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Cardiac tissue engineering: current state and perspectives

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TABLE OF CONTENTS

1. ABSTRACT

The goal of cardiac tissue engineering is to treat cardiovascular diseases through the implantation of engineered functional tissue replacements or the injection of cells and biomaterials, as well as to provide engineered cardiac constructs that can be used as an *in vitro* model of healthy or diseased heart tissues. This field is rapidly advancing with the new discoveries and improvements in stem cell technologies, materials science, and bioreactor design. In this review, some of the progress made in cardiac tissue engineering in the recent years, as well as the challenges that need to be overcome in future studies, will be discussed. The topics include the advances in engineering stem cell-derived cardiac tissues, the use of natural or synthetic polymers and decellularized organs as engineering scaffolds, the scaffold-free cell sheet engineering approach, the application of perfusion and mechanical or electrical stimulation in bioreactors, the organization of cardiac cells through microfabrication techniques, and the vascularization of engineered cardiac tissues *in vitro* and *in vivo*.

2. INTRODUCTION

Cardiovascular diseases are the main cause of death in the world. Furthermore, conventional treatment options for cardiovascular diseases are limited by the diminished ability of the myocardium to regenerate after myocardial infarction (MI), which may potentially lead to heart failure, as well as the shortage of donor organs for transplantation. This motivates the search for new therapeutic interventions. Cardiac tissue engineering may provide a novel approach to treat heart disease, by replacing, repairing or regenerating the damaged myocardium using tissue- and cell-based strategies.

3. CURRENT APPROACHES FOR CARDIAC TISSUE ENGINEERING: CELLS, SCAFFOLDS AND BIOREACTORS

One of the main goals in cardiac tissue engineering is to organize cardiomyocytes (CMs) into a functional tissue *in vitro*. The criteria for a functional cardiac tissue that is clinically relevant include a thickness

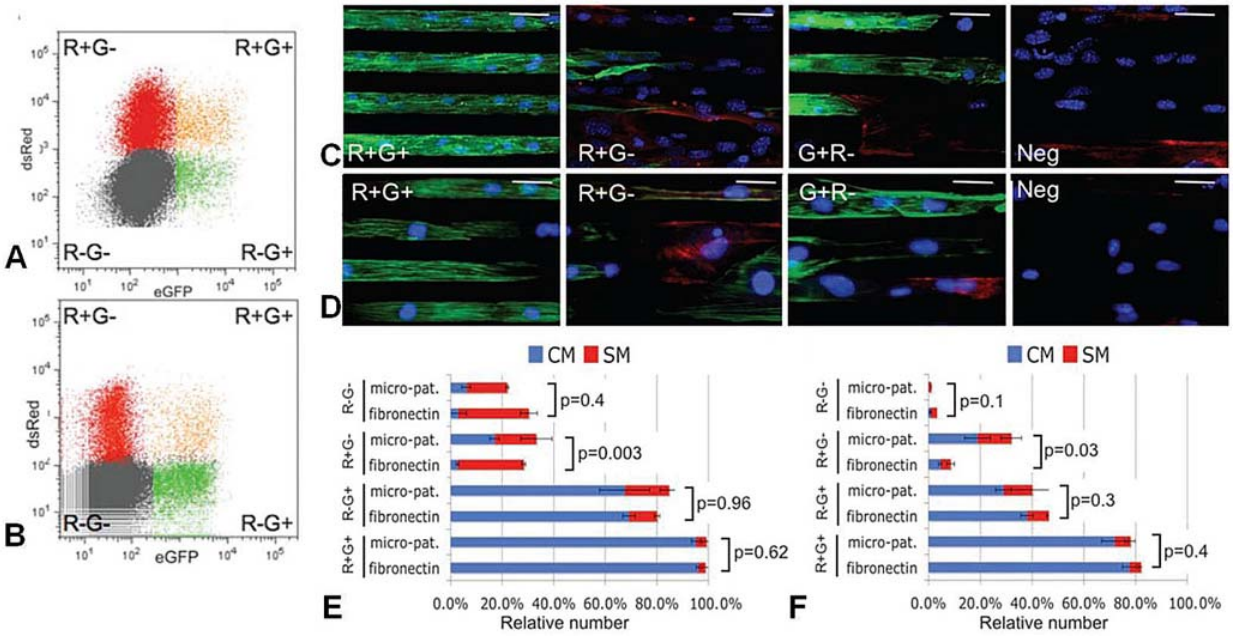

Figure 1. Cardiac tissue engineering based on stem cells. Embryonic stem cells with a defined myogenic identity were derived from double transgenic mice (Isl1-dsRed and Nkx-2.5-eGFP). The cells were differentiated and progenitor cells could be purified by FACS sorting. (A-B) Flow cytometry scatter plots showing distinct populations of cells (R+G+, R+G–, R–G+, and R–G–) from (A) Day 6 EBs and (B) E9.5 embryos. (C-D) Cells from the various derivative populations were grown on micropatterned lanes and stained for sarcomeric alpha-actinin (CM, green) or smooth muscle myosin heavy chain (smooth muscle, SM, red). In both (C) embryonic and (D) ES cell-derived progenitor cells, the R+G+ population most closely resembled the myogenic population while R+G- and R-G+ had a more smooth muscle-like phenotype. Negative controls (Neg) are shown for comparison. Nuclei are counterstained with DAPI (blue). (E-F) Relative CM/SM cell numbers were determined by counting the number of green and red stained cells, respectively, in each of the four subpopulations. R+G+ cells were the most myogenic population in both (E) embryonic or (F) ES cell-derived progenitors. (G) R+G+ progenitors were then used to grow contractile muscular thin films (MTF) that could be stimulated by field stimulation at 10V, 10ms and 0.5Hz to induce rhythmic deflections of a flexible substrate (images shown at 0ms, 120ms, 240ms, 360ms, and 480ms spanning diastole and systole) (18). Reproduced with permission from (18).

of ~0.5cm, a cell density of ~10^8 cells/mL, the ability to generate a force of 2-4mN/mm^2 during contraction, and the propagation of electrical signals at ~25cm/s. The engineered cardiac tissue should also have high viability and morphological properties similar to that of the native myocardium (1).

