step used to ensure HL-1 adhesion to microspheres during centrifugation. At two weeks, HL-1 viability in the bulk PEG hydrogels was low unless RGD density and degradation rate were finely tuned \((245)\). In the current study, HL-1 cells expressed functional markers and had a nine-fold increase in cell density at two weeks. While a long-term comparison between the systems has not yet been performed, HL-1 cells in PEG microsphere-based scaffolds may be at a long-term advantage due to increased porosity and nutrient diffusion.

However, the current scaffolds still need to be improved to be useful for cardiac tissue engineering. While dense cardiomyocyte packing is important for conduction, localized HL-1 proliferation caused clustering within the pores of the scaffold, and the cells lacked the alignment and highly organized gap junctions (Figs. 2.6 and 2.7) typical of natural myocardium. Furthermore, the frequency of spontaneous transients in the scaffolds was lower than those reported for confluent HL-1 monolayers \((246)\) and seen in our own two-dimensional cultures, but similar to what is reported for non-confluent
monolayer cultures (239). The lower frequency and lack of synchronization in spontaneous transients probably indicates low electrical conduction between cell clusters in the scaffold. Functionalizing some of the PEG microspheres with enzymatically degradable sites may enhance porosity and help the cells to remodel their environment, similar to what is seen in collagen gels (161, 229). Coupling enzymatic degradation with mechanical (230) and/or electrical (247) stimulation may further improve cellular alignment and electrical conduction.

Finally, replacing the HL-1 cells with an autologously derived cardiomyocyte source will be necessary before the engineered tissue can be used in a clinical setting. Recent studies have found that matrix stiffness (159, 164, 248) and topography (101, 107, 110) have profound influence on cell differentiation and maturation, so it is possible that the mechanical differences observed in this study would affect cell phenotype. However, the microstructural differences between PEG microsphere scaffolds and those described by other groups limit our ability to hypothesize on the outcome of mechanical and topographical changes. Our future research will likely include variation of microsphere size and stiffness and its effects on stem cell differentiation and cardiomyocyte phenotype.

2.6 Conclusions

In this study, previously published modular scaffold fabrication methods were improved, developing stronger, more porous PEG scaffolds by crosslinking microspheres in the phase separated state. Scaffolds were assembled around HL-1 cardiomyocytes, and cells were cultured in the scaffolds for over a month. The HL-1 cells proliferated, expressed cardiomyocyte markers, and retained electrical activity.
2.7 Acknowledgements

The authors are grateful to Dr. William Claycomb for the gift of the HL-1 cells and Prof. Rebecca Kuntz-Willits for use of the rheometer. We would like to thank Kristy Wendt and Matthew Stork for technical assistance, and Megan Flake, Casey Donahoe, Di Lang, and Christina Ambrosi for helpful discussions. Financial support was provided by the NIH R01 HL085364 (DLE), R01 HL085369 (IRE), and the Lucy and Stanley Lopata endowment (IRE).

2.8 Supplementary Figures

Supplementary Figure S2.1 HL-1 cells are well distributed throughout the thickness of a microsphere scaffold crosslinked in 20% dextran. Cell nuclei (yellow) in sections from a day 1 scaffold were counted and the average number per mm² of microspheres (blue) was calculated for three scaffold regions (each representing 550 µm of scaffold thickness). (A) Images of sections used for cell counts. (B) A bar graph presenting the average cell density in the top, center and bottom of the scaffold. Error bars represent standard deviation between 3 sections in one scaffold region.
Supplementary Figure S2.2 Macroscopically visible HL-1 clusters are present after 10 days of culture in a microsphere scaffold crosslinked in 20% dextran. A phase contrast image of an HL-1 loaded scaffold, 10 d after scaffold formation, compared to a scaffold without cells. White arrows indicate visible clusters of HL-1 cells.
CHAPTER 3*

Direct reprogramming of mouse fibroblasts to cardiomyocyte-like cells using Yamanaka factors on engineered poly(ethylene glycol) hydrogels

3.1 Abstract

Direct reprogramming strategies enable rapid conversion of somatic cells to cardiomyocytes or cardiomyocyte-like cells without going through the pluripotent state. A protocol recently described by Efe et al. (Nature Cell Biology, 2011, 13:215) couples Yamanaka factor induction with pluripotency inhibition followed by BMP4 treatment to achieve rapid reprogramming of mouse fibroblasts to beating cardiomyocyte-like cells. The original study was performed using Matrigel-coated tissue culture polystyrene (TCPS), a stiff material that also non-specifically adsorbs serum proteins. Protein adsorption-resistant poly(ethylene glycol) (PEG) materials can be covalently modified to present precise concentrations of adhesion proteins or peptides without the unintended effects of non-specifically adsorbed proteins. Here, we first reproduced the Efe et al. protocol to reprogram adult mouse tail tip mouse fibroblasts (TTF) and mouse embryonic fibroblasts (MEF) to cardiomyocyte-like cells that demonstrated striated sarcomeric \( \alpha \)-actinin staining, spontaneous calcium transients, and visible beating on Matrigel-coated TCPS. We then designed poly(ethylene glycol) culture substrates to promote MEF

* Chapter 3 is adapted from a manuscript accepted for publication in Biomaterials May 23rd, 2013. DOI: 10.1016/j.biomaterials.2013.05.050
adhesion via laminin and RGD-binding integrins. PEG hydrogels improved proliferation and reprogramming efficiency (evidenced by beating patch number and area, gene expression, and flow cytometry), yielding almost twice the number of sarcomeric α-actinin positive cardiomyocyte-like cells as the originally described substrate. These results illustrate that cellular reprogramming may be enhanced using custom-engineered materials.

### 3.2 Introduction

Heart failure is one of the most common health problems in the United States. It will eventually affect ~20% of all 40-year-old Americans (11). Innovative solutions are necessary to reduce the costs of heart failure, which currently stand at ~$39.2 billion per year (11). Cell-based therapies, or introduction of healthy cells into cardiac scar tissue, have modestly improved various measures of cardiac function (60, 249, 250). The best donor cell source is still under debate, with much room for optimization across cell types (58). Current cardiomyocyte derivation strategies include differentiation from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), resident cardiac progenitor or stem cells, or direct reprogramming, which bypasses the pluripotent state. ESC and iPSC (collectively, PSC)-derived cardiomyocytes are expandable, and iPSC-derived cardiomyocytes could be autologously sourced. However, their populations must be carefully purified to avoid risks of teratoma formation, and chromosomal defects arising over long periods in culture add to tumorogenic risks (46). Currently, iPSC derivation is relatively inefficient (0.01-1.4%) (78). The full derivation, expansion, and purification of pluripotent stem cell derived cardiomyocytes can take several months (36, 79).
Several direct reprogramming strategies (75, 76, 82, 84) have reported bypassing the pluripotent state, enabling autologous derivation of cardiomyocyte-like cells in a fraction of the time needed for iPS cell production. This has been achieved by inducing transcription factors important for cardiac development (for example; Tbx5, Mef2c, Gata4, Myocd and/or Hand2) (75-77) or using microRNAs (82) to control signaling pathways. In addition to implanting cells reprogrammed in vitro, similar techniques have been used to reprogram scar tissue to cardiomyocytes in vivo (77, 80-82). The direct reprogramming protocol described by Ding and colleagues (Efe et al. (84)) combines a carefully timed induction of Oct4, Sox2, Klf4, and c-Myc (OSKM), inhibition of the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pluripotency pathway, serum withdrawal, and BMP-4 mediated cardiogenic induction, resulting in beating patches of cells in as little as 11 days after OSKM induction (84). The rapid derivation process is especially relevant in cases of myocardial infarction, as the inflammatory response begins within 24 hours (86) and scar formation progresses quickly from there (87).

Extracellular matrix (ECM) proteins in the cell microenvironment affect pluripotent differentiation via integrin signaling, and are well known to direct differentiation down varied pathways (100). However, the optimal surface density and types of proteins are often determined by trial and error. Efe et al. found that Matrigel (~60% laminin-1, ~30% collagen IV, ~8% entactin, ~2% other proteins according to product literature) improved cardiac direct reprogramming over Geltrex, gelatin or fibronectin coatings, but no mechanism was proposed (84). In contrast, Thomson and colleagues revolutionized PSC culture by measuring ESC growth factor receptor
expression and tailoring the media formulation accordingly (251, 252). Recently, Koche et al. analyzed global gene expression of partially reprogrammed mouse embryonic fibroblasts (MEFs, 3 days into OSKM induction), reporting upregulation of laminin-binding integrin subunits α6 (4.0-fold) and β4 (13.3-fold), downregulation of collagen IV binding subunits α1 (4.3-fold), and little changes in expression of many RGD motif-binding integrin subunits (e.g. α5 and αV), with the exception of α8, downregulated 4-fold (253). Based on these data, we hypothesized that laminin alone would promote the direct reprogramming of MEFs to cardiomyocyte-like cells at least as well as Matrigel. The use of laminin also avoids the batch-to-batch variation in protein content seen with Matrigel (254, 255). We also hypothesized that the addition of RGD peptide would enhance cell adhesion on engineered substrates due to the continued expression of αV during the early reprogramming process.

In the original direct reprogramming protocol, Matrigel was adsorbed to tissue culture polystyrene (TCPS) prior to cell seeding (84). However, adsorption may cause unfolding of ECM proteins, resulting in altered cell signaling (256-259). Serum proteins from the media also adsorb to TCPS, competing with the intended ECM proteins (260) and activating unintended signaling pathways that may vary by batch of serum. Poly(ethylene glycol) (PEG) is resistant to non-specific protein adsorption, but may be covalently modified to present bioactive molecules at precise quantities and in their native conformations (261, 262). For this study, we engineered PEG cellular microenvironments that potentially provide better control over adhesion molecule concentration, conformation, and stability than is possible with TCPS. We demonstrate that these engineered hydrogel substrates lead to higher efficiency of direct
reprogramming to cardiomyocyte-like cells than the protocol initially described by Efe et al. (84)

3.3 Materials and Methods

3.3.1 Mouse protocols and fibroblast culture

All animal protocols were approved by Washington University Institutional Animal Care Use Committee. A pair of mice homozygous for doxycycline-inducible OSKM factors (Jackson Labs, stock number: 011011) was bred to start a colony for tail tip (TTFs) and embryonic fibroblasts (MEFs) (263). To harvest tail-tip fibroblasts (TTFs), adult tails were sterilized in 70% ethanol, skinned, and minced thoroughly. Minced pieces were incubated in 3 mL of 0.05% trypsin-EDTA (Invitrogen) at 37°C for 30 min then neutralized with 7 mL of fibroblast media (FB media: DMEM+10% fetal bovine serum+1% ABAM). Tail pieces were collected by centrifugation, resuspended in 3 mL of FB media, and transferred by tweezers to gelatin coated wells (1 mL/well at 0.1 mg/mL, preincubated 1 h at room temperature) at a ratio of one tail per 2 wells of a 6 well plate. Care was taken to dry the well surfaces by aspiration prior to placing the pieces, and to evenly space the pieces in the well. After allowing 4 h for tails to adhere at 37°C, 0.2 mL of FB media was added per well. The next day, 3 mL/well of FB media were added. Once TTFs became confluent, they were trypsinized, strained through a 70 µm cell strainer, and replated (2 wells/T75 flask). Once confluent, the flask was trypsinized and frozen at -80 (1 cryovial per initial mouse tail).

For preparing mouse embryonic fibroblasts (MEFs), day 13.5 embryos were harvested and the visceral organs removed to minimize the presence of cardiac precursor
cells. Embryos were minced, incubated at 37°C for 30 min in 7 mL of 0.05% trypsin-EDTA and then triturated using a 10 mL pipet. Trypsin was neutralized with 23 mL of FB media, divided into T75 flasks (2-3 embryos/flask), and cultured in 15 mL of FB media. Once confluent, cells were passaged 1:3 and the passage 2 cells were trypsinized and frozen at -80°C (3 cryovials/confluent flask).

