

Micro- and Nanoscale Control of the Cardiac Stem Cell Niche for Tissue Fabrication

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Advances in stem cell (SC) biology have greatly enhanced our understanding of SC self-renewal and differentiation. Both embryonic and adult SCs can be differentiated into a great variety of tissue cell types, including cardiac myocytes. *In vivo* studies and clinical trials, however, have demonstrated major limitations in reconstituting the myocardium in failing hearts. These limitations include precise control of SC proliferation, survival and phenotype both prior and subsequent to transplantation and avoidance of serious adverse effects such as tumorigenesis and arrhythmias. Micro- and nanoscale techniques to recreate SC niches, the natural environment for the maintenance and regulation of SCs, have enabled the elucidation of novel SC behaviors and offer great promise in the fabrication of cardiac tissue constructs. The ability to precisely manipulate the interface between biopolymeric scaffolds and SCs at *in vivo* scale resolutions is unique to micro- and nanoscale approaches and may help overcome limitations of conventional biological scaffolds and methods for cell delivery. We now know that micro- and nanoscale manipulation of scaffold composition, mechanical properties, and three-dimensional architecture have profound influences on SC fate and will likely prove important in developing the next generation of “transplantable SC niches” for regeneration of heart and other tissues. In this review, we examine two key aspects of micro- and nanofabricated SC-based cardiac tissue constructs: the role of scaffold composition and the role of scaffold architecture and detail how recent work in these areas brings us closer to clinical solutions for cardiovascular regeneration.

Introduction

HEART FAILURE REPRESENTS an enormous clinical problem, affecting 23 million people worldwide with a projected annual health cost burden of \$27.9 billion in the United States alone.¹ Despite important advances in both pharmacological and nonpharmacological therapies (e.g., mechanical support devices, organ transplantation), limitations of these approaches in the management of end-stage heart failure have driven the search for stem cell (SC)-based techniques to repair and regenerate heart muscle. While SC-based therapies have already entered clinical trials, data concerning the fate of implanted cells and their functional contribution to overall myocardial contractility remain inconclusive.²⁻⁴ If the field of myocardial repair and regeneration is to advance, new techniques must be sought to help surmount some of the existing challenges. These challenges include precise control of implanted cell phenotype, stage of lineage commitment and differentiation, as well as proliferation and differentiation potential, survival, integration, and function upon subsequent implantation into the heart.

Most SC types used in cardiogenic differentiation studies are either multi- or pluripotential such as bone marrow-derived mesenchymal SCs (MSCs) or embryonic stem (ES) cells. One must therefore regulate the balance between self-renewal and lineage commitment/differentiation. This is particularly important to avoid tumorigenesis that is observed following implantation of ES cells into ventricular muscle.⁵ In addition, it is well known that there are multiple possible sublineages within the heart that evolve during development and which one can derive using *in vitro* systems.⁶ Some of these are adapted for contractile cardiac function, while others include pacemaker, conduction-type, and supporting cells. Control of phenotype at the cellular level is therefore a key aspect to avoid arrhythmias—a further major problem observed in clinical trials of SC cardiac implantation, as well as to maximize contractile properties for optimal functional improvements.

A highly promising approach to regulate these SC behaviors is to engineer tissue constructs using micro- and nanoscale techniques. These constructs incorporate synthetic, transplantable SC niches, which can modulate SC responses

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TABLE 1. FACTORS INFLUENCING STEM CELL BEHAVIOR THAT CAN BE CONTROLLED OR IMPACTED BY MICRO/NANOFABRICATED NICHE PROPERTIES

<i>Micro- and nanofabricated niche properties</i>	<i>Factors influencing stem cell behavior</i>	<i>Examples (references)</i>
Cell scaffold composition	Surface chemistry	Laminin (50) Fibronectin (49) RGD (51–53)
	Mechanical properties	Elasticity (14, 61, 63) Anisotropy (26,42)
	Mechanical environment	Cyclic strain (20, 62–64) Electrical stimulation (66, 67, 69–71)
	Soluble factors	Morphogenetic proteins (55, 56, 68) Vascular endothelial growth factor (30, 57) Activin A (68)
Scaffold architecture	Surface topography/cell shape	Size/shape (12, 15, 65) Topography (38)
	Cell–cell contacts	Electrical coupling (6, 66, 67) Contact (65)

such as proliferation, cell fate, differentiation, and apoptosis in manners similar to the niches found *in vivo*. In addition, these constructs can protect the cells from adverse mechanical and biochemical influences within damaged recipient heart muscle. Micro- and nanoengineered constructs create an interface between cells and biomaterial substrates that can be precisely controlled to regulate cardiac SC behavior. An additional potential of these fabricated SC niches is the ability to integrate with host cardiac muscle through ingress of host-derived cells and capillaries and/or egress of construct-derived cells and capillaries preengineered into the graft. Finally, fabricated, transplantable niches can be functionalized to derive smart constructs that can release factors, such as anti-apoptotic, pro-angiogenic, and matrix-modifying molecules, in a defined spatiotemporal manner to modify the behavior of both host- and construct-derived cardiac cells.

In this review, we examine two key aspects of micro- and nanofabricated SC-based cardiac tissue constructs: the role of scaffold composition and the role of scaffold architecture (Table 1). In addressing these issues, we highlight cell behaviors that can be modified and how this could help toward overcoming current hurdles toward myocardial regeneration and repair. We also discuss new insights gained regarding SC behaviors *in vitro* which have been uncovered using micro- and nanoscale technologies. Finally, we discuss emerging approaches for creating macroscale, implantable engineered tissues from assembly of micro- and nanoscale-engineered tissue modules.