In the classical cardiac tissue engineering approach, cardiac cells are combined with scaffolds or hydrogels, and cultivated in bioreactors. Current studies involve the use of stem cells in the engineering of cardiac tissues (Section 3.1). The scaffolds are fabricated using natural polymers such as collagen, chitosan and alginate (2-5), or synthetic polymers such as poly (glycerol sebacate), poly (glycolic acid) and poly (lactic acid) (6-9) (Section 3.2.1). More recent studies utilize scaffolds derived from decellularized native tissues, or scaffold-free approaches (Section 3.2.2-3.2.3). Bioreactors (Section 3.3) are used to provide perfusion, mechanical stimulation and electrical stimulation during cultivation of engineered cardiac tissues (2, 10-11).

3.1. Cells: stem cell-based tissue engineering of the myocardium

The engineering of cardiac tissues *in vitro* requires large numbers of cells to achieve physiological cell densities (~10^5cells/cm^3) in order to ensure contractile function. Adult CMs have minimal proliferative capacity (12) since they are terminally differentiated. As such, alternative cell sources including 1) resident stem cells, 2) embryonic stem (ES) cells, and 3) induced pluripotent stem cells (iPSC) have been used to derive and provide millions of CMs for tissue regeneration.

Several resident stem cell populations have been used for heart regeneration (13-15). For example, cardiac progenitor cells expressing stem cell antigen-1 (Sca-1) were found in the adult mouse myocardium (16), and were involved in cardiac homing and differentiation post-MI. In another study, the injection of lin (-) c-kit (+) cells into an ischemic heart supported myocardial regeneration (17). Resident Islet-1+ (Isl1+) cardiac progenitors were also shown to differentiate into CMs (13). Domian *et al.* used a double fluorescent reporter system to derive CMs from Isl1+ cardiac progenitor population, in order to engineer cardiac tissues from progenitor cells with a defined myogenic identity (Figure 1A-F). These resulting CMs were then used to generate beating thin films, demonstrating their potential for contractile force generation (Figure 1G) (18). However, generating millions of cells required for tissue engineering from these resident stem cells may be a problem, since they