3.3.2 PEG hydrogel fabrication and functionalization of culture surfaces

All experiments were performed using 24-well plates. Thirty minutes prior to gel fabrication, wells were treated with a plasma cleaner (Harrick Plasma) set to medium (350 mTorr for 5 min) to reduce meniscus formation in the wells during gel formation. Plates were then UV-sterilized for 30 min and gel precursor solutions were added immediately. PEG derivatives were synthesized as previously described (213, 214, 264). For gel fabrication, PEG₈-Vs and PEG₈-Am solutions (both 20% w/v in PBS, pH 7.4) were sterile-filtered (0.22 µm filter) and diluted in 0.03 M NaOH in PBS (pH 8.75, NaOH enhanced reaction kinetics and further reduced meniscus formation) for a final PEG concentration of 12.43% (w/v). The precursor mix was aliquoted at 200 µL/well and allowed to crosslink in a humidified incubator (37°C) for 1-2 days.

Protein and peptide surface concentrations are reported as 1x or 5x. When referring to Matrigel or laminin, 1x is the approximate protein concentration of Matrigel used in Efe et al. The concentration used here may be slightly different than Efe et al. as they reported the Matrigel dilution factor (1:40) instead of the coating concentration (84), and so their surface concentration will vary by Matrigel batch (265). Here, we standardized the 1x coating protein concentration at 11.4 µg/cm² (the 1:40 dilution of our
first Matrigel stock when used at 200 µL/well). The 1x concentration of laminin was also set to 11.4 µg/cm². When referring to the RGD peptide, 1x is the approximate surface density of RGD on the PEG microspheres previously tested with HL-1 cardiomyocytes (266).

For functionalization with Matrigel (BD Biosciences, 356234) or laminin-1 (Sigma, L2020), protein stocks were first diluted to 108.3 (1x) or 541.5 µg/mL (5x) in PBS. If RGD peptide (GCGYGRGDSGP, Genscript) was included, it was diluted in the same tube as the protein at 3.36 (1x) or 16.82 (5x) mg/mL. Each well received 200 µL of protein/peptide solution. For TCPS conditions, (non-plasma treated) 24-well plates were incubated at room temperature for 1 h to permit protein adsorption (according to Matrigel product literature). The pre-formed PEG hydrogels were incubated with the protein/peptide mixtures overnight at 37°C to permit the slower, covalent Michael-type reaction to occur. Fibroblast seeding occurred the next day.

### 3.3.3 Cell culture and reprogramming

All reagents were purchased from Invitrogen unless otherwise noted. Passage 3 fibroblasts were seeded at day -1 (minus one) onto the functionalized 24-well plates at 3,600 MEFs or 9,700 TTFs/cm² and incubated overnight in FB media to ensure complete adhesion. Reprogramming commenced the following day (day 0), and media was changed every 24-48 hours. Media formulations and reprogramming timelines were adapted from Efe et al. (84). Reprogramming timeline #4 (Fig. 3.1 a) was the most efficient at producing beating in initial testing with MEF, and was used in all experiments thereafter. From days 0-5, cells received knockout DMEM (KO DMEM, 10829018) + 2
μg/mL fresh doxycycline (Sigma, D9891) + 0.5 μM JAK inhibitor I (JI1, EMD, 420099) + 15% embryonic stem cell qualified FBS (ES-FBS, 10439-024) + 5% knockout serum replacement (KSR, 10828028) + 0.1 mM β-mercaptoethanol (21985-023), + 1% Glutamax (35050-061) + 1% non-essential amino acids (11140-050) + 1% embryonic stem cell qualified nucleosides (Millipore, ES-008-D). For days 6-8, doxycycline was removed, ES-FBS concentration reduced to 1%, and KSR concentration increased to 14% (all other components remained the same). From days 9-14, cells were given chemically defined medium (CDM) with BMP4: RMPI-1640 (21870-084) + 20 ng/mL BMP4 (Stemgent, 03-0007) + 0.5x N2 (17502-048) + 1x B27 w/o vitamin A (12587-010) + 0.05% BSA fraction V (15260-037) + 0.5% Glutamax + 0.1 mM β-mercaptoethanol.

From day 15 onward, cells were given CDM without BMP4. RW.4 mouse embryonic stem cells (ATCC), a gift from the Sakiyama-Elbert laboratory, were cultured according to previously described methods (267).

3.3.4 Cell counts and quantification of beating patches

Phase contrast microscope images (10x magnification) were taken with a CCD camera (Magnifire, Olympus) and used to perform cell counts at days 0 and 3 for analysis of initial adhesion and proliferation. Cell counts for three randomly selected images were averaged to determine the cell density of a given well. For beating patch quantification, 2x images of the entire well were taken by phase microscopy. Wells were then carefully examined at 4x for beating patches, and these were outlined on the 2x images using Microsoft Paint. Outlined beating patches filled with white using Adobe Photoshop, and any bright regions of the image not associated with beating patches were filled with
black. Images were then thresholded and quantified in Matlab (Mathworks) to determine the number and size of the beating patches and total beating area of the well. For late stage cell counts, cells were enzymatically dissociated using trypsin at days 15 (n = 3-6) and 18 (n = 3-6) and counted using a hemocytometer.

### 3.3.5 Live-cell calcium imaging

Calcium-sensitive dye Fluo-2 (medium affinity, 10 mM in dimethyl sulfoxide, excitation/emission 488/515 nm, TefLabs) was mixed (1:1 volume ratio) with 20% (w/v) Pluronic F-127 (Invitrogen), diluted to 10 µM in DMEM, and applied to cells for 30 min at 37°C/5% CO₂. Cells were washed three times in PBS and incubated for 15 min in DMEM to allow complete de-esterification of the Fluo-2. Cells were imaged in Tyrode’s solution (1.33 mM CaCl₂, 1 mM MgCl₂, 5.4 mM KCl, 135 mM NaCl, 0.33 mM NaH₂PO₄, pH 7.4) containing 5 mM glucose and 5 mM HEPES by fluorescence microscopy (IX70, Olympus) using a Magnafire camera (Optronics, 10 frames/second). The resulting images were analyzed using a custom Matlab script that tracked fluorescence localization over different time intervals. Noise was removed through a Prewitt edge detection filter and a series of structuring element filters (2-pixel lines at 90, 0, then 45 degrees). A 10 x 10 pixel averaging filter was then used to remove biased pixels that were unrelated to the signal of interest. Finally, activation events were detected in each pixel by setting a fluorescence threshold. An activation map was formed by overlaying temporal data with a gradient color scheme.

### 3.3.6 Quantification of gene expression
Gene expression was evaluated by quantitative real time RT-PCR using an Applied Biosystems StepOnePlus or ViiA machine. On day 18 of reprogramming, cellular RNA was purified with TRIzol reagent (Invitrogen, 15596-026) by following the manufacturer’s instructions. RNA concentration and quality (260/280 nm absorbance ratio) was verified using a NanoDrop spectrophotometer. Genomic DNA was removed and RNA was reverse transcribed to cDNA (10 ng RNA/µL) using the QuantiTect Reverse Transcription Kit (Qiagen, 205313). Pre-validated primers for mouse GAPDH, sarcomeric α-actinin (Actn2), cardiac troponin T (Tnnt2), connexin 43 (Cx43), and Nanog (Nanog) transcripts were obtained from Qiagen (Quantitect Primer Assay). Forward and reverse primer sequences designed using IDT software and experimentally validated were β-actin (ActB), sarcomeric α-actinin (Actn2), myosin light chain-atrial isoform (Mlc2a), potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channels 1 and 4 (Hcn1, Hcn4), T-box transcription factor Tbx3 (Tbx3), and contactin-2 (Cntn2). Primer sequences are in Supplementary Table S3.1. cDNA was amplified in triplicate reactions using Quantitect SYBR Green PCR Kit (Qiagen) following manufacturer’s instructions. Expression levels were normalized to GAPDH or β-actin internal control genes, or to sarcomeric α-actinin to exclude effects from non-cardiomyocytes, using the $2^{-\Delta\Delta CT}$ method (268).

3.3.7 Immunocytochemistry

For immunocytochemistry, cells were fixed in 4% paraformaldehyde (500 µL/well) for 15 min. Triton-X 100 (Sigma) was diluted 1:1000 in blocking solution (10% normal goat serum + 1% BSA in PBS) for permeabilization (300 µL/well, 30 min at
room temperature). Sarcomeric α-actinin primary antibody (mouse IgG, Sigma A7811) was diluted 1:1600 in the blocking solution and applied to the cells (300 μL/well, overnight at 4°C). Cells were washed 3x in PBS (5 min each) before incubation in secondary antibody diluted 1:1000 in blocking solution (300 μL/well, 1 h at room temperature, Alexa Fluor 594 goat anti-mouse, Invitrogen, A-11005). Cells were washed 3x more in PBS before application of DAPI as a nuclear counterstain (100 ng/mL, 300 μL/well, 30 min at room temperature). Wells were washed once in PBS then imaged in PBS.

### 3.3.8 Flow cytometry

At day 18, cells were enzymatically dissociated into a single-cell suspension using 0.25% trypsin-EDTA (Invitrogen), fixed in 1% paraformaldehyde in PBS at 4°C with agitation for 20 min, permeabilized with 1% saponin (Sigma, S4521) in PBS at 4°C with agitation for 20 min, and blocked with 5% normal goat serum (NGS) + 0.5% saponin in PBS at 4°C with agitation for 20 min. Sarcomeric α-actinin primary antibody (mouse IgG, Sigma A7811) was applied to the cells for 30 min at 4°C with agitation (1:1600 in 2% NGS + 0.5% saponin in PBS). Negative controls had no primary antibody at this step. AlexaFluor 647 goat anti-mouse secondary IgG (Invitrogen, A21235) was applied to the cells for 30 min at 4°C with agitation (1:200 in 2% NGS + 1% saponin in PBS). DAPI (1:125 in PBS) was applied as a nuclear counterstain. Data were acquired on a FACSCanto II (BD Biosciences), with at least 10,000 counts per condition. Data were analyzed using FlowJo software (Tree Star). Negative controls were used to determine gating parameters.
3.3.9 Statistics

Two-condition comparisons were made using student’s t-test, whereas multiple comparisons were made using ANOVA with post-hoc Tukey HSD. In figures, data are presented as mean ± standard error of the mean. Text references to data are mean ± 1 standard deviation.

3.4 Results

3.4.1 Replicating direct reprogramming to cardiomyocyte-like cells on Matrigel-coated TCPS

To verify that direct reprogramming using OSKM could be replicated in our hands, we first used adult mouse TTFs to test three possible interpretations of the published protocol (84) (Fig. 3.1 a). Cells from all groups demonstrated noticeable morphological changes, notably a decrease in cytoplasm/nucleus ratio by day 6. By day 12, large cell clusters had formed, and were most prevalent in wells from timeline 4 (Fig. 3.1 b). By day 14, beating patches were noted under timelines 3 and 4 only. Timelines 3 & 4 gave similar numbers of beating patches by day 18, with timeline 4 having a slightly higher number. Given that timeline 4 was also shorter and less complex, it was used in all subsequent experiments.
Figure 3.1: Effects of varied induction timelines on adult tail tip fibroblasts. A) Multiple reprogramming media timelines were tested to clarify the protocols described by Efe et al. Media with doxycycline (dox) promoted expression of the Yamanaka factors Oct4-Sox2-Kit4-c-Myc (OSKM) in tail tip fibroblasts (TTFs) derived from transgenic mice. Timeline 4 produced the most beating clusters. In timeline 4, six days of dox-induced OSKM were coupled with pluripotency inhibition by Jak-Stat Inhibitor (J1) in high serum medium. This was followed by three days of recovery in low serum medium also containing J1, and then six days of induction of cardiomyocyte differentiation in chemically defined (no serum) medium containing bone morphogenetic protein 4 (BMP4). Abbreviations: Fibroblast (FB) medium, Knockout (KO) medium, Chemically Defined (CD) medium, ES-qualified fetal bovine serum (FBS). B) TTFs became smaller and more rounded under the influence of OSKM, and cells in timeline 4 produced clusters of very small cells by day 12 that ultimately began beating.
The beating patches demonstrated spontaneously oscillating calcium transients, which were most prevalent at the outer edges of differentiated clusters or in patches of cells surrounding the clusters (Fig. 3.2 a-c). Beating patches derived from TTFs stained positively for striated sarcomeric α-actinin, a cardiac-specific marker (Fig. 3.2 d-e). In agreement with the location of calcium transients, the most intense α-actinin staining was

![Figure 3.2: Characterization of cardiomyocytes directly reprogrammed from adult tail-tip fibroblasts. Reprogrammed cells formed beating patches visible by phase contrast microscopy (A, day 19). (B) A time-based map of an activation wavefront from the beating patch in (A) was made using the calcium-sensitive dye Fluo-2. The wavefront starts at the right side of the cluster (dark red) and travels through the perimeter of the cluster (demonstrated by color changes to orange and then yellow). Calcium traces from individual regions of interest (ROIs) are shown in (C). (D and E) Immunocytochemistry of day 18 TTF-derived cardiomyocytes revealed expression of sarcomeric α-actinin (red) within the beating patches and in cells attached to the adjacent substrate.](image-url)
found in cells surrounding the differentiated clusters.