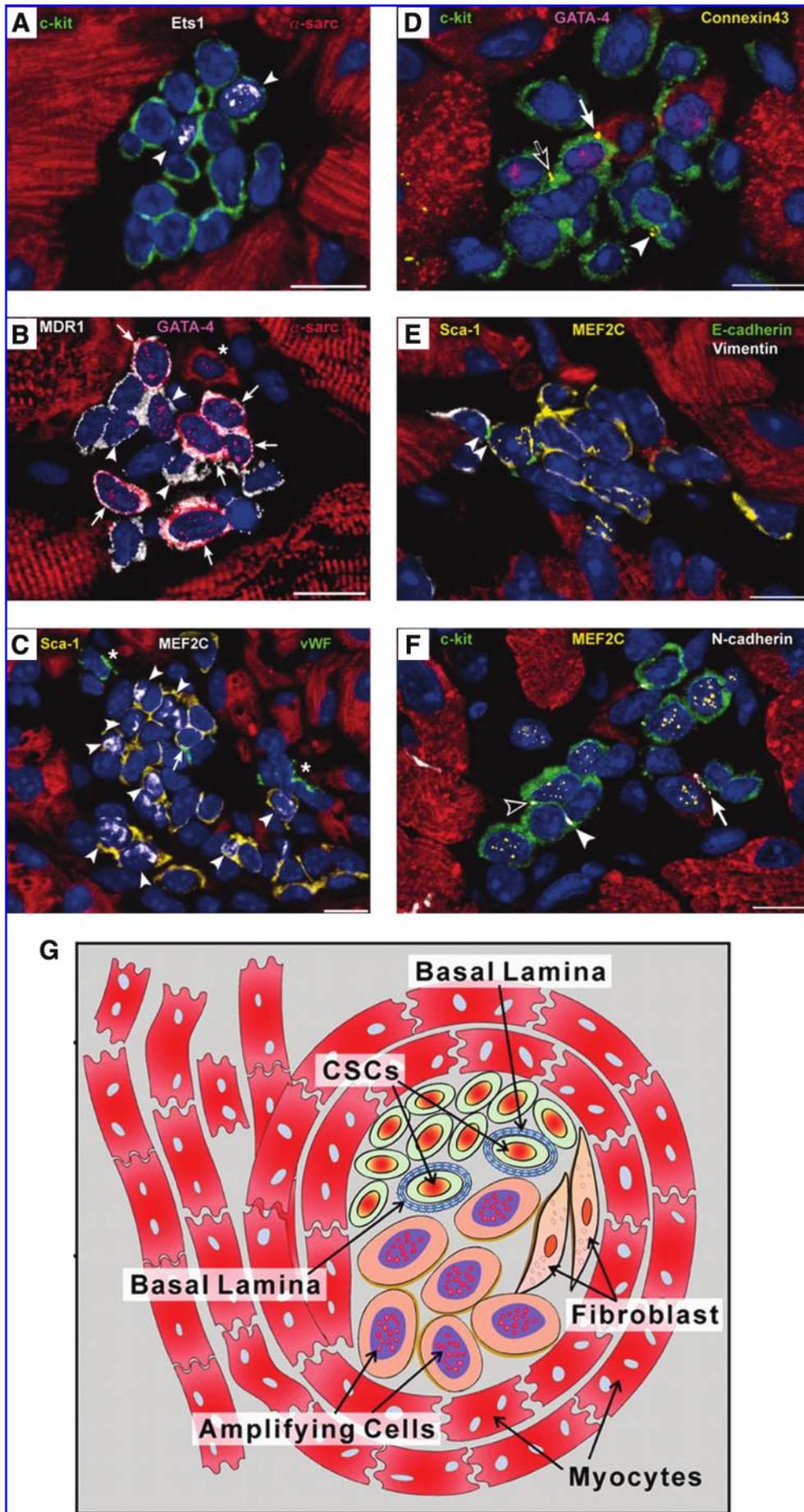
Micro- and Nanoscale Control of the Cardiac SC Niche

One cannot necessarily extrapolate from the mesoscale to the nanoscale to predict how cells will respond to subcellular

micro- and nanoscale features.⁷ Precise control of directed SC differentiation is therefore likely to require a better understanding of the SC responses to these environmental cues, such as cell–cell signaling and extracellular matrix (ECM) binding. SCs have been recently isolated from the adult mammalian myocardium, and a putative cardiogenic niche for these cells has been identified (Fig. 1).^{2,4,8} While these niches have been identified through location of SC aggregates in cardiac tissues, the precise microarchitectural and biochemical features of these niches is still an area of active investigation.⁹ SC niches are found throughout the body; however, the differences between niches of different cell and tissue types are likely important factors in directing SC behavior, proliferation, and differentiation. Multipotent, undifferentiated cardiac progenitor cells (CPCs) reside within these niches in intimate association with lineage-committed cells and supporting cells, with gap and adherens junctions at the interfaces of the various cell types specific to the niches.^{10,11} Microscale cues have been shown to be important in inducing specific SC fates independent of soluble cues.¹² Although still at its earliest stage, strategies for creating *ex vivo* SC niches by using microscale approaches are currently under development.^{13–16} Initial steps to creating a designer cardiogenic niche may therefore involve selection of compatible materials then applying micro- and nanoscale techniques to manipulate cellular interactions with the surrounding substrate, extracellular signaling molecules, and neighboring cells.