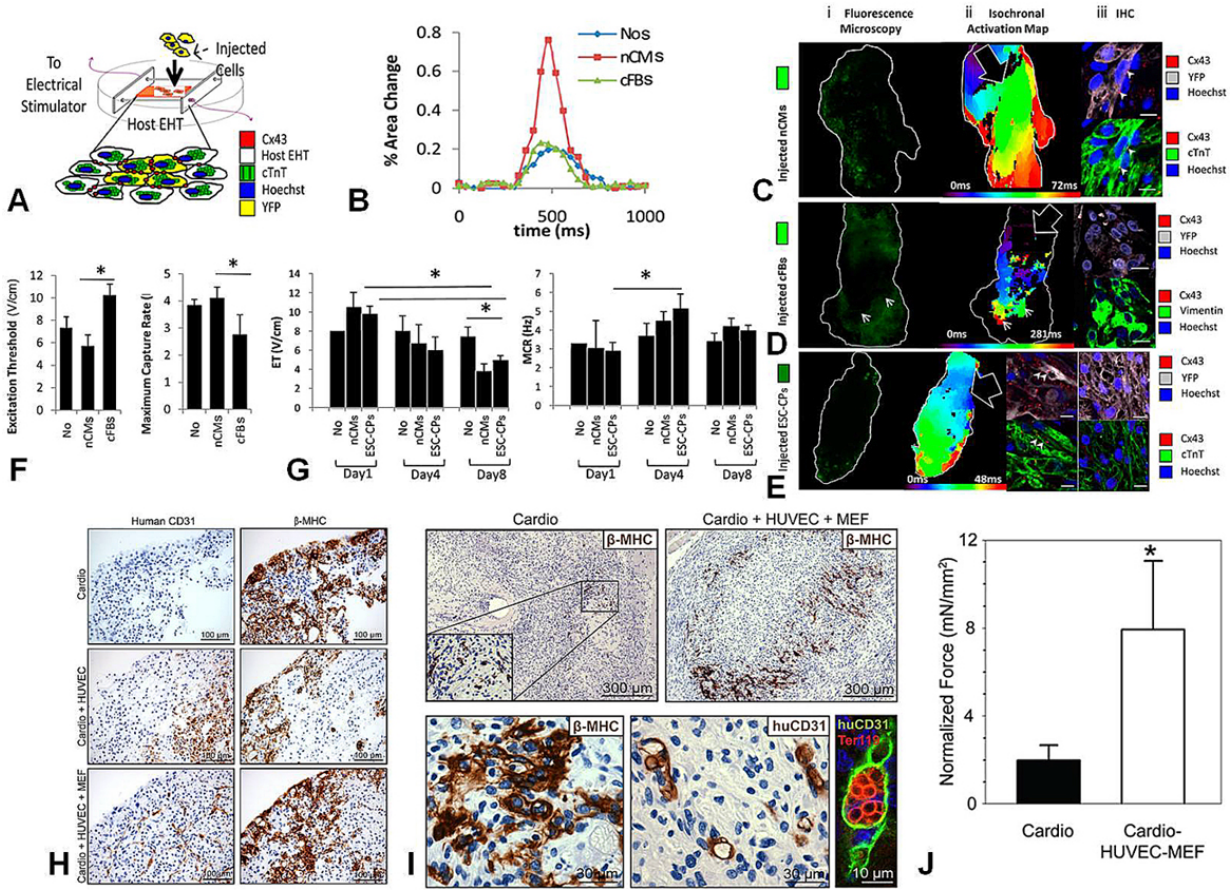

Figure 2. Growth of functional stem cell-based engineered heart tissues. (A-G) Engineered heart tissues (EHTs) were used as an *in vitro* model to evaluate the integration of injected ES cell-derived cell populations with the host tissue (25). (A) Schematic diagram showing the EHT in a stimulation chamber to be used as a model for the injection of YFP+ cells in the host tissue. (B) Time-dependent fractional area change was diminished by the addition of cardiac fibroblasts (cFBs) compared to the neonatal cardiomyocytes (nCMs)-injected group. (C-E) Isochronal activation maps show that impulse propagation is uniform in (C) EHTs injected with nCMs but is blocked by (D) the addition of cFBs. (E) Left, Isochronal activation map showing the speed of electrical impulses through the EHT. Embryonic stem cell-derived cardiac progenitors (ESC-CPs) improved conduction velocity and impulse propagation in EHTs. Right, Immunohistochemical staining of the injected EHT showing YFP expression and mature expression of Connexin-43 (Cx43) and cardiac troponin T (cTnT). (F) Functional properties (Excitation Threshold, ET, and Maximum Capture Rate, MCR) were not markedly improved when nCMs were injected but were worsened by the addition of cFBs. (G) EHT injected with ESC-CPs resulted in improvement in both ET and MCR at Day 4 and Day 8 compared to nCMs or no cells. (H-J) Scaffold-free cardiac patches were engineered using human ES cell-derived cardiomyocytes (hESC-CM) cultivated alone or in co- or tri-culture with either human umbilical vein endothelial cells (HUVECs) or hESC-endothelial cells (hESC-ECs) and mouse embryonic fibroblasts (MEFs) (24). (H) Histological sections show that hESC-CM-only patches contained almost no evidence of human CD31+ ECs, while hESC-CM/HUVEC patches show that human CD31+ cells were clumped at the center of the patch and appeared necrotic with CMs (beta-MHC, brown) at the periphery. Conversely, patches engineered with hESC-CM/HUVEC/MEFs showed vascular network formation and a healthier overall appearance. Similar data were found with hESC-ECs (not shown). (I) After implantation into rat skeletal muscle, the patches survived and anastomosed with the host vasculature (as evidenced by Ter119+ red blood cells infiltrating human CD31+ vessels). (J) Normalized contractile force generation was higher in tri-cultured hESC-CM/HUVEC/MEF patches than in patches engineered using hESC-CMs alone. Reproduced with permission from (24).

exist in low numbers. In contrast, pluripotent stem cells such as ES cells and iPSC can give rise to millions of bona fide CMs.

Early efforts at stem cell-based engineered cardiac tissues focused on the mouse system. As an example, Guo *et al.* generated ES cell-derived myocardium by using serum differentiation with embryoid bodies to attain CMs from mouse ES cells. The derived CMs were seeded into circular molds with collagen I and Matrigel to produce engineered heart tissues, which were subsequently subjected to unidirectional cyclic stretch at 10% strain and 2Hz (19). We have used engineered heart tissues (EHTs) as surrogate models for host myocardium to evaluate functional integration of injected Flk1+/PDGFR-alpha+ progenitors and progenitor-derived CMs into the heart (Figure 2A-G) (25). Recent work

by several groups has also shown promise with human ES cell-derived CMs for engineering cardiac tissues (20-24).

The ability to derive CMs from human ES cells and iPSC lines allowed cardiac tissue engineering to move towards human cell work (20-24). Stevens *et al.* (23-24) engineered scaffold-free cardiac patches containing human ES cell-derived CMs tri-cultured with mouse embryonic fibroblasts (MEFs) and ES cell-derived endothelial cells or human umbilical vein endothelial cells (HUVECs), showing that these patches were far superior to CM-only patches in their ability to form a vascular bed, generate contractile force, and anastomose with the host vasculature (Figure 2H-J). It was found that the co-culture of CMs with endothelial cells and mesenchymal cells (i.e. fibroblasts) was essential for the survival and integration of the engineered cardiac tissue with the native myocardium (22, 26). The donor-derived blood vessels were functionally integrated with the host coronary vasculature, thus enhancing the survival of the engineered graft.

The remaining ethical and immunogenic issues impede the therapeutic use of engineered cardiac tissues based on human ES cells, thus motivating the use of autologous adult stem cells. Adult stem cells, such as cardiac stem cells, as well as hematopoietic and mesenchymal bone marrow cells can self-renew and differentiate into specific lineages. For example, adult bone marrow cells have been used in the regeneration and functional improvement of the myocardium (27-30). The improvement in cardiac function through bone marrow cell transplantation is thought to be due to the secretion of soluble factors by these cells, rather than transdifferentiation (31). However, the transplantation of bone marrow cells for the treatment of infarcted myocardium faces problems such as their possible formation of bone and cartilage.

Adult cardiac progenitors that are isolated from explant cultures of human endomyocardial biopsies can be expanded *in vitro* in forms of cardiospheres (32-33). These progenitors played a role in cardiac regeneration in mice with myocardial infarction through direct differentiation, as well as paracrine mechanism by the secretion of vascular endothelial growth factor, hepatocyte growth factor and insulin-like growth factor-1, which increased capillary density and lowered apoptosis (34). Using magnetic targeting, Cheng *et al.* (35) attracted cardiosphere-derived cells labelled with superparamagnetic microspheres to accumulate around the ischemic area of the heart after injection, in turn significantly improving cell retention and engraftment.