After demonstrating that the protocol is effective with adult somatic cells, MEFs harvested from the same mice containing the doxycycline-inducible OSKM cassette were utilized because they could be expanded to greater numbers in culture and reprogramming efficiency could be directly compared to the results of Efe et al. At day 21, the number of beating patches generated with MEFs on Matrigel-coated TCPS was similar to the number previously reported by Efe et al. (245 ± 83 in this study, versus 257 ± 17 beating patches per initial 100,000 MEFs seeded).

3.4.2 Adhesion and proliferation of MEFs undergoing reprogramming on PEG hydrogels

To verify that direct reprogramming could be performed on PEG hydrogels, MEFs were cultured on surfaces presenting covalently immobilized Matrigel, laminin or RGD at varied concentrations (referred to as 1x or 5x: for detailed explanations of the surface concentrations see the methods section or Supplementary Table S3.2). As noted in previous studies (264), cells did not adhere to PEG surfaces without adhesion molecules. It is well known that the protein content of Matrigel varies by batch (~60% laminin, ~30% collagen IV, ~8% entactin and ~2% various growth factors and other proteins according to product literature) (265). Consistent with this, we found that the ability of cells to adhere to Matrigel-functionalized PEG was Matrigel batch-dependent (data not shown). However, cells consistently adhered to laminin-functionalized PEG. By 24 hours after seeding (day 0 of reprogramming), cell densities were similar on PEG-RGD(5x) and TCPS coated with Matrigel or laminin. The densities were statistically
higher than all other PEG groups and uncoated TCPS (Fig. 3a), indicating higher rates of initial adhesion and/or proliferation on these surfaces.

Early proliferation dictates the ultimate number of cells available for cardiac differentiation. To analyze the role of the adhesion molecules on proliferation of cells on PEG conditions during the first 3 days of OSKM induction, we quantified cell densities at day 3 and applied the following equation to determine the number of doublings per day:

$$d = \frac{\log_2 \left( \frac{N_t}{N_0} \right)}{t}$$

where $d =$ doublings/day, $N_0 =$ number of cells at day 0, $N_t =$ number of cells after $t$ days, and $t =$ number of days (Fig. 3.3 b). Day 3 was chosen as the endpoint because cells on PEG conditions reached confluency by days 4-5 and thus could not be readily counted. Rates could not be determined for TCPS conditions as they had reached confluency by day 3. For PEG conditions presenting RGD alone, the RGD concentration had an effect on cell proliferation ($0.84 \pm 0.07$ doublings/day for 1x RGD versus $1.14 \pm 0.15$ for 5x RGD, $p = 0.01$ by t-test). PEG conditions presenting laminin alone at 1x or 5x concentration exhibited proliferation rates similar to the 5x RGD condition.

Over the course of reprogramming, cells on PEG-RGD(5x) continued to proliferate rapidly compared to other PEG groups, resulting in an extremely high cell density by day 9 (Fig. 3.3 c). Interestingly, when laminin was added to PEG hydrogels that also contained 5x RGD, cell densities were noticeably lower. By day 12, the dense cell layer on PEG-RGD(5x) began peeling off the gel forming compact aggregates at the edges of the wells (Fig. 3.4 b), and could not be analyzed further. Other conditions enabled better long-term adhesion, but by day 18, cells of the 1x laminin, 1x RGD and, to
Figure 3.3: Substrate effects on early stage cell adhesion and proliferation. The culture substrate had dramatic effects on cell adhesion and proliferation during the first 9 days of the protocol. A) Although surfaces were seeded equally, the efficiency of cell adhesion and/or proliferation was not the same. One day after MEF seeding (day 0 of reprogramming), similar cell densities were seen on TCPS surfaces (red bars) coated with Matrigel or laminin. Cell densities on PEG surfaces did not vary with low (blue bars) versus high (green bars) concentrations of adhesion molecules (#not statistically different at α=0.95 by Tukey’s HSD test), but some conditions were lower than the coated TCPS groups (* p<0.05 versus TCPS-Mat(1x) and TCPS-lam(1x)). B) Cell counts from PEG hydrogels containing only RGD or only laminin were used to calculate proliferation rates over days 0-3. The proliferation rate was positively influenced by RGD concentration (*p<0.05 by t-test), but not laminin concentration. C) When RGD was used alone at 5x (lower left), an exceptionally high cell density was noticeable by phase microscopy by day 9. Densities were not as high on PEG-RGD(1x) or any PEG group where laminin was included.
a lesser degree, 5x laminin conditions had also begun peeling off the gel in isolated areas. Long-term adhesion problems were completely resolved by using both laminin and RGD. No long-term adhesion issues were noted on any TCPS conditions.

Cells were counted by hemocytometer on days 15 and 18 for TCPS-Mat(1x), PEG-lam(5x), PEG-lam(5x)-RGD(1x), and PEG-lam(5x)-RGD(5x) to assess proliferation at a later stage of the reprogramming timeline. The cell density between the two days was not different for any condition, suggesting a decrease in proliferation. This finding is consistent with the decrease in progenitor marker (Mesp1 and Isl1) expression reported by Efe et al. for that time frame, which suggests a transition from a proliferative progenitor to a less proliferative immature cardiomyocyte (84). Counts also did not vary between PEG-lam(5x) with 1x versus 5x RGD. Counts were therefore pooled to increase the number of samples for a statistical analysis of late stage cell density (n=6-12). PEG-lam(5x)-RGD(1x or 5x) gels had a ~25% increase in cell density compared to the original condition used in Efe et al., or PEG-lam(5x) without RGD (Fig. 3.4a, p<0.05 by ANOVA with post-hoc Tukey HSD). Cell density on PEG-lam(5x) with RGD increased by a factor of 136.2 ± 23.7 compared to initial MEF seeding density. Densities on PEG-lam(5x) without RGD and TCPS-Mat(1x) increased by factors of 109.1 ± 20.5 and 108.3 ± 16.7, respectively.
Figure 3.4: Cell density and morphology at late stages of reprogramming protocol. Addition of RGD to PEG hydrogels boosted cell proliferation. A) Cells were trypsinized and counted by hemacytometer at days 15 and 18. Within the same condition, the cell density did not vary by day. Counts also did not vary between PEG-lam(5x) with 1x or 5x RGD. Counts from these conditions were therefore pooled to increase the number of samples for statistical analysis (n = 6-12). PEG-lam(5x) gels with RGD yielded ~25% more cells per square centimeter than TCPS-Mat(1x) or PEG-lam(5x) without RGD (*p<0.05 by ANOVA with post-hoc Tukey HSD test). B) Phase contrast images were stitched together to give a macroscopic view of the individual wells. Without laminin, cells on the RGD(5x) gel rolled off the plate forming a large clump at the edge of the well (white arrow). On gels with laminin, more clusters were seen on gels that also presented RGD at 5x. Clusters on gels with 5x laminin but no RGD had a more spread morphology than those on gels presenting RGD (bottom image).
3.4.3 Functional assessment of direct reprogramming to cardiomyocyte-like cells on PEG surfaces

Many of the laminin receptors are upregulated during the first 3 days of OSKM reprogramming, whereas collagen receptors are downregulated (253). Thus, we hypothesized that reprogramming would be equally as efficient on laminin versus Matrigel. Numbers of differentiated clusters were visibly increased on PEG-lam(5x)-RGD(5x) compared to PEG-RGD(1x), PEG-lam(1x)-RGD(1x) and PEG-lam(5x)-RGD(1x) (Fig. 3.4 b).

Beating assays provide a simple yet well-accepted method for initially estimating cardiac reprogramming efficiency (32, 84, 269-272). On TCPS (Fig. 3.5 a, red columns,

Figure 3.5: Effects of culture substrate on cell beating at day 18 of reprogramming. The extent of cell beating, quantified manually by phase contrast microscopy, was influenced by the culture substrate. A) Beating patches are defined as synchronously contracting. PEG hydrogels with high surface densities of adhesion molecules (green bars) had greater numbers of beating patches than gels with low surface densities (blue bars) or any TCPS surface (red bars) (*p<0.05 versus PEG-lam(5x)-RGD(5x); #p<0.05 against PEG-lam(5x) by Tukey’s HSD test). The numbers of beating patches on TCPS surfaces (red bars) were similar regardless of adhesion molecule type or concentration. Unlike the PEG gels, it was not possible to increase beating on TCPS by increasing the laminin concentration. B) PEG conditions presenting laminin at 5x (with or without 5x RGD) also had greater coverage of beating cells than all TCPS conditions or PEG-lam(1x) (*p<0.05 by Tukey’s HSD test).
Matrigel and laminin-1 coatings led to similar numbers of beating patches per cm$^2$ at day 18 (9.04 ± 3.05 versus 9.44 ± 2.42 respectively; p = 0.71 by t-test). When PEG was functionalized with 1x RGD, 1x laminin, or both (Fig. 3.5 a, blue columns), beating patch numbers were similar to the TCPS conditions. A non-significant increase was noted when PEG-lam(1x) gels also presented 5x RGD. This trend was also seen on PEG-lam(5x) gels presenting 1x or 5x RGD (Fig. 3.5 a, green columns). PEG-lam(5x)-RGD(5x) had more beating patches than all TCPS conditions and PEG with lower adhesion molecule concentrations. PEG-lam(5x) (no RGD) also promoted more beating than several of these groups, including the PEG-lam(1x) (no RGD) condition. Increasing laminin concentration from 1x to 5x on TCPS did not result in more beating, suggesting that adsorbed amounts of laminin were already near maximal at 1x solution concentration. On TCPS, laminin saturates around 850 ng/cm$^2$ (273), which is 13.4 and 67.1 times lower than our 1x and 5x concentrations. Therefore, the actual density of the laminin layer adsorbed on TCPS is likely similar for our 1x and 5x concentrations. Similar trends were noted when considering the total beating area instead of the number of beating patches (Fig. 3.5 b). Reprogramming efficiency on Matrigel-functionalized PEG was difficult to assess given the aforementioned adhesion inconsistencies, and Matrigel-functionalized PEG gels were not studied further.
3.4.4 Gene and protein expression of directly reprogrammed cultures

Gene expression in day 18 reprogrammed cells was evaluated by reverse transcription quantitative real-time PCR (qRT-PCR) (Fig. 3.6 a-b). Similar to what was seen in the beating patch counts, gene expression levels from PEG hydrogels with 5x laminin and 1x, 5x, or no RGD were statistically similar. Likewise, no differences were

![Figure 3.6: Effects of culture substrate on gene expression at day 18 of reprogramming. A) Levels of cardiac (Actn2, Tnn1, Cx43) and pluripotency (Nanog) markers were analyzed by quantitative real time RT-PCR. Markers were normalized to the GAPDH internal control gene. TCPS condition levels are scaled to 1 to present fold induction. Levels of Actn2 were higher on PEG-lam(5x) substrates (all RGD levels, green bars) than TCPS (all laminin levels, red bars) (*p <0.05 by t-test). Nanog levels were decreased (*p<0.05 by t-test). N = 9-18 wells. Samples were pooled to increase the number of samples, with measurements for individual substrates presented as supplementary materials. No statistical differences or trends were found between any of the pooled conditions. B) Expression levels of cardiac sub-type genes were normalized to sarcomeric α-actinin (Actn2) to exclude effects from non-cardiomyocytes. Expression level of the atrial marker Mlc2a stays constant, while markers for cardiac nodal and conduction cell phenotypes (Hcn1, Hcn4, Tbx3, Cntn2) are generally downregulated on PEG-lam(5x) conditions when compared to TCPS-Mat(1x) (the condition in Efe et al.). TCPS+Mat(1x) (n=3) is normalized to 1 to present fold change of the PEG-lam(5x) conditions. No statistical differences or trends were seen in PEG-lam(5x) gels presenting 5x RGD (n=3) or no RGD (n=3), so the conditions were pooled for the purpose of statistical testing (n=6). (*p<0.05 by Student’s t-test.)
seen in expression levels on TCPS with 1x or 5x laminin (supplementary Fig. S3.1). Due to inherent high variability in qRT-PCR, data from similar conditions were pooled to increase sample numbers for statistical analysis. Results for each surface are presented individually as supplementary materials (Fig. S3.1).