Porous polymers and hydrogels have been used successfully for many cardiac tissue engineering applications due to their mechanical and cell viability properties. Hydrophilic hydrogel environments are advantageous for maintaining cardiac cell viability and achieving even cell distributions in engineered cardiac tissues.^{17–19} Cardiac cells can also be seeded on a porous polymer support structures for added

FIG. 1. Schematic representation of the cardiac stem cells (CSC) niche. Cardiac niches are prevalently located in the atria and apex and consist of differentiated myocytes that surround clusters of CSCs and highly dividing amplifying cells. The amplifying cells are committed cells and express transcription factors of endothelial (Ets1, vWF, **A**, **C**), cardiac (GATA-4, **B**, **D**), and myocyte (MEF2C, **C**, **E**, **F**) lineages. The interaction among CSC, myocytes, fibroblasts, amplifying cells, and the basal lamina is depicted in (**G**). Reprinted with permission from (**A–F**) Urbanek *et al.*, 2006¹¹ and (**G**) Leri *et al.*, 2005.⁹ Color images available online at www.liebertonline.com/ten.



mechanical strength, both with and without hydrogels for improved cell retention.^{18,20,21} These porous polymers can be made through a number of techniques such as electrospinning^{22,23} or weaving²⁴ to create fibrous networks or porogen leaching to create highly porous sponge-like materials.²⁵ The goal is to create a synthetic environment that emulates the native ECM environment to better control and direct cell behavior. This is especially important in recreating the cardiac SC niche, as the interactions between stem and helper cells and the surrounding micro- and nanoenvironment can dictate cell proliferation, differentiation, and function.¹¹ Current techniques can recreate some of these structural and biochemical features on the micro- and nanoscale to better recapitulate the native cardiac SC niche.

Since many interactions between cells and their surrounding environment occur on the micro- and nanoscale, current research has been focused on creating scaffolds with specific micro- and nanoscale features to better direct cell behaviors. Typical ECM fiber sizes are at the nanoscale, therefore techniques to create fibrous scaffolds have been aimed at creating fiber widths similar to those of natural ECM to emulate the native cell–ECM environment.²³ Electrospinning uses a high-voltage differential to send a jet of prepolymer liquid between two electrodes forming nanometer sized fibers, which can be collected in random or aligned mats.^{22,23} Inducing fiber alignment is of critical importance in cardiac tissue engineering as this alignment drives cell alignment and mechanical anisotropy to optimize contractile function.²⁶ Fibers for some polymers can also be made through extrusion then woven to form a fibrous, porous scaffold with specific three-dimensional (3D) mechanical properties and controllable anisotropy.²⁴ Hydrogel components have also been varied on the nanoscale to improve the inability of encapsulated cardiac cells to bind,

elongate, degrade, or migrate through the network. Research has investigated new hydrogel formulations, such as altering the chemical composition of the polymer networks,^{19,27} incorporating binding and degradation sites,^{28,29} and localized delivery of growth factors,³⁰ to encourage cell spreading and migration and exert better control on cell behavior at the nanoscale. Techniques that can combine these attributes with other microscale techniques for controlling the microarchitecture of engineered tissues can better recapitulate the cardiac SC niche.

Cell–cell interactions are vital to cardiac SC tissue engineering, leading to investigation of techniques to control the microarchitecture of engineered cardiac tissues to better direct cell behavior. One such technique is soft-lithography, which is a series of techniques that uses elastomeric stamps, made of poly(dimethylsiloxane), to print molecules or to mold structures on substrates^{31–33} (Fig. 2). Poly(dimethylsiloxane) is a transparent, nondegradable and nontoxic elastomer that can be cured on patterned silicon or SU-8 wafers to generate molds with microscale resolution that can be used to pattern substrates or hydrogels including polyethylene glycol (PEG),^{34,35} methacrylated hyaluronic acid (HA),^{34,36,37} poly(lactic-co-glycolic acid) (PLGA), poly(L-lactic acid) (PLLA), and polycaprolactone (PCL).³⁸ Lasers have also been used to create microscale holes of specific design in scaffolds to create pores for perfusion and directed cell seeding in cardiac tissue engineering.^{26,39} Importantly, the biomaterials used in conjunction with micropatterning and microfluidic techniques can in turn be precisely defined in terms of chemistry and elasticity, which can also influence SC fate decisions.^{13,14,40} The overall aim of these approaches is to generate 3D constructs that recapitulate the complex organization of native cardiac muscle and SC niche and the intimate association between cardiac myocytes and the

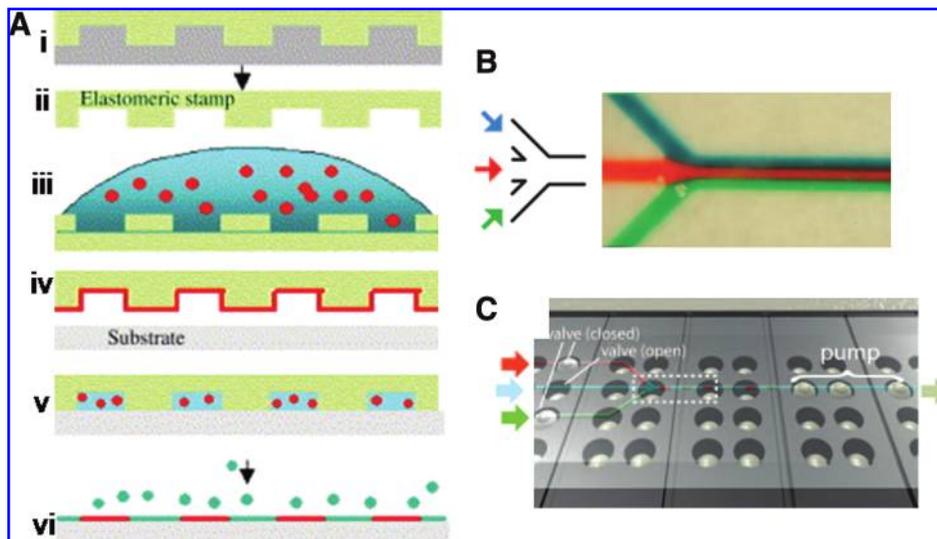


FIG. 2. Microscale technologies are useful for controlling stem cell (SC) behavior. (A) Soft-lithography can be used to generate elastomeric poly(dimethylsiloxane) stamps (i, ii) that can be “inked” with molecules (iii, iv) to generate micropatterns directly or indirectly through fabrication of microchannels (v, vi). (B) Microfluidic technologies can also be used to generate multiple, parallel laminar streams of flow to control distribution of fluid phase components and shear stresses to cells within microchannels. Further control over such fluid flows may be achieved by using a computer-controlled Braille display of pins (C). (A) Reprinted with permission from Falconnet *et al.*, 2006³¹ and (B, C) from Gu *et al.*, 2004.³³ Copyright National Academy of Sciences USA. Color images available online at www.liebertonline.com/ten.

microvasculature.⁴¹ The ability to control material properties both in chemical and biological characteristics, as well as defining the micro- and nanoarchitectural aspects of the materials provides a multifaceted approach for regulating cell behavior and tissue fabrication.