Another possible source of autologous adult stem cells is adipose tissue. Adipose tissue is an abundant and dispensable tissue that can be harvested using less invasive procedures and has a significantly higher stem cell density (5%) in comparison to bone marrow (0.01%) (36). Adipose-derived cells isolated by digestion with collagenase are a heterogeneous cell population containing multipotent stem cells ($CD44^+$ and $CD105^+$), cells of hematopoietic lineage ($CD34^+$, $CD45^+$ and $CD11b^+$), smooth muscle (smooth muscle actin positive) and endothelial ($CD31^+$) cells (37). These different cell populations present in the adipose tissue could contribute to different aspects of cardiac tissue regeneration. Both heterogeneous and homogeneous (marker-based cell selection) adipose-derived cell populations have been shown to improve cardiac function after myocardial injury in different animal models (37-39). Adipose-derived cells have been shown to improve myocardial regeneration by both secretion of paracrine factors and direct differentiation (37). Importantly, there have not been any reports of tumorigenesis in studies using the adipose-derived cells in myocardial regeneration, suggesting that these cells would be good candidates for autologous cardiac cell therapies.

3.2. Scaffolds

3.2.1. Polymeric and naturally-derived fibrous and porous scaffolds

Cardiac tissues have been engineered using both polymeric and naturally-derived scaffolds. Natural scaffolds are made of proteins or carbohydrates from plants and animals (40). Some natural materials used as scaffolds for cardiac tissue engineering include alginate (41-44), chitosan (45-46), collagen (47-50), fibrin (31, 46, 51-56), gelatin (43, 57-58), and glycosaminoglycan (50, 59). While naturally-derived scaffolds are biocompatible and biodegradable, they can be easily degraded by enzymes and have relatively weak mechanical properties (60). Polymeric materials such as glycolic acid and lactic acid derivatives can be modified to have precisely controlled physical and biological properties, including pore size, hydrophobicity, degradation rate, and incorporation of biomolecules.

Fibrous scaffolds mimic the nanoscale structure of the native extracellular matrix (ECM), consisting of adhesive collagen, elastin, fibronectin and laminin fibers. The fiber structure and diameter, as well as the porosity, of fibrous scaffolds can affect cell behaviour. Oriented biodegradable non-woven poly (lactide) scaffolds were fabricated using electrospinning, and used to cultivate neonatal rat CMs (9). The cultivated CMs developed contractile apparatus and showed electrical activity. In a separate study, Rockwood *et al.* (61) also created polyurethane scaffolds with aligned microfibers by electrospinning. Cardiac ventricular cells cultivated on these scaffolds were organized along the aligned fibers after two weeks. These cells exhibited a more mature phenotype according to a lower content of atrial natriuretic peptide compared to cells grown on unaligned fibers.

Porous scaffolds support host cell infiltration and vascularization, and provide a large surface area for cell adhesion, differentiation and proliferation, thus making them advantageous for growing functional three-dimensional tissues (62-63). In a study by Li *et al.* (64), rat CMs were seeded into gelatin scaffolds and cultivated for 7 days prior to implantation of the constructs onto the myocardial scar tissue in rats. Upon implantation, native blood vessels infiltrated into the scaffold and the seeded CMs formed junctions with the native cardiac cells. Fetal rat cardiac cells cultivated on porous alginate scaffolds