Cells reprogrammed on PEG hydrogels presenting 5x laminin (with varied levels of RGD) had higher sarcomeric α-actinin (Actn2, 1.7-fold, p =0.03) levels than those reprogrammed on TCPS conditions (with varied levels of laminin) (Fig. 3.6 a). Levels were also higher for cardiac troponin T (Tnnt2, 1.3-fold, p =0.08) and connexin 43 (Cx43, 1.6-fold, p= 0.06), although these changes were not statistically significant. Nanog expression was lower on 5x laminin PEG groups (with all levels of RGD) than on TCPS groups with all laminin concentrations (2.4-fold decrease, p= 0.0001), and the pooled 5x laminin PEG groups were ~700 fold lower than Nanog levels of RW.4 mouse embryonic stem cells.

Cardiomyocyte-like cells resulting from direct reprogramming strategies have not been well characterized with regard to the cardiomyocyte subtype generated, however, the ability to generate different types of cardiomyocytes will likely have important therapeutic consequences. We compared expression of atrial and conduction-enriched genes on PEG-lam(5x) (with and without 5x RGD) to the original condition described in Efe et al. (TCPS-Mat(1x)). Markers for conduction and nodal cell phenotypes (Hcn1, Hcn4, Tbx3, and Cntn2) were normalized to α-actinin to take into the account the differences we see in reprogramming efficiency in these conditions (Fig. 3.6 b). Interestingly, expression of conduction and nodal genes was generally higher on the TCPS-Mat(1x) condition, suggesting that there may be subtle but important roles that the
microenvironment plays in the reprogramming processes to generate these different subtypes of cells.

Immunocytochemistry was used to quantitatively compare cardiac marker sarcomeric α-actinin expression on PEG conditions. Actinin/DAPI-positive pixel ratios for PEG hydrogels presenting 5x laminin with varied RGD concentration were double those presenting 1x laminin with varied RGD concentration (0.02 ± 0.01 versus 0.01 ± 0.01, respectively. p < 0.05 by t-test) (Fig. 3.7 a-c). Data for individual conditions can be found in supplementary Fig. S3.2.

![Graph and images](image)

**Figure 3.7:** Immunocytochemical comparison of cardiac marker expression on PEG conditions with 1x versus 5x laminin. Wells were stained for the cardiomyocyte marker sarcomeric α-actinin, with DAPI as a nuclear counterstain. A) The number of α-actinin positive pixels was normalized to the number of DAPI positive pixels for each well. PEG conditions presenting laminin at 5x had a higher α-actinin/DAPI ratio than conditions presenting laminin at 1x (*p < 0.05 by t-test). (B and C) Sample images from the quantitative analysis performed in (A). Samples were pooled to increase the number of samples, with measurements for individual substrates presented as supplementary materials.
Reprogramming efficiency was measured by flow cytometry for sarcomeric α-actinin (Fig. 3.8 a). No significant differences were found between any of the PEG-lam(5x) groups, though averages for groups containing RGD were slightly higher. The percentage of sarcomeric α-actinin-positive cells was 1.72-fold higher on PEG-lam(5x)-RGD(1x or 5x) compared to TCPS-Mat(1x) (Fig. 3.8 b, 6.35 ± 0.69% versus 3.70 ± 1.03%, p<0.05 against TCPS-Mat(1x) by ANOVA with post-hoc Tukey HSD test).

![Figure 3.8: Quantification of α-actinin expression by flow cytometry. PEG-lam(5x) gels with RGD promoted higher efficiency reprogramming. A) Histograms for fluorescence intensity of sarcomeric α-actinin IgG negative controls (black) versus stained cells (grey) demonstrated well-separated peaks between stained cells and the negative control. At least 10,000 events are presented per condition. B) The average percentage of α-actinin positive cardiomyocytes in PEG-lam(5x) gels containing RGD was ~1.72-fold higher than the condition originally described in Efe et al. (*p<0.05 against TCPS-Mat(1x) condition by ANOVA with post-hoc Tukey-HSD). C) Combined with the proliferation data, the increased efficiency gave greater numbers of cardiomyocyte on PEG-lam(5x) gels containing RGD than PEG-lam(5x) gels without RGD or TCPS-Mat(1x) controls (*p<0.05 by ANOVA with post-hoc Tukey HSD).]
Coupled with the late-stage cell density data (Fig. 3.4 a), the PEG-lam(5x) conditions containing RGD yielded 7.73 ± 0.78 cardiomyocyte-like cells per originally seeded MEF, which is ~2-fold higher than TCPS-Mat(1x) and ~1.5-fold higher than PEG-lam(5x) without RGD (Fig. 3.8 c, p<0.05 against both conditions by ANOVA with post-hoc Tukey HSD).

### 3.5 Discussion

In this study, we demonstrated that the efficiency of direct reprogramming to cardiomyocyte-like cells can be nearly doubled by using custom-tailored PEG culture substrates. By using protein-resistant PEG, we were able to specifically probe the effects of covalently bound ligands on direct reprogramming without the confounding effects of adsorbed serum proteins. Ultimately, this led to the development of a culture surface that produced about twice as many cardiomyocyte-like cells as the originally described substrate. This type of precise control over microenvironment definition is a promising frontier in the field of undifferentiated PSC expansion (274). Global gene expression analyses have also allowed researchers to specifically tailor media to meet the needs of pluripotent stem cells (251, 252), and this strategy should have a similar impact on the design of materials to control cell microenvironment. To date, many laboratories still use Matrigel to trigger integrin signaling pathways and define the cell microenvironment. Replacement of Matrigel with synthetic surfaces (275, 276), biologically inspired peptides (277-279) or purified whole proteins has allowed or enhanced PSC expansion in multiple studies. When ECM components are analyzed individually, laminin (100, 280), vitronectin (102), and E-cadherin (281) have shown to promote undifferentiated human
PSC expansion on TCPS, while fibronectin and collagen IV have been reported to cause their differentiation (100). Interestingly, while the laminin-111 isoform (used in this study) allows undifferentiated expansion of human PSCs (280), it has also caused differentiation of mouse PSCs (282), suggesting differences in integrin expression/signaling/ligand interactions between species.

While many groups are studying substrate designs for differentiating PSCs to cardiomyocytes, the cells of this protocol start as “pre-iPSCs”, which are phenotypically different from iPSCs (283). The cardiac direct reprogramming literature has not yet examined the importance of ECM signaling, making comparisons between various protocols rather complicated. As mentioned earlier, we began with the insight that Koche et al. (253) provided into pre-iPSC integrin expression 3 days after OSKM induction. Taking that study into account, we designed the PEG substrates to have a high concentration of laminin (without the collagen IV that is present in Matrigel). However, laminin alone was not sufficient to support cell adhesion to PEG during the full 18 days of our protocol, possibly due to matrix metalloproteinase degradation. Because RGD-binding integrins were not strongly affected by OSKM induction (253), we incorporated RGD to promote long-term cell adhesion. While the presence of RGD positively affected cell proliferation on PEG hydrogels, its effects on cardiac gene or protein expression were not significant.

Recently, MacLellan and colleagues demonstrated that the timing of ECM protein presentation is important for cardiac maturation. First, they performed an immunohistochemical analysis of developing mouse and human hearts. This showed distinct ECM differences between stem cell niches (mostly collagen IV and laminin) and
the surrounding myocardium (mostly collagen I and fibronectin) into which the maturing
cells migrate (124). Using this information, they differentiated mouse ESCs on TCPS
and found upregulation of Flk1 (a VEGF receptor but also a cardiac progenitor marker)
and ultimately higher expression of the α-MHC (cardiomyocyte marker) on collagen IV
and laminin coatings (as compared to collagen I or fibronectin). By isolating Flk1+
progenitor cells and re-plating them onto new ECM protein coatings, they showed that
fibronectin was better at promoting cell cardiomyocyte maturation than collagen IV or
laminin. Interestingly, Kamp and colleagues have demonstrated that cardiogenesis from
iPSCs is robustly increased by providing “Matrigel overlays” at several timepoints during
differentiation (284), further indicating the importance of timing in ECM presentation.
We expect that by providing ECM proteins to match the timing of integrin expression
during direct reprogramming, we could further improve cardiac cell differentiation
efficiency.

Hydrogel substrates allow manipulation of multiple variables beyond what was
examined in this study. In addition to ligand type and density, one could also study
effects of hydrogel chemistry, nanotopography, degradation/remodeling, and elastic
modulus. Here, we compared effects of two substrates that differ drastically in stiffness.
The storage modulus of this type of gel is ~7 kPa (200), which is similar to developing
heart tissue (164) and has been shown to support embryonic cardiomyocytes (109) and
ESC differentiation to cardiac progenitors (159) better than substrates of drastically
different stiffnesses. While the adhesion protein density dependence (seen only on PEG)
confirmed that integrin-dependent pathways are in some part responsible for the
improvement, it is possible that the heart-like elastic modulus also played a role. Further
studies should be undertaken to examine the interplay of these two factors in greater detail.

To compare our data to Efe et al., we terminated our studies at day 18. However, many factors are known to affect the maturation of PSC-derived cardiomyocytes, including duration in culture (34) embryoid body size and culture condition (285, 286), and growth factor signaling (287). Electrophysiological analysis and global gene expression analyses could be used to assess the maturity at timepoint and later time points (288). Based on the aforementioned studies, we expect that the mechanical and integrin-activation properties and could be adjusted to fine-tune specific cardiac phenotypes yielded by the protocol.

Of the currently reported cardiac direct reprogramming strategies, we chose to work with the Efe et al. protocol for several reasons, many of which are also outlined in a recent review by Morris and Daley (74). The ability to expand cells in culture is important for treating large myocardial injuries. In the Ieda et al. method, cells are converted directly to non-proliferating cardiomyocytes. In the Efe et al. protocol, the cells travel through a proliferative progenitor state, yielding greater numbers of cardiomyocytes (84). Indeed, in this study we were able to double the number of cardiomyocytes produced, in part due to enhanced proliferation on the PEG-lam(5x) gels containing RGD. The progenitors formed by this protocol may also have vascularization potential (84, 85). Indeed, brief OSKM induction combined with VEGF stimulation has separately been shown to directly reprogram human pre-iPSCs to endothelial cells (289). Finally, the Efe et al. method reduces the time to beating (~2 weeks versus 4-5 weeks)
compared to the Ieda et al. method, which may be important clinically as the cardiac remodeling cascade starts immediately following infarction (86, 87).

On the other hand, several groups have reported in vivo infarction scar reprogramming since their original in vitro paper (77, 80-82). This feat has not yet been performed using the Efe et al. protocol, and it can be argued that teratoma risks are higher for the Efe protocol because the cells are more likely to revert to a pluripotent state. However, the OSKM induction in this protocol is transient, and further studies may allow development of methods that ameliorate the risk of teratoma formation (290). In this study, we were able to reduce the already low Nanog expression 2.3-fold by using defined substrates. This leads us to believe that it will ultimately be possible to prevent the formation of pluripotent cells. However, it will also be important to demonstrate the effects of cell microenvironment on the efficiency of the Ieda protocol.