Effects of Scaffold Composition and Mechanical Properties on SC Behavior

Scaffold chemistry, binding motifs, and immobilized soluble factors

The ECM of the myocardium constitutes an important milieu during homeostasis, development, and disease.^{42,43} The SC niche is supported within this matrix. While collagen types I and III comprise much of the adult cardiac interstitium⁴⁴ and have been used to support SC grafts, their use is complicated through limitations of quality control, reproducibility, control of precise composition, varying rates of biodegradability, lack of ability to modify the substrate, and less flexibility with respect to manipulation and interfacing with micro- and nanoscale fabrication techniques. Biomaterials with similar properties to native ECM, but with more reliable reproducibility, tunable properties, and biocompatibility, may be used for micro- and nanoscale fabrication, allowing for more precise control of SC proliferation, survival, differentiation, adhesion, and organization. Further, functionalization of these scaffolds can be achieved by immobilizing growth factors and cytokine molecules, such as cell-binding motifs like arginine-glycine-aspartic acid (RGD), to better control SC behaviors within a scaffold and to better mimic the cardiac SC niche.

A variety of biopolymers have been used as scaffolds for both SCs and fetal and neonatal primary cardiac myocytes, which serve as parallels for cardiac differentiation and maturation. Examples of such polymers are PLLA, PLGA, and PCL,³⁸ as well as hydrogels such as PEG³⁴ and HA.^{36,45,46} These polymers may be patterned with cells either seeded on the surface of porous or fibrous scaffolds or embedded within hydrogels, in precise spatial configurations using techniques such as soft-lithography. Surface topography and architecture can have dramatic effects on SC behaviors, both in concert with, and independent of, surface chemistry. Concerning chemistries, MSC and CPC show differential adhesion responses to different substrates, with CPC adhering best to PLGA and PLLA compared with PCL—in contrast to MSC, all while maintaining their multipotency.³⁸ Defining fundamental SC responses such as these are a key first step to manipulating these materials by micro- and nanopatterning and substrate modifications on the road to developing functional cardiac grafts.

An alternative class of substrate scaffold that can be used for SC-based grafts is a hydrogel derived from self-assembling peptides. These peptide gels are composed of oligopeptides with alternating hydrophobic and hydrophilic residues that self-assemble depending on the salt concentration of solution. Human endothelial cells embedded in RAD 16-I or (RADA)₄ RAD 16-II or (RARADADA)₂ hydrogel scaffolds have been shown to form interconnected capillary-like networks that did not occur with other formulations,⁴⁷ while cardiomyocytes elongated and formed interconnected networks when seeded on RAD 16-I hydrogels containing embedded endothelial cell networks.⁴⁸ When seeded to-

gether inside RAD 16-I hydrogels, embedded cardiomyocytes and cardiac fibroblasts formed large, multicellular aligned cell networks.¹⁹ These effects were likely mediated through cell adhesion effects as RAD 16-I and RAD 16-II hydrogels have demonstrated improved adhesion of cardiac myocytes and various SC types,^{48,49} while also demonstrating compatibility with matrix metalloproteinase (MMP)-mediated remodeling.¹⁹

The micro/nanoenvironment of hydrogels can also be altered by changing the chemical characteristics of the hydrogel components, improving cell morphology and function. Genova and colleagues demonstrated that RAD 16-I structures modified with sequences derived from the endogenous ECM molecule laminin induced spreading and formation of capillary-like structures of human endothelial cells.⁵⁰ In similar studies, Gelain *et al.* modified neural SC viability, proliferation, and organization using RAD 16-I nanofiber scaffolds functionalized with motifs derived from fibronectin (RGD) and laminin, suggesting that RAD 16-I nanofiber scaffolds functionalized with ECM mimicking the cardiac SC niche could perform similarly with cardiac cells.⁴⁹ Similarly, modification of PEG to include bound ECM/integrin sequences, such as RGD, has been demonstrated to greatly improve cell elongation and migration in hydrogels.^{51–53} Lastly, chemical modification of esters of HA substrates using butyric and retinoic acid residues has been shown to significantly enhance expression of cardiac transcription factors Nkx 2–5 and GATA-4 in ES cells.⁴⁵ These studies suggest that by altering the cell-binding environment of cell-laden hydrogels, researchers can have better control of cell behavior and phenotype, making these systems appealing for improving control in cardiac SC differentiation and function applications.

Classic work in developmental biology has elucidated the importance of morphogens and their gradients in embryogenesis.⁵⁴ A great deal is already known about soluble factors and their signaling pathways in cardiac differentiation during development and in SC-based systems, with important roles defined for bone morphogenetic proteins, fibroblast growth factors, wingless-Int proteins, and retinoic acid.^{55,56} As reviewed by Dellatore *et al.*, molecules such as leukemia inhibitory factor, fibroblast growth factor, and vascular endothelial growth factor can be covalently or noncovalently bound to a variety of materials including PEG and PCL to drive ES cell proliferation, differentiation, adhesion, and spreading.⁵⁷ PEG hydrogels can be functionalized with heparin to influence MSC survival and differentiation as can gels with incorporated RGD motifs modify pluripotent P19 embryonic SC fate.⁵⁸ As well as RGD-binding motifs, scaffold compositions may incorporate MMP-sensitive degradation sites.^{28,30,58–60} In one example, cardiac differentiation of ES cells was shown to be enhanced in 3D PEG hydrogels by controlling the concentrations of RGD and MMP degradation sites as well as the mechanical stiffness.⁵⁸ MMP-sensitive hydrogels have also demonstrated the ability for targeted, cell-responsive release of growth factors, such as vascular endothelial growth factor, to improve vascularization in engineered constructs.³⁰ Use of temporally variant and cell-induced delivery of these and other molecules in a targeted, cell-initiated manner has the potential to control cell proliferation, phenotype, and integration leading to more effective engineered tissues.