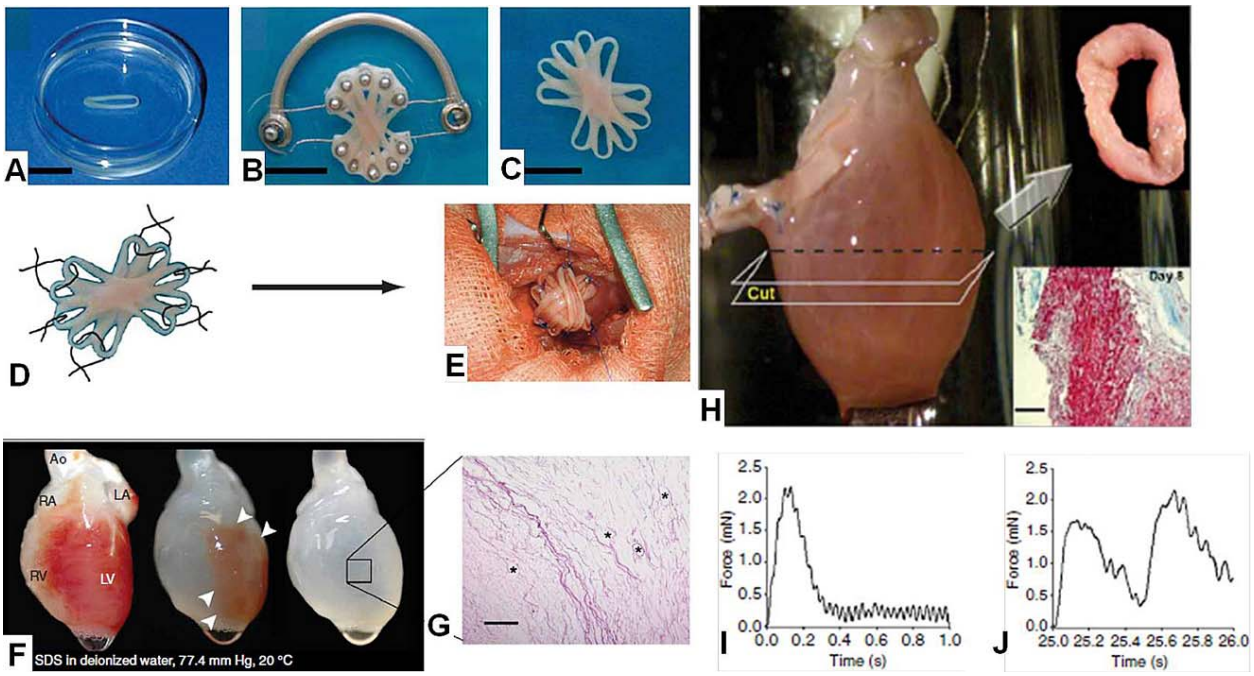

Figure 3. Current approaches for cardiac tissue engineering and repair. (A-E) Five single EHTs (A) were stacked on a device that allowed EHT fusion and contractions under auxotonic load (B). This approach resulted in synchronously contracting multiloop EHTs (C) that could be used for *in vivo* engraftment. (D) Six single-knot sutures were used to fix multiloop EHTs on the recipient hearts (E). Reproduced with permission from (87). (F-J) Decellularization of cadaveric rat hearts causes them to appear more translucent as cellular material is washed (with SDS) out from the right ventricle, then the atria and finally the left ventricle (F). (G) Hematoxylin and eosin staining of a thin section of SDS-treated heart showing no intact cells or nuclei, but maintaining large vasculature conduits (black asterisks). Scale bar, 200μm. (H) Recellularized whole rat heart at Day 4 of perfusion culture in a working heart bioreactor (Upper Insert showing cross-sectional ring harvested for functional analysis at Day 8, and Lower Insert showing Masson's trichrome staining of a thin section of the ring which contains cells throughout the thickness). Scale bar, 100μm. (I-J) Graphs showing force generation in the left ventricular rings with the application of electrical stimulation at 1Hz (I) and 2Hz (J). Reproduced with permission from (66).

were spontaneously beating in aggregates at Day 4 of cultivation (3). Neonatal rat CMs seeded into collagen sponges also formed spontaneously contracting tissues at Day 3 of cultivation, and maintained contractility for 12 weeks (65). This shows that porous scaffolds, amongst other different types of scaffolds, can support the growth of spontaneously beating cardiac tissues.

3.2.2. Decellularized matrix as an engineering scaffold

Engineered cardiac tissues were also generated using scaffolds that were derived from decellularized native tissues (66-67). Pig decellularized matrix hydrogels were found to improve maturation of human ES cell-derived CMs compared to gelatin (68). In addition, Gaudette *et al.* (69) showed that the treatment of a canine full thickness right ventricular defect could be improved using an FDA-approved extracellular matrix from porcine urinary bladder compared to the use of poly (ethylene terephthalate), which was previously used to restore the normal ellipsoidal shape of the ventricle (70). More interestingly, recent studies involved the decellularization of whole adult cadaveric rat hearts by coronary artery perfusion with detergents (66, 71-72) to obtain whole heart scaffolds (Figure 3F-J). The process preserved the underlying extracellular matrix, and produced an acellular scaffold with perfusable vascular architecture, competent acellular valves and intact chamber geometry. A beating heart can be generated by reseeding the scaffolds with neonatal rat CMs or endothelial cells and subsequently culturing the organ in a perfusion bioreactor. Decellularized pig and human hearts were also generated (71-72).

3.2.3. Scaffold-free cell sheet engineering

Alternatively, functional engineered cardiac tissues can be generated without scaffolds. In this cell sheet engineering approach, individual cell monolayers or cell sheets were stacked to create thick cardiac tissues (73). Monolayers of CMs or autologous skeletal myoblasts were first grown on poly (N-isopropylacrylamide)-grafted polystyrene dishes (Figure 4A-J). The temperature sensitive polymer allowed the attachment of cells at 37^{o}C, and the release of the monolayer by lowering the temperature to 20^{o}C, at which point the polymeric surface turned from being hydrophilic to hydrophobic. Single cell or composite double-stacked cell sheets were transplanted onto infarcted rat hearts to improve cardiac performance (74-75). However, the stacked cell sheets were limited in thickness due to the lack of vascularization and oxygen transport to the center of the tissue. To address this issue, a polysurgery approach with serial re-operations and implantations of multiple cell sheets was used to create 1cm thick myocardium (76).