Some groups have reported difficulties reproducing the Ieda et al. protocol (291), and to our knowledge no studies have yet been published using the Efe et al. protocol. Robust induction and proper stoichiometry of the reprogramming factors are highly important for achieving reprogramming using the Ieda et al. protocol (292) and presumably the Efe et al. protocol as well. This can be difficult to achieve using viral induction methods. The availability of mice carrying a doxycycline-inducible polycistronic OSKM cassette (263) eliminated variability between experiments, providing an excellent cell source for probing cell-material interactions in direct reprogramming. While demonstration of direct reprogramming in human cells is more clinically relevant, the genetic homogeneity of cells from transgenic mice is a powerful
advantage, especially when differences in microenvironments may change a number of measures by less than two-fold.

The original publication also reported ~40% cTnT-positive cells, which was an additional reason we initially selected this protocol over the others. However, we found cells cultured on TCPS-Mat(1x) to be only ~4% α-actinin-positive. This discrepancy may be attributed to one or several of the following: 1) temporal variations in the expression of cTnT versus α-actinin during reprogramming, 2) differences in the robustness of the cTnT antibody versus the α-actinin antibody, 3) subtle differences in culture protocols between laboratories, or 4) variations in flow cytometry analysis between laboratories.

The field of cellular reprogramming has progressed at lightning speed since the discovery of induced pluripotency in 2006 (28). Unlike traditional PSC differentiation strategies, direct reprogramming has the potential to eliminate the need for in vitro cell expansion, differentiation, and cell transplantation. Biomaterials that control the cellular microenvironment and eliminate confounding signals caused by adsorbed serum proteins have the potential to enhance our understanding of the requirements for both in vitro and in vivo direct reprogramming.

### 3.6 Conclusions

PEG culture substrates were designed to match integrin expression during the initial stages of OSKM-mediated direct reprogramming of fibroblasts to cardiomyocyte-like cells. Through a combination of increased reprogramming efficiency and increased proliferation, PEG hydrogels presenting high concentrations of laminin and RGD peptide
yielded twice as many cardiomyocyte-like cells as the originally reported substrate (Matrigel-coated TCPS). RGD peptides enabled better cell adhesion, stimulated proliferation, and did not impede reprogramming. The general methodology, using gene expression to guide materials design, may be applicable to a wide variety of reprogramming strategies.

3.7 Acknowledgements

The authors would like to thank Drs. Jeanne Nerbonne, Shelly Sakiyama-Elbert, Robert Mecham, and Guy Genin for helpful discussions relating to these results. We would also like to thank Mitchell Manar for cell culture assistance, and Casey Donahoe, Patrick Blanner, Dr. Scott Marrus, Dr. Nithya Jesuraj, Laura Marquardt, and Hao Xu for technical advice. This work was funded by an American Heart Association predoctoral fellowship 11PRE7690043 (AWS), the National Institutes of Health NIH R01 HL085364 (DLE), R01 HL085369 (IRE), the Lucy and Stanley Lopata endowment (IRE), the Career Award for Medical Scientists from the Burroughs Wellcome Fund (SR), and K08 HL107449 (SR).
3.8 Supplementary Tables and Figures

Table S3.1: Primer sequences. Primer sequences used in the analysis of cardiac sub-types.

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Table S3.2: Substrate conditions table. Surface densities and method of binding for adhesion molecules on various substrates. *Matrigel and laminin-1 surface densities (1x) are based on approximate Matrigel surface protein concentration reported in Efe et al. **RGD surface density (1x) based on the approximate RGD surface density of PEG hydrogel microspheres in Smith et al.

<table>
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<th>Matrigel Surface Density (µg/cm²)</th>
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<th>RGD Surface Density (µg/cm²)</th>
<th>Molecule-Substrate Binding Method</th>
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Figure S3.1: Expanded gene expression data. Sarcomeric α-actinin (Actn2), Cardiac troponin T (Tnnt2), Connexin 43 (Cx43), and Nanog (Nanog) gene expression levels were acquired by quantitative real-time RT-PCR and normalized to those of the internal control gene GAPDH. *p<0.05 by Tukey’s HSD test against the undifferentiated MEF control.
Figure S3.2: Immunocytochemical comparison of cardiac marker expression on PEG gels
Wells were stained for cardiomyocyte marker sarcomeric α-actinin and DAPI as a nuclear counterstain. The number of α-actinin positive pixels was normalized to the number of DAPI positive pixels for each well. *p<0.05 against PEG-RGD(1x) by Tukey’s HSD test.
CHAPTER 4

Effects of 3D microenvironmental variables on direct reprogramming of mouse fibroblasts to cardiomyocyte-like cells

4.1 Abstract

Direct reprogramming to cardiomyocytes, or conversion of somatic cells to cardiomyocytes without going through the pluripotent state, has so far only been investigated using two-dimensional culture substrates. However, optimizing three-dimensional culture protocols will bolster the scalability for clinical use. In this study, we analyze the effects of materials, topography, diffusion, and remodeling on a 3D direct reprogramming protocol adapted from Efe, et al. (Nature Cell Biology, 2011, 13:215). Compared to 2D culture, cardiac gene expression (Actn2 and Tnnt2) was downregulated when cells were reprogrammed within 3D PEG microsphere scaffolds and 3D collagen scaffolds but was not statistically different for 3D collagen-PEG microsphere hybrid scaffolds. Sarcomeric α-actinin expression was lowest within PEG microsphere scaffold interiors, suggesting that diffusion and remodeling restrictions may be at play. Cardiac gene expression was also downregulated when cells were cultured on 2D layers of PEG microspheres as compared to flat 2D PEG surfaces, suggesting that the topography of the microspheres may be inhibiting efficient cardiac reprogramming. These analyses provide the groundwork for designing 3D scaffolds to permit high efficiency direct reprogramming to cardiomyocytes, particularly with regards to substrate topography and cellular remodeling capacity.
4.2 Introduction

Two-dimensional (2D) cell culture models have led to countless medical breakthroughs. However, the clinical transition of autologous cell transplantation research will demand a scalability that 2D culture cannot easily provide. For example, if all of the cells of the average human heart were to be cultured in 2D, it would require \( \sim 3,500^2 \) feet of culture area. Three-dimensional (3D) culture techniques address scalability, but many differentiation protocols are still being optimized for 2D culture, including those related to the new and rapidly growing field of direct reprogramming to cardiomyocytes (75, 76, 82, 84). Direct reprogramming is the process by which a fully differentiated cell (i.e. a skin fibroblast) can be converted to another cell type without entering a pluripotent state. In this study, we focus on the protocol initially described by Efe et al., which is faster and more efficient than taking somatic cells through induced pluripotency before reaching cardiomyocytes (84). Like other direct reprogramming protocols (75, 76, 82), it has only been optimized for 2D culture.

Cells adhered to 3D materials exhibit dramatic differences compared to their 2D counterparts (119-123). Adhesion-mediated processes begin at integrin receptors in focal adhesions, and are transmitted through the cell via mechanically-sensitive cytoskeletal networks (293-296). DeSimone (297), Guilak and colleagues have recently provided thorough reviews on adhesion mediated processes, which are well known to affect cell division, apoptosis, migration, and differentiation. Various groups have demonstrated the importance of substrate mechanical properties (108, 109, 159), substrate topography (101, 107), and the shape of adhesive areas (110, 298, 299) on cytoskeletal morphology and cell behavior. Recently, Schenke-Layland et al. investigated specific properties of the
natural cardiac stem cell niche, and designed culture substrates accordingly. They found that differentiation of mouse embryonic stem cells (ESCs) to cardiac progenitors was enhanced in a 3D electrospun scaffold compared to routine, flat 2D culture (124). Differentiation to cardiac progenitors was further enhanced when the scaffolds were coated with type IV collagen and gelatin as opposed to gelatin alone.

We have previously demonstrated that direct reprogramming efficiency can be increased using 2D poly(ethylene glycol) (PEG) substrates that present precise concentrations of adhesion molecules instead of 2D tissue culture polystyrene (TCPS) substrates (300). Separately, we have also demonstrated that modular PEG microsphere-based scaffolds enable HL-1 cells to retain certain aspects of their cardiomyocyte phenotype for long periods in culture (266). In this study, we encapsulate cells in PEG microsphere scaffolds, collagen scaffolds, or collagen-PEG microsphere hybrid scaffolds, and compare the 3D materials in terms of direct reprogramming efficiency. We also examine the effects of dimension (2D vs. 3D), diffusion into the 3D scaffold, and topography of the culture substrates using PEG materials.
4.3 Materials and Methods:

4.3.1 Cell culture and direct reprogramming

The direct reprogramming protocol used to generate cardiomyocytes is adapted from Efe et al. (84). Extended versions of this protocol, including validation with adult somatic cells, are published elsewhere (84, 266). All experiments in this study used mouse embryonic fibroblasts (MEFs) homozygous for doxycycline-inducible Yamanaka factors (Oct4, Sox2, Klf4 and c-Myc) (28, 263). Animal protocols were approved by Washington University Institutional Animal Care Use Committee. A schematic of the culture timeline is shown in Fig. 4.1. MEFs were plated onto 2-dimensional substrates (3,600/cm²) or seeded into 3-dimensional scaffolds one day prior to beginning the

![Timeline schematic](image)

**Figure 4.1:** Direct reprogramming media and supplements timeline. The direct reprogramming protocol was adapted from Efe et al. (and verified in Smith et al.). Six days of dox-induced OSKM were coupled with pluripotency inhibition by Jak-Stat Inhibitor (JI1) in high serum medium. This was followed by three days of recovery in low serum medium also containing JI1, and then six days of induction of cardiomyocyte differentiation in chemically defined (no serum) medium containing bone morphogenetic protein 4 (BMP4). Abbreviations: Fibroblast (FB) medium, Knockout (KO) medium, Chemically Defined (CD) medium, ES-qualified fetal bovine serum (FBS).
protocol (day -1). Seeding densities were 10 million/mL for PEG scaffolds (unless otherwise noted) and 1 million/mL for collagen-containing scaffolds (lowered to account for cell density increases upon collagen gel compaction (301)). Reprogramming commenced the following day (day 0), and media was changed every 24-48 hours. From days 0-5, cells received knockout DMEM (KO DMEM, 10829018) + 2 µg/mL doxycycline (made fresh for each experiment, Sigma, D9891) + 0.5 µM JAK inhibitor I (JI1, EMD, 420099) + 15% embryonic stem cell qualified FBS (ES-FBS, 10439-024) + 5% knockout serum replacement (KSR, 10828028) + 0.1 mM β-mercaptoethanol (21985-023), + 1% Glutamax (35050-061) + 1% non-essential amino acids (11140-050) + 1% embryonic stem cell qualified nucleosides (Millipore, ES-008-D). For days 6-8, doxycycline was removed, ES-FBS concentration reduced to 1%, and KSR concentration increased to 14% (all other components remained the same). From days 9-14, cells were given chemically defined medium (CDM) with BMP4: RPMI-1640 (21870-084) + 20 ng/mL BMP4 (Stemgent, 03-0007) + 0.5x N2 (17502-048) + 1x B27 w/o vitamin A (12587-010) + 0.05% BSA fraction V (15260-037) + 0.5% Glutamax + 0.1 mM β-mercaptoethanol. From day 15 onward, cells were given CDM without BMP4. Cells in scaffolds received 3 mL of media/day, while 2D cultures were given 1 mL/well. RW.4 mouse embryonic stem cells (ATCC), a gift from the Sakiyama-Elbert laboratory, were cultured according to previously described methods (267).

4.3.2 Materials

For 2D control conditions, experiments were performed in 24-well plates. For PEG substrates, plates were first treated with a plasma cleaner (Harrick Plasma) set to
medium (350 mTorr for 5 min) to reduce meniscus formation in the wells during gel formation. Plates were then UV-sterilized for 30 min and gel precursor solutions were added immediately. PEG derivatives were synthesized as previously described (213, 214, 264). For gel fabrication, PEG₈-Vs and PEG₈-Am solutions (both 20% w/v in PBS, pH 7.4) were sterile-filtered (0.22 µm filter) and diluted in 0.03 M NaOH in PBS (pH 8.75; NaOH enhanced reaction kinetics and further reduced meniscus formation) for a final PEG concentration of 12.43% (w/v). The precursor mix was aliquoted at 200 µL/well and allowed to crosslink in a humidified incubator (37°C) for 1-2 days.