Scaffold matrix elasticity and deformability

Recent work has identified substrate mechanical properties to be an important biophysical determinant of SC fate¹⁴ and engineered cardiac tissue function.²⁶ Using a defined biomaterial system to enable control over substrate elasticity, Discher and colleagues were able to isolate the effects of substrate elasticity and deformability on the microscale from those of surface chemistry on MSC differentiation (Fig. 3).¹⁴ Atomic force microscopy was used to quantify the strains within individual cells on substrates of differing elasticity to demonstrate that cells detect and respond to stiffer substrates through increases in intracellular strain forces. These data suggest that microscale control of the mechanical properties in engineered tissues could be employed to drive targeted SC differentiation. More recently, in a paradigm for SC-based cardiogenesis, embryonic cardiac myocytes were shown to be sensitive to substrate elasticity.⁶¹ Possessing mechanical characteristics that mimic the developing heart on the cellular level could thus provide the optimal cues for myofibrillar organization and beating frequency in SC-derived engineered cardiac tissues. Further investigation of controlled variation of matrix elasticity in SC-based cardiogenesis is therefore important both for the studies of differentiation and fate of cells implanted into the failing myocardium that has a matrix composition and elasticity unfavorable for cardiac differentiation.³ Further, if SCs could be preconditioned to thrive in this hostile environ-

ment, the potential for long-term survival and integration could be greatly improved.

While consideration of the static mechanical properties of biopolymeric scaffolds such as elasticity clearly can influence SC fate, one must also consider the responses of these materials and associated cells when subjected to dynamic forces within the contracting myocardium. As a preliminary means to examine these effects, a number of *in vitro* responses to the effects of dynamic strain on elastomeric polymers micropatterned with cells have been studied. Micropatterned, maturing cardiac myocytes cultured on deformable elastomeric substrates can be subjected to strains applied along different axes relative to the orientation of cells and which may be varied cyclically.⁶² Cyclic strain can also modulate SC fate and has been studied in models of SC-based vascular tissue engineering⁶³ and neonatal myocyte-based cardiac tissue constructs.^{20,64} Anisotropy of forces is an important concept to explore with respect to SC cardiogenesis as forces continually change during development from the perigastrulation stage when heart fields are first specified through looping and later stages of morphogenesis,⁴² and the mature myocardium has specific anisotropic features to optimize contractile function. Recent studies have demonstrated improved contractile properties using scaffolds with micropatterned anisotropic pore structures similar to those found in native adult myocardium.²⁶ These results suggest one application of micro/nanoscale technology that could be