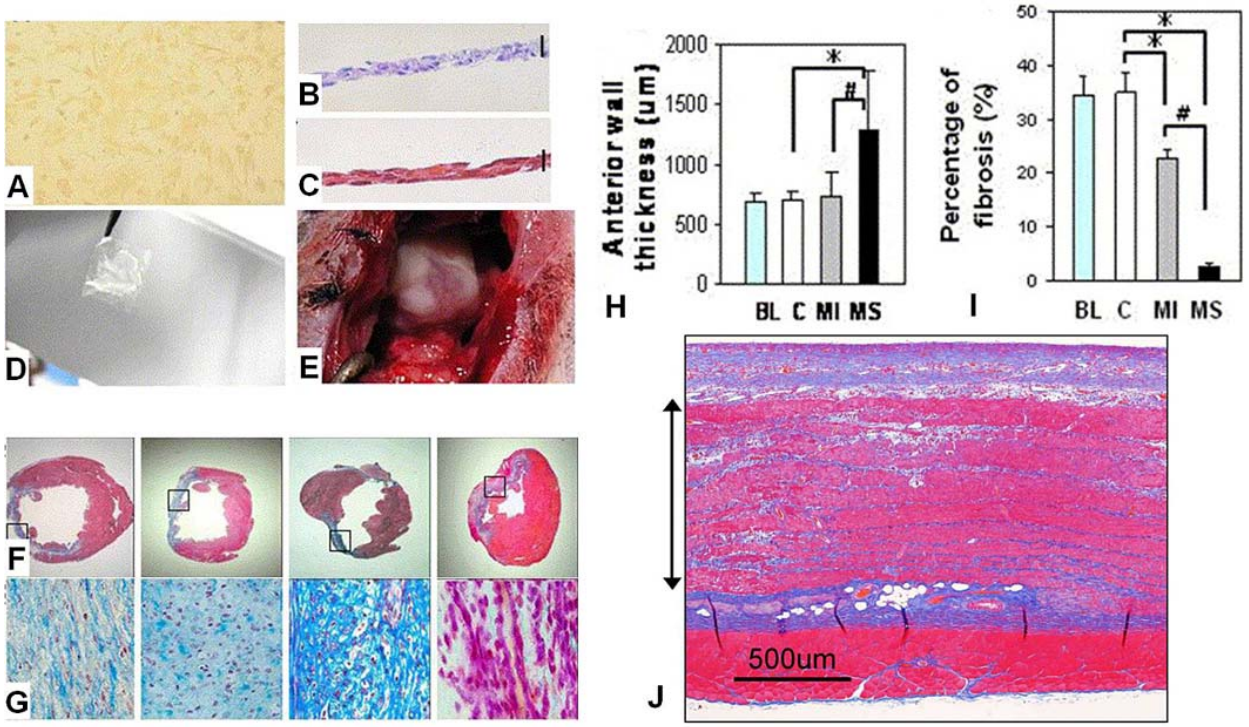

Figure 4. Cell sheet engineering and its application in cardiac repair. (A-J) Autologous skeletal myoblast cell sheets were created and imaged after immunohistochemical staining (A; 40× magnification), showing roughly 60% to 70% of cultured cells were desmin-positive muscle lineage cells. Hematoxylin and eosin (B) and Masson's trichrome staining (C; 400× magnification) indicated high cellularity and low fibrous content of the myoblast sheets. Scale bar, 50μm. Two sheets were piled to make a composite sheet graft (D) and then implanted to the infarcted area of a rat heart (E) to evaluate the direct injection of skeletal myoblasts to the site of infarct in comparison to the cell sheet method. Macroscopic (F, 10x) and microscopic (G, 400x) view of hearts from baseline (BL, far left), control (C, middle left), myoblast injection (MI, middle right), and myoblast sheet implantation (MS, far right) groups are shown. The baseline hearts that were excised 2 weeks after left anterior descending coronary artery ligation showed complete scar formation in the infarcted area, and the area in the control group showed similar findings. Histology was also similar in the MI group, although dispersed cellularity in the scar area was observed. The infarcted areas in the MS group were occupied with highly cellular tissues. (H) The anterior wall was significantly thicker in the myoblast sheet group compared with the injection and control groups. (I) The degree of myocardial fibrosis was similar in the baseline and control groups, and it was reduced in the MI group. The percentage of fibrosis in the MS group, however, was markedly less compared with that in the MI and control group. *P < 0.05 versus control group; #P < 0.05, MS group versus MI group (75). Reproduced with permission from (75). (J) In further work, Azan staining shows that by using 10-times polysurgery with 1 day intervals, multilayer cell-dense myocardium with well-organized microvessels can be created. Bidirectional arrow indicates viable myocardial cell sheet layers. The measured thickness of the grafts was 0.84 ± 0.16mm (76). Reproduced with permission from (76).

In two separate studies, Sca-1-positive resident cardiac progenitor cells and embryonic stem cell-derived SSEA-1-positive cardiac progenitors were used to generate cell sheets which were then transplanted into the infarct area of mouse and rhesus monkey hearts respectively (77-78). The transplanted cells differentiated into CMs and improved cardiac function after myocardial infarction. More importantly, cell sheets engineered from autologous skeletal myoblasts were used in a clinical trial in Japan, which successfully treated a patient with dilated cardiomyopathy (79).

3.3. Bioreactors

3.3.1. Perfusion

Due to a high oxygen demand of CMs and the limited oxygen diffusion within the tissue, the thickness of viable engineered cardiac tissue is often limited to ~200μm during *in vitro* cultivation (80). Perfusion bioreactors containing culture medium that was supplemented with an oxygen carrier were used to provide sufficient oxygen supply during *in vitro* cultivation to produce thick and compact cardiac constructs (81). In addition, channelled poly (glycerol sebacate) scaffolds were used within the perfusion bioreactors, thus further improving oxygen transport (81). Electrical stimulation (82) and mechanical stimulation (83-84) were incorporated into new designs of perfusion bioreactors such that the grown tissue can be perfused and stimulated simultaneously.

3.3.2. Mechanical stimulation

Previous studies also demonstrated the importance of physical stimuli for the morphology, mechanical properties and function of engineered cardiac tissues. Eschenhagen and Zimmermann created an EHT by

seeding a mixture of collagen I, Matrigel and neonatal rat CMs into lattice or circular molds. After one to two weeks of cultivation, synchronously contracting ring-shaped EHTs were formed due to spontaneous remodelling of the mixture and the application of cyclic mechanical stimulation (11, 85-86). The EHTs were then stacked into a flower-like construct and cultivated under auxotonic mechanical stimulation to create thick cardiac tissues that were implanted in a rat MI model (Figure 3A-E). Electrical coupling with the native tissue and improved diastolic and systolic function were achieved compared to sham control (87).

3.3.3. Electrical stimulation

It is essential that cells within engineered cardiac tissues are capable of synchronously responding to electrical pacing in order to develop proper excitation-contraction coupling. Bioreactors that provide electrical field stimulation can be used to induce synchronous beating of CMs in engineered cardiac tissues. We previously demonstrated that the use of physiologically relevant electric field stimulation during culture of neonatal CMs on porous collagen scaffolds or Matrigel led to the formation of mature myocardium with elongated and aligned cells (2, 88-89). The stimulated cardiac constructs had a high level of ultrastructural organization, and demonstrated increased amplitude of contractions. By applying electrical field stimulation with biphasic pulses, we also achieved contractile cardiac organoids with multiple cell types including CMs, fibroblasts and endothelial cells (89). Electrical field stimulation using symmetric biphasic square pulses showed improvements in Connexin-43 expression and success rate for achieving synchronous contractions in the engineered cardiac tissues, as compared to the use of monophasic square pulses with the same total amplitude and duration.