On 2D surfaces, protein and peptide surface concentrations are given as 1x or 5x as previously reported (300). When referring to Matrigel (BD Biosciences, 356234) or laminin-1 (Sigma, L2020), 1x is the approximate protein concentration of Matrigel used in Efe et al. (11.4 µg/cm²). The 1x concentration of laminin was also set to 11.4 µg/cm². When referring to the RGD peptide, 1x (354 µg/cm²) was empirically determined to promote robust cell adhesion and reprogramming on 2D substrates (300). For functionalization with Matrigel or laminin-1, protein stocks were first diluted to 108.3 (1x) or 541.5 µg/mL (5x) in PBS. If RGD peptide (GCGYGRGDPG, Genscript) was included, it was diluted in the same tube as the protein at 3.36 (1x) or 16.82 (5x) mg/mL. Each well received 200 µL of protein/peptide solution. The pre-formed PEG hydrogels were incubated with the protein/peptide mixtures overnight at 37°C to permit the covalent Michael-type reaction to occur. For TCPS conditions, (non-plasma treated) 24-well plates were incubated at room temperature for 1 h to permit protein adsorption (according to Matrigel product literature).
Microspheres were fabricated as previously described (199, 200, 266). Briefly, 20% (w/v) PEG$_8$-VS and PEG$_8$-Am were mixed (either 2:1 or 1:1 PEG$_8$-Vs:PEG$_8$-Am) and pre-reacted for 70% of the total gel time needed to reach gel point. RGD (1 or 5 mM, sequence GCGYGRGDSPG, Genscript) was added to the prereacted solution and reacted at 37°C for an additional 30 min. The pre-reacted solution was diluted to a final concentration of 2% PEG and ~0.5 M Na$_2$SO$_4$ and brought to 37°C to induce phase separation. The Na$_2$SO$_4$ concentration needed to achieve cloud point is sensitive to fluctuations in pH, temperature, Na$_2$SO$_4$ and PEG stock concentrations (199), and should be empirically verified any time a new stock solution is introduced. PEG was reacted in the phase-separated state for 45 min (for 1:1 OVs:OAm microspheres) or 55 min (for 2:1 OVs:OAm) to form microspheres, Na$_2$SO$_4$ solution was replaced with PBS via centrifugation (14,000 RCF for 2 min), and microspheres were washed once more then resuspended in PBS. Post-swelling microsphere diameters were 10.2 µm for 1:1 OVs:OAm and 20.1 µm for 2:1 OVs:OAm. Microsphere volumes were determined by measuring the total volume of solution after the addition of 500 µL of PBS.

For reaction of adhesion proteins to remaining OVs groups on microsphere surfaces, microspheres were incubated overnight at 37°C on a slow, horizontal rocker with laminin in PBS at a 2:5 microsphere:laminin solution volume ratio. The concentration of laminin reacted to the microspheres was adjusted to approximate the equivalent protein spacing as the cells experienced in 2D:

$$[lam]_{3D} = \left(\sqrt{[lam]_{2D}}\right)^3$$

So 1x (11.4 µg/cm$^2$) in 2D becomes 38.5 µg/mL microspheres in 3D. To fluorescently label microspheres, 1 µL of Dylight-633 NHS Ester (10 mg/mL, Thermo Scientific,
46414) was added per microliter of pre-formed 2:1 OVs:OAms microspheres. To create 2D microsphere layers on TCPS, 20 µL of pre-functionalized microspheres in PBS were suspended per well of a 24 well plate, and centrifuged at 300 RCF for 15 min. Microspheres remained adhered to the plate by electrostatic interactions throughout the 18 day protocol, forming a continuous layer upon which cells could be cultured.

Encapsulation of cells during the crosslinking of PEG microsphere scaffolds has also been described elsewhere (266). Pelleted MEFs were resuspended in 50 µL of warmed 20% dextran (M_r = 100,000, Sigma Aldrich, 09184) in DMEM, and this solution was used to resuspend a microsphere pellet of equal volume (50 µL) in a 1.5 mL Eppendorf tube. The tube was centrifuged (500g for 3 min) to promote separation of the PEG and the dextran, and incubated at 37ºC for 1 hr to allow microsphere-to-microsphere crosslinking around the MEFs. Cylindrical, crosslinked scaffolds (~5.9 µm in diameter, 1.8 µm in height) were then transferred to 35 mm diameter non-tissue culture treated plastic petri dishes and routine cell culture techniques commenced (3 mL media/day).

For collagen-based scaffolds, 60 µL of collagen (5 mg/mL, Cultrex Rat Tail Tendon Collagen I, R&D Systems, 3447-020-01) was diluted on ice in 73.6 µL sterile double distilled water, 15 µL of 10X concentrated DMEM, and 1.4 µL of 0.34 M NaOH. Pelleted MEFs were quickly resuspended in 50 µL of serum free DMEM and added to the collagen mixture for a final collagen concentration of 1.5 mg/mL. For collagen-PEG hybrid gels, 20 µL of pre-functionalized microspheres were added with the cells. Collagen was then carefully pipetted onto a 35 mm non-tissue culture treated plastic petri dishes. After crosslinking at 37ºC for 1 hr, gels were carefully detached from the surface and routine cell culture techniques commenced (3 mL media/day).
4.3.3 Quantification of gene expression

Gene expression was evaluated by quantitative real time RT-PCR using an Applied Biosystems StepOnePlus machine. On day 18 of reprogramming, cellular RNA (n=6 samples per condition) from 2D samples was purified with TRIzol reagent (Invitrogen, 15596-026) by following the manufacturer’s instructions. For 3D samples, each scaffold was homogenized (Tissue Miser, Fisher Scientific) on ice in a 50 mL centrifuge tube containing 2.6 mL of ice cold TRIzol and 137 µL of glycogen (5 mg/mL, Invitrogen, AM9510) for 1 min. The suspension was divided into two 1.5 mL Eppendorf tubes and centrifuged at 4°C for 2 min at 12,000 RCF to remove solid material. The supernatant was transferred to two new 1.5 mL Eppendorf tubes, and routine TRIzol extraction methods ensued. The final RNA pellet was resuspended in 12 µL of 1 mM sodium citrate buffer (pH 6.4, Invitrogen, AM7000). RNA concentration and quality (260/280 nm absorbance ratio) were verified using a NanoDrop 2000 or Implen Nanophotometer P-Class. Genomic DNA was removed and RNA was reverse transcribed to cDNA (10 ng RNA/µL) using the QuantiTect Reverse Transcription Kit (Qiagen, 205313). Pre-validated primers for mouse GAPDH (Gapdh), sarcomeric α-actinin (Actn2), cardiac troponin T (Tnnt2), connexin 43 (Cx43), Nanog (Nanog), and CD31 (Cd31) transcripts were obtained from Qiagen (Quantitect Primer Assay). cDNA was amplified in triplicate reactions using Quantitect SYBR Green PCR Kit (Qiagen) following manufacturer’s instructions. Expression levels were normalized to GAPDH and then to the 2D PEG control condition by the \(2^{-\Delta\Delta C_T}\) method (268).
4.3.4 Scaffold remodeling assay

Images of scaffolds were taken by brightfield microscopy (2x objective, IX70, Olympus) using a Magnafire camera (Optronics). For scaffolds larger than the field of view, multiple images were stitched using Adobe Photoshop. Scaffold diameters were measured using ImageJ (National Institutes of Health) (4 equally spaced diameters were averaged for each scaffold). Average diameters for each day were then normalized to the diameter from the day of seeding.

4.3.5 Immunofluorescence and image processing

Sarcomeric α-actinin expression within reprogrammed scaffolds was analyzed by quantitative immunofluorescence. Day 18 scaffolds were fixed in 3.7% formaldehyde for 45 min at room temperature, treated with 30% sucrose solution for 3.5 h at room temperature for cryoprotection, then frozen in OCT (Tissue-Tek®, Sakura) at -80º C for 30 min (or more for storage). Scaffolds were cryostat sectioned with careful attention to depth of sectioning. After sectioning, slides were blocked for 30 min at room temperature using 10% normal goat serum/1% bovine serum albumin in PBS (also containing 0.1% Triton-X 100 for permeabilization). Sarcomeric α-actinin primary antibody (mouse IgG, Sigma A7811) was diluted 1:1600 in the blocking solution and applied to the slides overnight at 4ºC. Slides were washed 3x in PBS (5 min each) before incubation in secondary antibody diluted 1:1000 in blocking solution (1 h at room temperature, Alexa Fluor 594 goat anti-mouse, Invitrogen, A-11005). Slides were washed 3x in PBS before application of DAPI as a nuclear counterstain (100 ng/mL, 30 min at room temperature). Slides were washed once more and coverslips were applied.
For quantification of sarcomeric α-actinin expression, images were acquired by fluorescence microscopy (IX70, Olympus) using a Magnafire camera (Optronics). Each section was imaged in its entirety using a 10x objective. Images were analyzed in Matlab, with the number of α-actinin positive pixels normalized to the number of DAPI positive pixels for each image to create an α-actinin-DAPI ratio. Ratios were then averaged with respect to condition or depth within the scaffold. For the cell density analysis, at least 20 images were averaged per scaffold. For the depth analysis, at least 6 images were averaged per depth region, per scaffold.

4.3.6 Statistics

Two-condition comparisons were made using student’s t-test, whereas multiple comparisons were made using ANOVA with post-hoc Tukey HSD. Data are presented as mean ± standard error of the mean.

4.4 Results

4.4.1 Effects of dimension and 3D scaffold material on gene expression of directly reprogrammed cells

To assess the effects of dimension and 3D scaffold material on the phenotype of reprogrammed cells, we tested three 3D conditions and normalized gene expression to cells cultured on 2D PEG-lam(5x)-RGD(5x) substrates (300). Microspheres used in scaffolds within this experiment were also PEG-lam(5x)-RGD(5x) (2:1 OVs:OAm).
Cardiac troponin T (Tnnt2) and sarcomeric α-actinin (Actn2) are well-established cardiomyocyte markers, while connexin 43 (Cx43) is a gap junction protein that is present in ventricular cardiomyocytes and many other cell types (including ESCs (302)). Nanog (Nanog) is a pluripotent cell marker, which is minimally expressed by cells directly reprogrammed on 2D PEG-lam(5x)substrates (~700-fold lower than RW.4

**Figure 4.2:** Effects of dimension and 3D scaffold material on gene expression. Expression of cardiac markers Actn2, Tnnt2, and Cx43, pluripotency marker Nanog, and endothelial cell marker Cd31 was assessed for various 3D materials (100% PEG- green columns, collagen/PEG hybrid- red columns, 100% collagen I- blue columns) and normalized to levels from 2D PEG culture substrates (with laminin and 5 mM RGD) by the $2^{-\Delta\Delta C_{T}}$ method. PEG microspheres used in 3D materials were also functionalized with laminin and 5 mM RGD. Gene expression was compared using a nonparametric Kruskal-Wallis test with post-hoc Tukey HSD. The collagen/PEG hybrid material presented no statistical differences for any gene compared to the 2D control. However, expression of cardiac markers Actn2 and Tnnt2 was downregulated for the 3D 100% PEG microsphere and 100% collagen I scaffold conditions (*p<0.05). Cx43 expression was slightly upregulated for all conditions, possibly due to the presence of other cell types (#p<0.05 between 100% PEG-microsphere and 100% collagen I). Nanog expression was not significantly different for any of the tested conditions. Cd31 expression was downregulated in the 100% PEG microsphere scaffolds compared to the 2D control (*p<0.05), but changes in the collagen and collagen/PEG hybrid were not significant.
mouse ESCs (300)). Since the originally described protocol reported a progenitor that may have potential to form vascular cell types as well as cardiomyocytes (84), we also looked at the endothelial cell marker CD31 (Cd31).