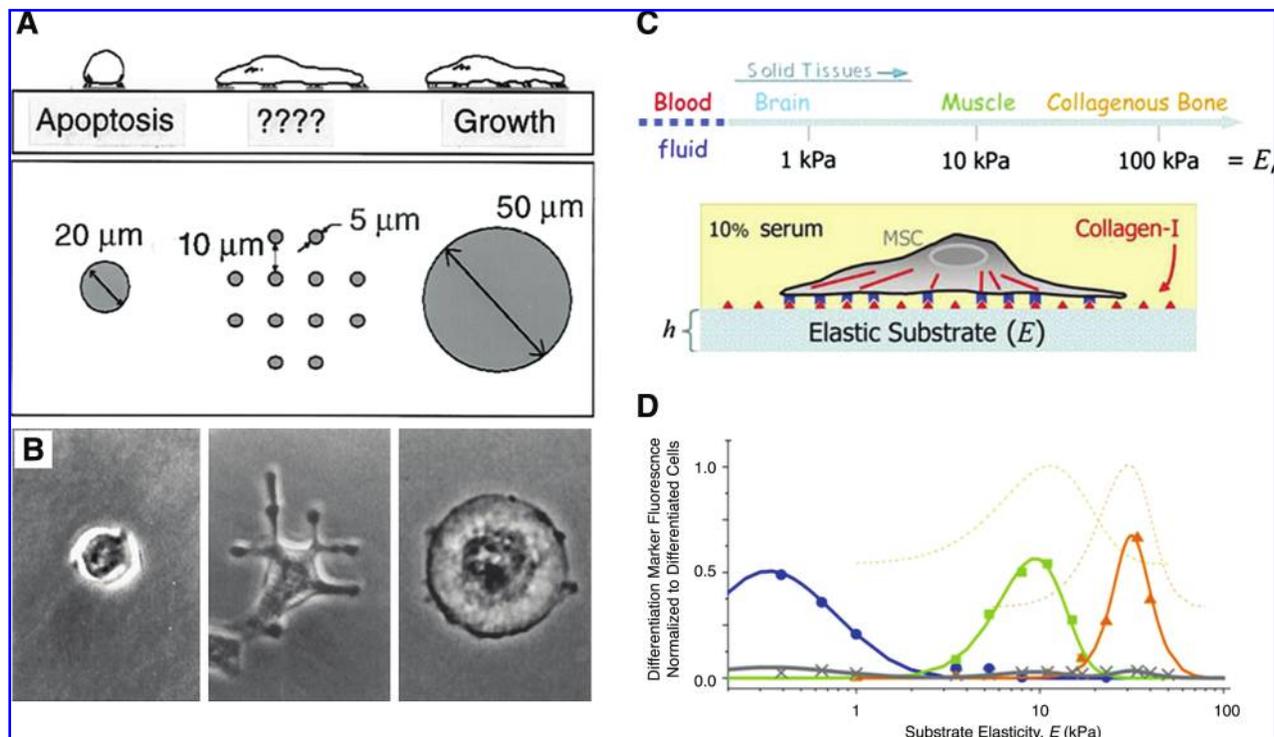


FIG. 3. Effects of surface topography and elasticity on cell behavior. Cell shape as determined by substrate topography of micropatterns regulates cell behaviors of apoptosis and growth. Different patterns of substrate (A) can induce changes in cell shape (B) with differential cell responses despite having similar total surface contact areas. (C) Substrate elasticity (E) is an independent determinant of SC fate and progressively stiffer substrates induce differentiation of mesenchymal SCs into brain (blue), muscle (green), or bone (orange) (D). (A, B) Reprinted with permission from Chen *et al.*, 1997⁶⁵ and (C, D) Engler *et al.*, 2006.¹⁴ Color images available online at www.liebertonline.com/ten.

employed to improve engineered tissue function through precise control of the mechanical and structural environment to recreate important features of the cardiac SC niche.

Micro- and Nanoscale Tissue Architectures

3D scaffolds can be engineered with specific topographical cues by virtue of the pattern and orientation of features within the scaffold matrix. In addition, one can control the size and distribution of pores within a scaffold and their interconnections with consequent control over cell–cell proximity and potential for coupling. First we consider the effects of surface topography and micropatterning on SC responses and cell–cell electrophysiological interactions, then we discuss approaches for creating 3D constructs incorporating these cues.

Micropatterns to elucidate effects of topography and cell shape

It had previously proven difficult to separate the effects of surface chemistry and cell–substrate contact area from cell shape. In an elegant study, Chen and colleagues decoupled the effect of cell shape and contact on cell function by micropatterning adhesive islands of defined size and shape separated by nonadhesive regions (Fig. 3).⁶⁵ Single capillary endothelial cells plated onto these micropatterned islands spread to different extents while maintaining a similar cell–substrate contact area. Cell shape was found to be an independent determinant of cell growth and in addition to independently dictating whether cells would undergo proliferation or apoptosis. More recent work using adhesive fibronectin islands micropatterned onto substrates found that cell shape is an important determinant of SC fate.¹² A round cell shape on small islands promoted adipogenic differentiation of MSC, while larger islands, which enhance cell spreading, led to osteogenic differentiation. These surface topography effects on cell shape are mediated by cytoskeletal tension and RhoA GTPase signaling.¹² Intriguingly, although MSC can be differentiated into cardiac myocytes, and recent work has shown that MSC and multipotent CPC can exhibit qualitatively divergent responses to a 3D microenvironment simply by altering the surface topography of biopolymeric matrices.³⁸

Micropatterning to control and study cell–cell coupling

Cell–cell interactions are important in developing an adult heart and as such it is desirable to define and manipulate these interactions to improve SC-based tissue regeneration. Although the effects of cell–cell interactions on cardiac SC fate are poorly understood, microscale techniques have enabled indirect control of these interactions within embryoid bodies (EBs) as well as more directly within precisely defined patterned arrays of cells. Electrophysiological coupling has been demonstrated between ES cell–derived cardiac myocytes and neighboring cells within EB outgrowths with GATA-6⁺ cardiac lineage cells being able to act as pacemakers for adjacent regions of contractile cells within the EB,⁶⁶ and the number of cells in an EB influences the cardiogenic potential of ES cells.⁶⁷ We have recently shown that microfabricated PEG microwells can be used to control the size, shape, and homogeneity of ES cell aggregates.¹⁵ This

approach is therefore one way to understand the *in vitro* differentiation and coupling of ES cells within EBs in a large-scale microarray format. Alternatively, LaFlamme and colleagues demonstrated the ability to drive ES cell differentiation toward a cardiac lineage without the EB step.⁶⁸ Directing ES cell differentiation through culture in an activin A and morphogenetic protein-4-rich environment improved efficiency in cardiac differentiation as well as retention and integration when implanted *in vivo*. As micropatterning has proven capable of controlling ES cell differentiation through precise control of microwell, and therefore EB, size, there is a great potential to use micropatterning to limit cell aggregation down to single-cell systems, as single-cell suspensions have proven successful in driving ES differentiation down various pathways.

While control of EB size is one technique for modulating and studying cell–cell interactions in cardiogenesis, it has also proven useful to study networks of SC-derived cardiac myocytes using microelectrode arrays (MEAs) generated using microfabrication techniques. Micropatterned arrays of SCs can be used to precisely control functional coupling between SC-derived cardiac myocytes. Similarly, functional synaptic coupling between embryonic hippocampal cells has been achieved by micropatterning.⁶⁹ Using MEAs, micropatterned SCs can be characterized electrophysiologically at various stages of differentiation. Cardiac myocytes change their profile of connexin expression and distribution during differentiation, with subsequent changes in their electrophysiology.^{6,70} This is important for two reasons: first to define the phenotype of cardiac cells at specific stages of differentiation; second to study mechanisms of arrhythmogenesis in populations of cells that are of importance for predicting how groups of SC-derived cardiac myocytes might behave upon implantation into the myocardium. This has proven to be a major limitation of current SC therapies for heart failure as demonstrated in preliminary clinical trials.⁷¹ MEAs can map extracellular field potentials of clusters of SC-derived cardiac myocytes to delineate the origin and route of propagation of depolarization waves as well as rhythmicity.⁷² MEAs used to characterize electrophysiological properties of SCs at different stages of differentiation along the cardiac lineage have revealed changes in the spontaneous beat frequency as well as responses to chronotropic agents over time.⁷⁰ Positioning of individual maturing neonatal rat cardiomyocytes into 30 μm microwells within a microfabricated agarose chip with defined interconnections has revealed interesting changes in the stability of the beat frequency of networks of cells according to the size of the cell community. These findings have important implications for characterization of SC-based cardiac lineage derivatives prior to implantation into the heart.⁷³

Scaffold fabrication strategies for creating tissue microarchitectures

The specific design of porosity and topographical guidance cues within micro/nanoscale pores and their interconnections offers a potentially powerful way to influence functional coupling and integration of myocardial tissue constructs. This is achieved by influencing the ingress/regress of myocytes, vascular endothelial, and other cells types such as matrix-synthesizing fibroblasts. Further,

through 3D regulation of myocyte–myocyte contact and proximity by design of 3D topography within the biopolymeric scaffold, electrical coupling activity of a myocyte syncytium within the scaffold construct could be modulated.