4. MICROFABRICATION FOR CARDIAC TISSUE ENGINEERING

The complex macro- to nano-scale structural organization of the native myocardium is essential for proper cardiac function. In particular, it is made of myocytes and myofibers that are aligned in parallel. The force of contraction and the velocity of impulse propagation are the highest along the long axis of the fiber (90). The orientation of the myofibers varies along the depth of the ventricular wall, and this orientation variation allows for the most efficient pump function. Previous work has been focused on improving the function of engineered cardiac tissues by mimicking the aligned structure of the native myocardium (Figure 5F-G) (90-94). Topographical cues can influence properties of CMs, including cell attachment, cell hypertrophy, binucleation, the remodelling of ion channels, the release of atrial natriuretic peptide, biomechanical stresses, and structural remodelling (95).

To enhance the orientation and elongation of CMs by providing anisotropic cues, strategies such as nanopatterning of poly (ethylene glycol) (PEG) hydrogels (90), rotary spinning of polymer nanofibers (96), and stamping of extracellular matrix proteins in lanes on thin films and hydrogels (90, 93, 97-98) have been used. Specifically, Kim *et al* (90) cultured ventricular myocytes on nanopatterned PEG hydrogels, which mimicked the nanoscale organization of the native extracellular matrix. Cells penetrated into the nanogrooves and this nanopattern-cell interaction placed spatial constraints on cellular organization, such as the direction and degree of actin polymerization and focal adhesion formation. In turn, cell spreading, cell-cell coupling and conduction velocity were improved. Badrossamay *et al.* (99) used rotary jet-spinning to fabricate anisotropic arrays of polymer nanofibers, and the seeded CMs used the aligned fibers to self-organize their contractile cytoskeleton to generate a beating tissue that is similar to the heart muscle in its laminar and anisotropic architecture. Engelmayr *et al.* (7) designed an accordion-like honeycomb poly (glycerol sebacate) scaffold by overlapping 200μm by 200μm squares at 45^{o} angles. These scaffolds have controllable stiffness and anisotropy, suitable for cardiac tissue engineering. By cultivating CMs on the accordion-like microstructure, cardiac cell alignment, contractility and mechanical properties comparable to the native adult rat right ventricular myocardium were achieved.

Feinberg *et al.* (98) cultured CMs on thin polydimethylsiloxane films with micropatterned ECM proteins to achieve muscular thin films that showed functional three-dimensional conformations when released from the thermally sensitive poly (N-isopropylacrylamide) substrate. The constructs could perform functions such as gripping, pumping, walking and swimming. Alford *et al.* (97) measured the stress of the cardiac muscle tissues that were engineered on anisotropically patterned fibronectin on the thin polydimethylsiloxane films to be 9.2±3.5kPa at peak systole, which was similar to that of the adult rat papillary muscle. Badie and Bursac (94) created high-fidelity models of ventricular cross-sections by projecting three-dimensional maps of local cardiac fiber directions in mouse ventricles onto two-dimensional masks to generate angled parallel lines on PDMS stamps (Figure 5A-E). The stamps were used to print fibronectin patterns that guided local cell alignment, achieving realistic anisotropic slice cultures.

Instead of controlling cell alignment through patterning of proteins on substrates, Bian *et al.* (54-55) guided three-dimensional cell alignment based on controlling the spatial pattern of gel compaction. In this approach, polydimethylsiloxane molds with arrays of mesoscopic posts were made and seeded with CMs in hydrogels (Figure 5H-K). The properties of the posts, such as size, elongation and spacing, can be controlled such that the desired local guidance of cell alignment can be achieved. Interestingly, the size of adherens junctions was found dependent on the mechanical tugging force, showing that the integrity of multicellular tissues requires the coordination of mechanical forces and cell-cell adhesive interactions (100). Micro-organoids were also cultivated using gel compaction around two posts (100-101). Studies by Black et al. (56), Huang et al. (102) and others also used the same principle – constrained cell induced compaction of a gel – to attain cell alignment.