Compared to the 2D PEG control, cardiac markers Actn2 and Tnnt2 were downregulated for the 100% PEG-microsphere and 100% collagen 3D scaffold conditions (Fig. 6). Cardiac gene expression from the 3D PEG/collagen hybrid scaffold did not differ statistically from the 2D control (though a 9.3-fold decrease in the mean was observed). Cx43 levels were slightly higher in 3D materials than in 2D, but this was not statistically significant. Cx43 upregulation in conjunction of downregulation of the more specific cardiac genes may reflect reprogramming into other cell types (for example, Cx43 is also expressed in ESCs (302)). For all 3D materials, Nanog expression remained similar to the 2D control. Cd31 was significantly downregulated for 100% PEG microsphere scaffolds but not for the 100% collagen or the collagen/PEG hybrid scaffold, suggesting these materials may be better suited for endothelial cell differentiation or certain hematopoietically derived cell types (neutrophils, platelets, monocytes, and some T-cells) that also express Cd31 (303).
4.4.2 Effects of cellular remodeling on 3D direct reprogramming to cardiomyocytes

In two dimensions, reprogramming cells begin to form clusters around day 10, and many of these begin beating by day 18. These clusters are often macroscopically visible, and can grow to be several millimeters wide (Fig. 4.3 a, (84, 300)). In 3D culture,

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**Figure 4.3:** Morphology of reprogrammed cells at scaffold surfaces compared to cells embedded within microspheres. Scaffolds were fixed at day 18, sectioned, and stained for sarcomeric α-actinin. A) On 2D substrates, reprogrammed cells form large clusters that may be several millimeters wide and sometimes beat spontaneously. B) Densely packed, large, beating clusters were occasionally seen on the surfaces of scaffolds. Sarcomeric α-actinin within these surface clusters had a striated appearance, indicating a mature contractile apparatus (white arrow in magnified inset). C) The largest cluster of α-actinin positive cells embedded within microspheres at a depth greater than 100 µm. α-actinin positive clusters within microspheres were rare and smaller than those on the surface. Most often, α-actinin positive cells occurred in groups of 1-3 cells if embedded within microspheres.
beating clusters were occasionally observed at scaffold edges, and these stained positive for large areas of striated sarcomeric α-actinin (Fig. 4.3 b). However, at greater depths, α-actinin positive cells almost always appeared in small groups of 1-3 cells (not shown). The largest α-actinin positive cluster observed surrounded by microspheres was ~100 µm wide (Fig. 4.3 c), but this was unusual and very small compared to beating clusters seen on 2D substrates. The decrease in reprogramming efficiency seen in the PEG materials may be partially attributed to the inability to form large clusters, as is seen in studies on embryoid bodies (285, 304-307). This would impact paracrine signaling and possibly interfere with reprogramming.

Cells release matrix metalloproteinases (MMPs) that cleave and remodel collagen

**Figure 4.4:** Cellular remodeling of 3D scaffolds. Decreasing scaffold diameter indicates ECM cleavage and remodeling. Scaffolds were fabricated 1 day prior to initiating reprogramming and their diameters were measured over the first 5 reprogramming days. Diameters were normalized to their diameter at day 0. All groups decreased in diameter, with collagen-containing scaffolds decreasing to a greater extent than 100% PEG microsphere scaffolds (*p<0.05 by ANOVA with post-hoc Tukey HSD). This indicates that cells are better able to remodel the collagen materials than the PEG microspheres.
gels, causing a decrease in the scaffold size over time (301, 308, 309). To highlight the differences in how reprogramming cells may react with scaffold materials, we took scaffold diameter measurements during the early stage of direct reprogramming (Fig. 4.4). Microspheres used in this experiment were PEG-lam(5x)-RGD(5x) (2:1 OVs:OAm). All scaffolds showed decreases in diameter, but the collagen-based scaffolds shrank much more than PEG microsphere scaffolds. This could be due in part to the greater susceptibility to MMP cleavage (p<0.05 by ANOVA with post-hoc Tukey HSD test at every day following seeding).

PEG microsphere morphology was retained throughout reprogramming in 100% PEG scaffolds, but few sphere-like PEG structures were found in collagen-PEG hybrid scaffolds after 18 days of reprogramming (Fig. 4.5). In Fig. 4.5, PEG in collagen-hybrid scaffolds was fluorescently labeled (red), whereas the PEG microspheres in 100% microsphere scaffolds were visible by phase microscopy. In the collagen/PEG hybrid scaffold, embedding PEG microspheres in the collagen gel enables a reaction between free PEG-VS groups and the surrounding collagen fibrils, such that the PEG microsphere becomes part of the polymer network. The cells, which use focal adhesions to reshape the collagen, are also pulling on the collagen-linked PEG. In the 100% PEG microsphere scaffolds, cells interact only with laminin or RGD-functionalized VS groups, which likely provides far fewer focal adhesions than the collagen/PEG hybrid scaffold. This may explain why the 100% PEG microsphere scaffolds decrease to a lesser extent than the collagen based materials. In addition to differences in the chemical structure of the scaffold materials, the differences in cellular interactions with the materials may help to
explain the trend towards more efficient cardiac reprogramming seen in the in collagen-based scaffolds (Fig. 4.2).

4.4.3 Effects of cell density and cell depth on 3D direct reprogramming to cardiomyocytes

To assess the possible effects of nutrient diffusion and cell density within the

**Figure 4.5:** Morphology of PEG in scaffolds with or without collagen after 18 days of reprogramming. Collagen-PEG hybrid scaffolds were fabricated with fluorescent PEG microspheres (red, left side). Microspheres in 100% PEG scaffolds (right side) were not fluorescent, but are clearly visible by phase contrast. Scaffolds were fixed on day 18, sectioned and imaged by either fluorescence or phase microscopy. By day 18, the PEG in collagen-PEG hybrid scaffolds no longer resembled microspheres, indicating that it had been remodeled by cells. Microspheres were still clearly defined in 100% PEG scaffold (lower right, white arrows).
scaffold, we assessed sarcomeric α-actinin expression by immunohistochemistry at various depths from the surface of PEG-RGD(1x) microsphere scaffolds (1:1 OVs:OAm).

Scaffolds were seeded at either 5 million (low density) or 25 million (high density) MEFs/mL. The seeding density had an inverse effect on reprogramming efficiency, with α-actinin:DAPI ratios for low cell density scaffolds 18.7-fold higher than high cell density scaffolds (p<0.05 by t-test) (Fig. 4.6 a). Similar results have been noted in iPSC derivation from MEFs using the inducible transgene system (310), and may reflect: 1) differences in paracrine signaling intensity, or 2) nutrient depletion at high cell densities. The effect of depth into the scaffold was less pronounced, but a decreasing trend in reprogramming efficiency was noted as depth increased (Fig. 4.6 b). When depths were normalized to the outermost region, the innermost region had a 1.62 fold decrease in the α-actinin/DAPI ratio as compared to the outermost region (Fig. 4.6 c) (p<0.05 by t-test). This could be attributed to multiple effects: 1) limitations in cluster size as mentioned above, or 2) lowered diffusion of nutrients.
**Figure 4.6:** Effects of cell density and cell depth on direct reprogramming. A quantitative immunohistochemical analysis of sarcomeric α-actinin expression was performed to analyze expression at various depths within PEG microsphere scaffolds initially seeded at two different cell densities (5 million/mL - light green columns and 25 million/mL - dark green columns). Scaffolds were fixed on day 18, sectioned and stained for the α-actinin and DAPI. Fluorescent images were analyzed in Matlab, with the number of α-actinin positive pixels normalized to the number of DAPI positive pixels for each image. 

A) Cell seeding density was inversely related to reprogramming efficiency (*p<0.05 by t-test), with a 5-fold increase in cell density resulting in an 18.7-fold decrease in α-actinin/DAPI ratio (n=3, >20 images analyzed per condition). 

B) Reprogramming was generally higher in the outer regions of the scaffold, though no statistical differences were seen (n = 2-3, >6 images analyzed per condition). 

C) Data from scaffolds of both cell densities were combined and the 151-300 and 301-450 depths were normalized to the outermost region to present the fold decrease in α-actinin/DAPI ratio. The ratio of the innermost region was 1.62-fold lower than the outermost region (*p<0.05 by t-test, n = 4-5, >12 images analyzed per condition).
4.4.4 Effects of substrate topography on direct reprogramming to cardiomyocytes

To investigate the effects of substrate topography while eliminating the variable of diffusion, we reprogrammed cells on a 2D layer of PEG-Mat(1x)-RGD(1x) microspheres (1:1 OVs:OAm), and normalized their gene expression to flat 2D PEG-lam(1x). In our previous study, reprogramming efficiency remained similar between these adhesion molecules at these concentrations (300), so we expect any changes to be more influenced by the variation in topography. Changes in mechanical stiffness of the culture substrate can have dramatic effects on cellular phenotype (108, 109). The flat, hydrated PEG gel is estimated to be 1.1 millimeters thick, while the microspheres are about 10.2 ± 0.2 µm thick (~100-fold thinner). Therefore, the relative stiffness experienced by the cells is likely much higher on the microsphere layer (closer to that of the TCPS substrate underneath). To demonstrate that variations from the flat PEG surface are not solely attributed to these mechanical differences, we also have included the gene expression data for TCPS-lam(1x). Culture on the PEG-microsphere layer caused dramatic decreases in Actn2 (21.2 ± 11.2-fold), Tnnt2 (5.0 ± 2.4-fold), and Cd3l (27.0 ± 4.4-fold) expression as compared to the 2D PEG gel surface (p<0.05 by t-test) (Fig. 4.7). These changes were more pronounced than variations between PEG-lam(1x) and TCPS-lam(1x), indicating that they were not solely attributable to mechanical variation. This finding also suggests that, in addition to diffusion limitations, the microsphere topography is not optimal for direct reprogramming to the cardiomyocyte phenotype as markers were downregulated despite unrestricted access to the media.
Figure 4.7: Effects of 2D PEG topography on gene expression of directly reprogrammed cells. In order to analyze responses to microsphere topography without the confounding effects of diffusion limitations seen in 3D, cells were reprogrammed on a thin layer of PEG microspheres at the bottom of a 2D well, and expression levels were normalized to a flat 2D PEG gel control condition. Cells on the thin layer of microspheres experience higher stiffness than those on the thicker flat PEG gel due to closer proximity to the TCPS surface underneath (~100 fold difference in gel thickness). Because of this, expression levels from TCPS were also included to help delineate effects of topography and mechanical properties. Expression of cardiac markers Actn2 and Tnnt2 were downregulated on the microsphere layer as compared to the 2D PEG gel (*p<0.05 compared to the 2D PEG control by t-test). Nanog was upregulated (*p<0.05), but still low in comparison to RW.4 mouse embryonic stem cells (not shown). Cd31 was also downregulated on the microsphere layer, indicating that the topography may be disruptive for formation of endothelial cells. Regulation patterns between the microsphere layer and the TCPS substrate varied (#p<0.05 for 2D microsphere layer versus TCPS), suggesting that expression differences between the thin layer of microspheres and the thick flat PEG gel cannot be solely attributed to mechanical differences.
4.5 Discussion

The role of the microenvironment on embryonic cells has been under investigation for nearly a century, when their orientation along a fiber was first described (4). Since then, we have become increasingly aware of the roles of cellular remodeling, diffusion, intercellular contacts, topography, and mechanical properties in embryonic development and stem cell differentiation protocols. Differentiating cells in 3D culture is an increasingly important goal for the field of regenerative medicine, as cell transplantation techniques will require vastly higher numbers of cells than 2D culture can practically provide.

The improved scalability of 3D culture systems can be leveraged to meet this demand. For example, in this study we cultured 200,000 cells in one collagen-based scaffold, using 3 mL of media per day. Culturing the equivalent number of cells in 2D required 28 mL/day (~10 times as many cells in 3D versus 2D per mL of media). While our initial studies show a modest decrease in cardiac gene expression in 3D for the collagen-based scaffolds (Fig. 4.2), the 3D culture may still yield more cardiomyocytes per mL of media. Furthermore, various modifications to the material could foreseeably improve efficiency, and they will be discussed herein. A review of the current cardiac direct reprogramming literature is beyond the scope of this discussion, but can be found elsewhere (300).