Traditional, statically cultured engineered cardiac tissues using scaffolds with random pore architecture are typically plagued by poor cardiomyocyte viability, elongation, and organization.^{18,21} To improve these properties on the cellular level, researchers have focused on mechanical and biochemical stimulation to induce cellular self-organization and alignment. Characteristics of pores and their interconnections within a 3D matrix can have a significant impact on seeded cell distribution, density, adhesion, and migration as well as the potential ingress of host-derived vascular cells. Vascular smooth muscle cells can align on porous or nonporous micropatterned scaffolds,⁷⁴ while fibroblasts can bridge across pores using neighboring cells, despite being smaller than endothelial cells that are not able to bridge across pore margins (Fig. 4).⁷⁵ This has important implications for studying SCs and their derivatives at different stages of the cardiac lineage within a porous scaffold. Also of note is that porous microtextured scaffolds with pores up to 100 μm diameter have demonstrated sustained adhesion and proliferation of cardiac myocytes at a seeding density of $\sim 10^8$ cells/ cm^3 .^{76,77} In comparison, the normal adult human left ventricular myocardium contains $\sim 5.5 \times 10^9$ cardiac myocytes.⁷⁸ In addition, topographical features of microtextured biopolymeric scaffolds can also influence cardiac myocyte cell movements and excitability.²³ Cardiomyocytes grown on nonporous microtextured polymeric scaffolds with 1 μm grooves demonstrated an aligned cell orientation and morphology,⁷⁹ which influences the electrophysiology of

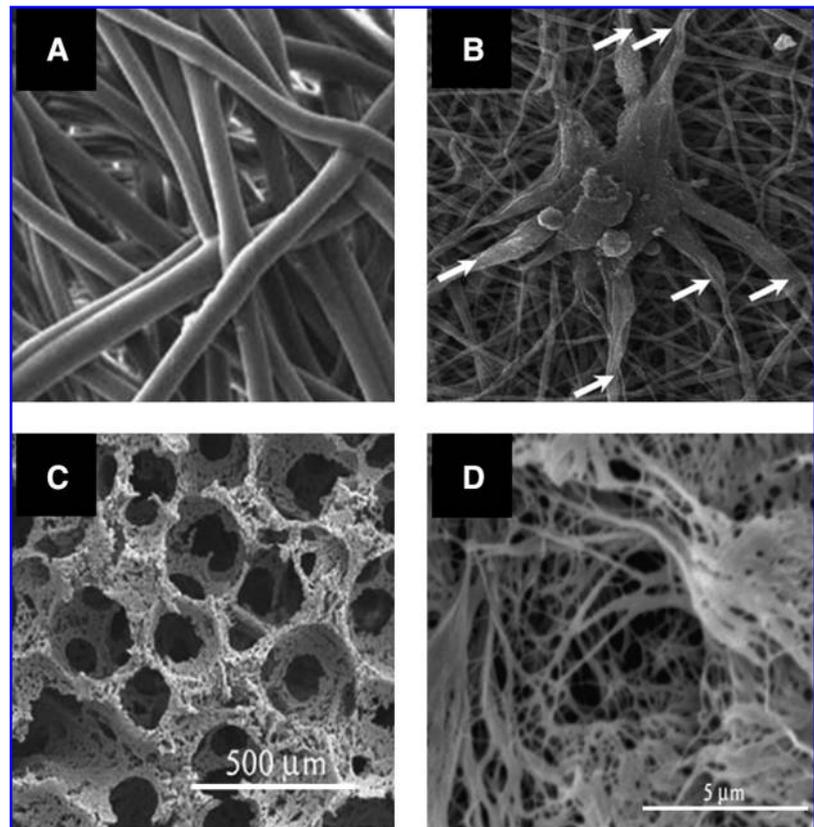
networks of cardiac myocytes.⁸⁰ Thus, balancing the mechanical and microstructural requirements while maintaining a physiologically relevant cell density is of paramount importance to controlling cell function and differentiation both *in vitro* and *in vivo*.

Additional control over surface topography within porous scaffolds can be achieved by using a nanofibrous matrix with precisely controlled pore size and interconnectivity. In combining a nanofibrous matrix with a porous structure, biomimetic scaffolds can be designed with nanofiber dimensions and an organization that parallels the arrangement of helical type I collagen fibers within ECM.⁸¹ Nanofibers can be produced using either electrospinning or directed self-assembly,⁸² and vascular smooth muscle cells grown on the surface of nanofibers exhibit excellent adhesion, alignment, and proliferation.²² Protein adsorption is significantly enhanced in nanofibrous porous scaffolds compared with scaffolds without a nanofiber network,⁸¹ and nanofibers coupled with defined epitopes can direct SC differentiation in a highly selective manner.⁸³ Interestingly, nanofiber components injected into the myocardium promote vascularization following self-assembly *in vivo*,²⁷ while nanofiber hydrogels also promote cardiomyocyte elongation and alignment *in vitro*.¹⁹ These studies demonstrate that specific control of nanoscale features can greatly improve cardiac repair, suggesting an even greater potential for controlling the *in vivo* function of SC-based cardiac tissues.

Strategies for tissue assembly and construction

To create macroscale tissues from microscale building blocks, microscale functional tissue units with specific

FIG. 4. Nano- and microfibrinous scaffolds. (A) Biopolymeric nanofibrous three-dimensional scaffolds can be generated and used to culture cardiac myocytes that extend filopodia-like extensions onto the scaffold (arrows, B). Scaffolds can be modified to incorporate a porous structure that has additional benefits in terms of mass transport properties of permeability and diffusion (C, D). SCs can thus be grown on such scaffolds for more precise control of cell behaviors. (A, B) Reprinted with permission from Zong *et al.*, 2005²³ and (C, D) Chen and Ma, 2004.⁸¹



microarchitectural features are assembled into the desired tissue structures. We have recently shown that microscale hydrogels can be generated as self-contained functional tissue units and that these modules can be assembled into organized tissue architectures.⁸⁴ The field of modular tissue engineering recreates biomimetic structures by designing microscale features to create building blocks to fabricate large, complex tissues such as the heart.⁸⁴ These modules can be created by self-assembled aggregation,⁸⁵ creation of cell sheets,⁸⁶ or microfabrication of cell-laden hydrogels.⁸⁷ Tissue assembly can be achieved by a number of methods, such as random assembly,⁸⁸ stacking of layers,^{89–91} or directed assembly.⁹² The inspiration for this bottom-up approach comes from nature as many tissues are comprised of repeating functional units, such as the lobule in the liver.⁹³ For the purposes of tissue assembly, one might also consider ventricular myocardium as composed of repeating subunits of muscle segments interlocked together to form the syncytium.

Recent work in modular tissue engineering has demonstrated the ability to create higher order structures with multiple cell types. For vascularization, coseeding with endothelial cells may be performed with these cells either within the hydrogel or on its surface. In one report, HepG2 hepatocyte-laden hydrogels with endothelial cell-coated surfaces were assembled together and perfused in a bioreactor with subsequent formation of vascularized channels.⁸⁸ Cell sheet technology has demonstrated the ability to create 3D tissues with clinically relevant dimensions and mechanical properties while controlling the microarchitectural features such as cell and ECM alignment. Large diameter blood vessels created using this technique have demonstrated native burst pressure, vasoactivity, cell and ECM alignment, and *in vivo* patency in primate and human trials.^{89–91} One major remaining challenge is better control of cell distribution within cell sheets, for example, coculture with endothelial cells to provide capillary networks similar to those demonstrated in hydrogels *in vitro*.⁴⁷ Recently, we have demonstrated that hydrogel modules can be assembled into 3D architectures using “lock-and-key” modular assembly, suggesting a simple method to create higher order structures and controlled cell distribution.⁹² Combining the knowledge and advantages of such strategies holds great promise for the future development of complex, vascularized tissues, with intricate microscale features such as myocardium.

Remaining challenges and future directions

While significant success has been achieved in cardiac tissue engineering, the challenge of translating this success to the clinic still remains significant. Reports demonstrating CPC in the adult myocardium are quite new, and therefore many of the successful techniques for creating engineered myocardium based on neonatal rat cells need to be validated using this new cell source. It is also likely that the protocols and techniques must be altered significantly to ensure success with adult, human SCs rather than with mammalian neonatal differentiated cells, while also requiring significant work to scale up to human length scales from rodent models. Some major challenges in this regard are improving the mechanical strength of hydrogel-based systems, perhaps through development of more robust materials or optimizing factors such as crosslinking density, increasing the resolution

of patterning techniques for polymeric scaffolds to allow for micro- and nanolevel control, as well as improving the interface between polymer scaffolds and hydrogel to create more functional hybrid scaffolds.

Considerable research remains to be completed demonstrating the specific chemical and microarchitectural composition and cell distribution of the cardiac SC niche. Micro- and nanoscale engineering techniques have proven successful in recreating known chemical and mechanical compositions; however, many of these attributes remain to be determined for the native SC niche.⁹ Recent results have determined that micropatterning technologies can reproduce attributes such as mechanical anisotropy, suggesting that micro and nanoscale control can recreate these complex environments and potentially determine the attributes necessary to direct cell behavior.²⁶ Lastly, while there are techniques, such as electrospinning, for controlling nanoscale fiber diameter and alignment, and micromolding for creating specific microarchitectural features, there currently is no single technique or technology that can simultaneously control the mechanical properties, microarchitecture, cell binding, and growth factor delivery on the micro- and nanoscales, presenting a considerable challenge. Combining current techniques, or creating new ones, will be critical for more completely recreating the cardiac SC niche and therefore being able to control CPC behavior *in vitro* and *in vivo*.

Conclusions

Micro- and nanoscale approaches have proven invaluable for identifying novel determinants of SC fate. A number of properties of the SC–biopolymeric scaffold interface can be manipulated using these techniques including surface chemistry, designer binding motifs, and immobilized cytokines and growth factors. Further, matrix elasticity, mechanical deformability, topography, and architecture can be tailored to direct and maintain specific cell phenotypes within a 3D context. These micro- and nanofabricated transplantable SC niches are thus a potentially powerful means to restrict SC fate to desired cardiac lineages and regulate the self-renewal/differentiation balance. This has important implications for maintaining graft cell number, phenotype, and functional integration and thus for avoiding undesirable effects such as tumor formation and arrhythmogenesis. Much work remains to be done in further exploring micro- and nanoscale influences on cardiac SC fate in both 2D and 3D contexts. The micro- and nanoscale techniques presented will facilitate the discovery of determinants of cardiac SC behavior contained within the cardiac SC niche, while continued exploration of approaches for tissue assembly offers exciting potential for fabrication of heart muscle segments of physiologically relevant dimensions.

Disclosure Statement

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