Our study highlights differences in ECM remodeling between collagen-based and 100% PEG-microsphere scaffolds. Collagen allows the cells to arrange their microenvironment, as demonstrated by the scaffold size changes (Fig. 4.4) and the differences in the day 18 PEG morphology when microspheres were embedded in
collagen versus part of a 100% microsphere scaffold (Fig. 4.5). Other studies have also highlighted the importance of remodeling in 3D environments. Recently, our collaborators showed greater PC12 cell aggregation in collagen-PEG microsphere hybrid scaffolds compared to 100% PEG microsphere scaffolds (311). The Seliktar group has studied neonatal rat cardiomyocytes cultured in PEG-fibrinogen hybrid materials and found that increasing the ratio of PEG to fibrinogen limited cellular remodeling (312) and resulted in a decrease in beating scaffolds (313). Similarly, the Hubbell group has found that inhibiting remodeling activity within a bulk PEG hydrogel caused P19 cells undergoing cardiac differentiation to stall at a progenitor state, whereas their uninhibited counterparts expressed markers of fully mature cardiomyocytes (159). It is therefore likely that remodeling constrictions within our 100% PEG microsphere scaffolds were partially responsible for the dramatic downregulation of cardiac gene expression compared to 2D surfaces.

Though they rarely exhibit a pluripotent phenotype (84), reprogramming cells on 2D surfaces form large clusters that morphologically resemble embryoid bodies (EBs). These clusters are macroscopically visible and can sometimes be several millimeters wide (Fig. 4.3 a). Several groups have demonstrated profound effects of EB size on cardiac differentiation efficiency (304-307). In 1987, Smith et al. noted that cardiac differentiation from P19 embryonic carcinoma cells was dependent on the initial size of the cell aggregates. They suggested a relationship between paracrine signals from an endodermal layer on the aggregate surface and ultimate cardiac differentiation efficiency (304). More recently, the Zandstra group has also noted that the optimal EB size for cardiac differentiation is 400 µm (as compared to 200 or 800) (285, 305). They
confirmed the relationship between cardiac differentiation and the endodermal surface layer, linking EB size to the relative strength of paracrine signaling (314). Taking these studies into account, it is possible that the relatively small pore size in our 100% PEG microsphere scaffolds (266) inhibited the large cluster formation seen in 2D and interfered with cardiac direct reprogramming processes.

We investigated the impact of topography on direct reprogramming by comparing gene expression from cells cultured on a 2D layer of PEG microspheres, a flat 2D PEG substrate, or a 2D TCPS substrate (Fig. 4.7). Substrate topographies are well known to have effects on cellular behaviors (103). For 2D cardiomyocyte culture, particular research emphasis has been placed on topographies that induce cellular alignment, as is seen in vivo. In natural myocardium, action potential propagation occurs fastest along the longitudinal axis of the rod shaped cardiomyocytes (315). The largest collagen fibrils run parallel to the long axis (316), likely helping cells to maintain their orientation (106). In 2D cardiac culture, elongated topographical features, such as physical fibers (10), grooves (9), or patterned lines of ECM molecules (317, 318), help to induce cellular alignment and conduction anisotropy (319). Recently, Levchenko and colleagues demonstrated that nanoscale variations in elongated 2D surface patterns influence cytoskeletal architecture and the resulting conduction speed and anisotropy (106). This research has also been translated to 3D, resulting in greater cellular alignment (227, 320), anisotropic conduction (321), and higher instances of beating within scaffolds (322). In contrast to elongated contact cues, the rounded surface of the microspheres does not appear to be favorable for efficient direct reprogramming to cardiomyocytes. This may
also further help to explain the better performance of the fibrillar collagen-based materials as compared to the 100% PEG-microsphere scaffolds (Fig. 4.2).

Many groups have noted a decrease in cell viability and differences in phenotype at scaffold depths greater than 200 µm due to decreased diffusion of nutrients (177-179). Depth within EBs also affects cardiac differentiation and cell necrosis (304). Future studies will need to address this facet of 3D reprogramming, possibly by using smaller scaffolds or improving nutrient transport using perfusion or hydrodynamic mixing. PEG microspheres may also improve diffusion within scaffolds made of more fibrous materials (like collagen or other naturally derived ECM). In a study comparing chondrogenic differentiation in ECM pellet cultures with and without microspheres, our collaborators found that inclusion of PEG microspheres reduced the incidence of a fibrous layer surrounding the pellet culture, and noted several variations in gene expression patterns (323). Since inclusion of PEG microspheres did not significantly hinder ECM remodeling in collagen scaffolds (Figs. 4.4 and 4.5) or alter gene expression (Fig. 4.2), they may be useful for delivering morphogen from within the scaffold. This has been shown to improve differentiation within EBs by more evenly distributing the delivery profile (324).

4.6 Conclusion

Future cell transplantation procedures will require more scalable approaches to stem cell differentiation and direct reprogramming. In this study, we investigated the effects of several factors on direct reprogramming to cardiomyocytes in 3D cultures. We
confirmed that material topography, diffusion, and cell density directly impact reprogramming efficiency in 3D. Our results also suggest that ECM remodeling must be taken into consideration when designing these 3D scaffolds. The results presented here lay the groundwork for development of materials that will promote efficient 3D direct reprogramming.

4.7 Acknowledgements

The authors would like to thank Drs. Jeanne Nerbonne, Shelly Sakiyama-Elbert, Robert Mecham, and Guy Genin for helpful discussions relating to these results. We would also like to thank Yun Qiao and Jake Hoyne for technical assistance, and Casey Donahoe, Patrick Blanner, Dr. Scott Marrus, Dr. Nithya Jesuraj, Dr. David Hoganson, Laura Marquardt, and Hao Xu for technical advice. This work was funded by an American Heart Association predoctoral fellowship 11PRE7690043 (AWS), the National Institutes of Health NIH R01 HL085364 (DLE), R01 HL085369 (IRE), and the Lucy and Stanley Lopata endowment (IRE).
CHAPTER 5

Conclusions and Future Directions

In this dissertation, I have explored PEG materials as they relate to cardiac tissue engineering, first as scaffolds for engineering a cardiac construct (Chapter 2), then as a base material for designing the stem cell niche (Chapters 3 and 4). In chapter 2, I designed a novel method for crosslinking PEG microspheres around cells to form a scaffold that could be easily handled and subjected to robust analyses. To verify that cardiac cell types would respond well to this material, we encapsulated HL-1 cardiomyocytes, which proliferated and maintained their phenotype over several weeks in culture. While the gap junction staining suggested that HL-1 cells were connected in small clusters, large-scale electrical synchrony was not observed within the scaffold. Certain improvements to the design and protocol may help to promote better organization and cellular alignment within the scaffold. Primarily, providing matrix-metalloproteinase degradable sites within the PEG mesh may enable better remodeling and allow for more cell-to-cell contact (159). Alternatively, the microspheres could be embedded around cells in a naturally derived hydrogel such as collagen or fibrin, similar to the collagen-PEG hybrids described in Chapter 4. This would retain the potential for drug release from PEG (200) and potentially improve diffusion within the natural hydrogel (167). Finally, electrical and mechanical stimulation might be helpful for coaxing cells into cardiac-like organization (230, 247).

The third chapter focused on a new cell type. The HL-1s provided an excellent model for preliminary biomaterial testing because they skirt many technical issues encountered with stem cell derived cardiomyocytes. However, their clinical relevance is
limited because they are a permanently dividing line. We were finishing the HL-1 studies when a new way of considering cell sourcing was emerging. Of the direct reprogramming strategies published, neither had yet considered the effects of the culture substrate on efficiency (75, 84). However, from pluripotent cell studies, we know this to be an important variable that can be engineered to our advantage (100, 124). The protein resistant properties of PEG ensure a clean slate for delivering the desired bioactive molecules while inhibiting non-specific adsorption (325, 326). An advantage of one of the direct reprogramming strategies was the potential to form vascular cells alongside cardiomyocytes (84, 85). Many in vitro and in vivo studies have also reported the positive influence of vascular cell types on cardiomyocyte viability (88) and cardiac function (89, 90) after implantation.

Using custom-engineered 2D PEG substrates, we demonstrated that reprogramming efficiency could be improved in ways that TCPS substrates would not allow. We also found RGD to promote proliferation during reprogramming, which ultimately provided more cardiomyocytes without diminishing efficiency. Interestingly, the Matrigel proteins that had been used with TCPS in the original publication were found to have high batch-to-batch variation in the ability to promote cell adhesion to PEG. Laminin was chosen as a potential alternative because its receptors are known to be upregulated during the first few days of reprogramming (253), and it makes up ~60% of the proteins in Matrigel (according to product literature). Because laminin can be recombinantly synthesized, this also represents a move away from animal derived materials and toward fully synthetic materials (though for our purposes we did use purified mouse-derived laminin-1).
While many questions could still be addressed using the 2D PEG substrates (differentiation to vascular cells, the effects of substrate elastic modulus, etc.), we were equally eager to analyze the effects of 3D culture during the direct reprogramming protocol (Chapter 4). This area of research has 2 main motivations. The first is scalability; 2D differentiation methods cannot reasonably provide enough cells. For example, in a recent clinical trial of human MSC injections, the authors injected $205 \times 10^6$ bone marrow cells (125). At a typical MSC culture density of 10,000 cells/cm$^2$ (327), that would require 20,500 cm$^2$, or ~270 typical T75 tissue culture flasks (for just one patient). While researchers may opt for large, thin, stackable 2D surfaces (“Cell Factories” with 636 cm$^2$ surface area per plate can be purchased from Thermo Scientific®) (328), the sheer media volume necessary brings up costs and routine upkeep becomes labor intensive. As discussed in Chapter 4, optimization of 3D differentiation methods could help with scalability issues.

The second motivation for Chapter 4 was to determine the effects of 3D culture on reprogramming efficiency. Since cells typically reside in a 3D environment, they are accustomed to receiving signals from three dimensions (116). In many cases, they take on different phenotypes in 2D versus 3D (119, 120), sometimes resulting in improved differentiation (121-124). Unfortunately, in our study we did not see an improvement in reprogramming efficiency. However, we did learn important details about how the reprogramming cells react to microsphere topography, encapsulation between microspheres, diffusion limitations, and variations in materials (collagen to PEG) that can help us to design better scaffolds for future studies.
In a broader sense, there are many future directions to this project. First, the direct reprogramming field moves at a rapid pace. Very recently (March of 2013), the first group published a protocol for directly reprogramming human cells to cardiomyocytes (83). We will need to consider the clinical relevance of continuing to work with mouse cell types in future materials studies. Secondly, a study of simultaneous reprogramming to vascular cells would be highly desirable as it may help with cardiomyocyte organization within the scaffold (88). For the 3D approaches, designing enzymatically degradable PEG microspheres may improve remodeling and organization within the scaffold, which may in turn improve reprogramming efficiency.

The collagen gels containing PEG microspheres may be the most promising of the three materials investigated in Chapter 4, based on the gene expression studies. Loading microspheres with bioactive factors may counteract diffusion limitations to improve reprogramming efficiency. Using microspheres to deliver retinoic acid from within EBs has shown to advance differentiation, likely by ensuring an even delivery profile instead of relying on diffusion from the outer surfaces (324). In the Efe et al. protocol, the pluripotency inhibitor is delivered for 9 days immediately following cell seeding. If we were to continue using this reprogramming protocol, one might design microspheres to incorporate controlled release of this or other factors.

Ultimately, diffusion limitations will need to be addressed by either incorporating perfusion or rotary culture into the methods, or making much smaller 3D constructs (~200-400 µm wide to completely avoid diffusion issues (177-179)). One such way would be to fabricate the scaffold as described, then to break it into smaller pieces such
that the cells were still encapsulated. In fact, such a strategy might lend itself well to \textit{in vivo} injections that deliver cells, bioactive factors and surfaces to prevent cell anoikis.
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