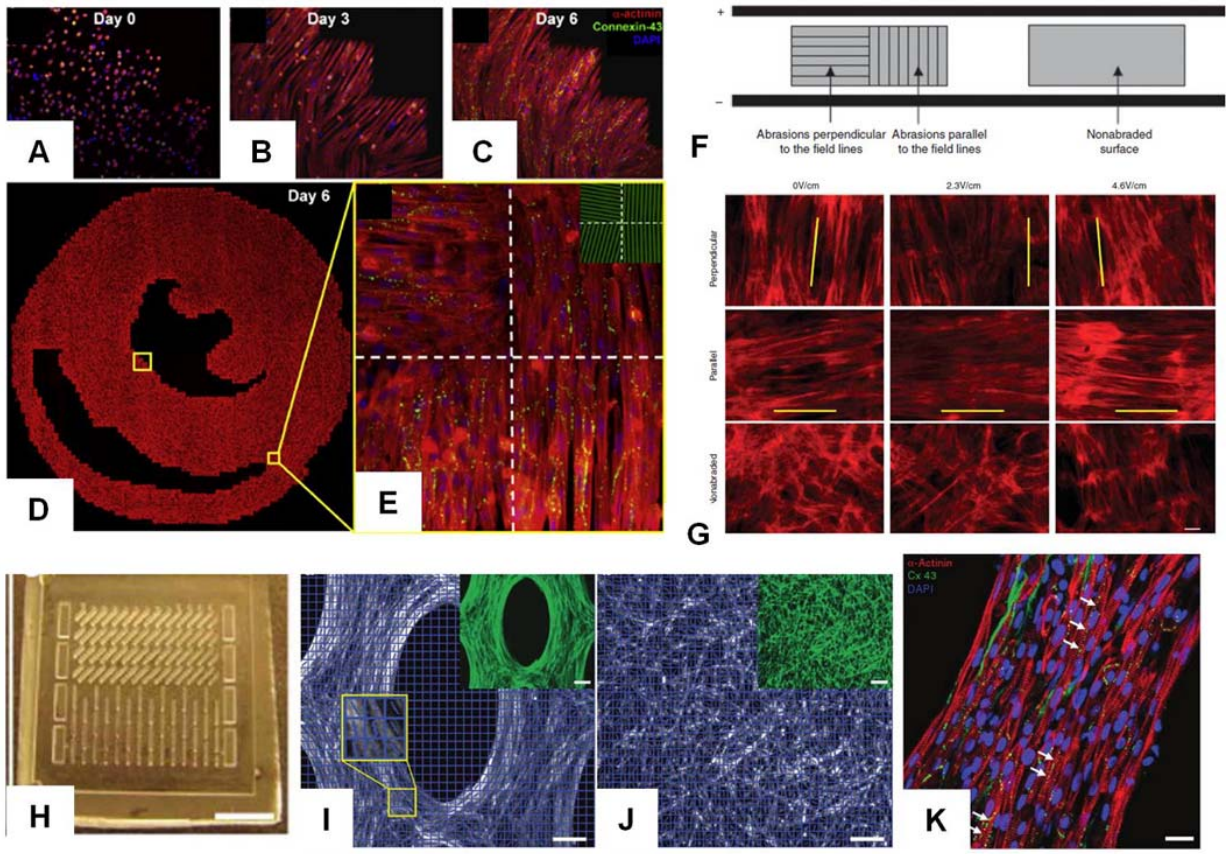

Figure 5. The alignment and elongation of cardiomyocytes in engineered cardiac tissues. (A-E) Creating realistic cardiac microstructure by the use of micropatterned anisotropic slice cultures that mimic the directions of fibers in native ventricular cross sections (94). Reproduced with permission from (94). Images showing (A) cell attachment, (B) cell alignment in the direction of micropatterned fibronectin lines, and (C) formation of confluent cardiac fibers. (D) A composite image showing the entire micropatterned slice culture. (E) Four sections of the slice culture with the underlying fibronectin pattern shown as an inset (green). (F-G) Engineering of synchronously contractile cardiac constructs by culturing cardiac cells with the application of electrical field stimulation and topographical cues via surface abrasion. Reproduced with permission from (88). (F) Schematic diagram of the setup with electrical field stimulation of cardiomyocytes seeded on abraded and non-abraded coverslips. (G) Phalloidin-TRITC staining of actin cytoskeleton shows elongation and alignment of cardiomyocytes on abraded surfaces, which were further enhanced by electrical field stimulation (scale bar represents 10μm, electrical field lines from left to right, yellow lines show the direction of abrasions). (H-K) Using spatial patterning of gel compaction to aid cell alignment by engineering three-dimensional muscle tissues in PDMS molds with arrays of mesoscopic posts (H) (54-55). More aligned cells shown in PDMS mold with posts (I) compared to that without posts (J, blue squares represent subregions, F-actin shown in inset). (K) Cardiomyocytes were densely aligned, striated and expressed Connexin-43 after 2 weeks of culture. Reproduced with permission from (54).

Au *et al.* (91, 103) studied the interactive effects of topographical cues and electrical field stimulation on CMs. Abraded polyvinyl surfaces with V-shaped abrasions of 13μm in width and 700nm in depth were seeded with fibroblasts or CMs and positioned between two carbon rod electrodes that were connected to the electrical field stimulation chamber (91). Fibroblasts and CMs on non-abraded surfaces were more elongated under electrical field stimulation. It was found that topographical cues had a more significant effect on the alignment of CMs as compared to the application of electrical field stimulation. To incorporate topographical cues and electrical stimulation into one cultivation system, cell culture chips consisting of microgrooves were created by hot embossing of polystyrene (103). In addition, two gold electrodes were electrodeposited at the ends of the chip at 1cm apart, with the microgrooves located between the electrodes. The cultivated CMs elongated and aligned in the direction of the microgrooves. The simultaneous application of electrical field stimulation led to the accumulation gap junctions at the ends of the cells. The CMs further elongated when the microgrooves were positioned parallel to the electric field.

Micro- and nanoscale techniques were also used to create stem cell niches to regulate their differentiation towards the myocardial lineage. Tay and colleagues (104) printed fibronectin onto poly (lactic-co-glycolic acid) film to form geometries for controlling the morphology of human mesenchymal stem cells. Cells grown on 20μm

micropatterned wide strips were highly elongated and expressed cardiac myosin heavy chain, indicative of differentiation towards the myogenic lineage. Sasaki *et al.* (105) made micropatterned surfaces with arrays of cell-adhesive circular micro-domains, and found an optimal diameter of 200μm for the micro-domains to drive cardiac differentiation in embryonic stem cell aggregates.

5. VASCULARIZATION OF ENGINEERED CARDIAC TISSUES

Although perfusion bioreactors can support the growth and viability of cardiac tissues with physiological thickness and high cell density *in vitro*, rapid vascularization and connection to the host vasculature is essential upon *in vivo* implantation. In the native myocardium, capillaries of ~7μm in diameter are spaced at distances of ~20μm, with each myofiber located between two capillaries (106). The main strategies for vascularization of engineered cardiac tissues include 1) cell tri-culture, 2) fabrication of proangiogenic scaffolds based on geometry and matrix components, 3) incorporation of growth factors and peptides, and 4) seeding of cells in subcutaneously implanted chambers around an arteriovenous loop. While some of the vascularization strategies described here have not been assessed in cardiac tissue engineering, they have important relevance as they can be potentially applied to create vascularized cardiac tissues.

5.1. Cell tri-culture

The co-culture of fibroblasts and endothelial cells (ECs) with CMs can improve vascularization of engineered cardiac tissues (22, 26). Lesman *et al.* engineered vascularized cardiac tissue constructs by a tri-culture of CMs with fibroblasts and endothelial cells, and transplanted them to rat hearts (22). There was increased formation of donor and host-derived vasculature in tri-culture constructs compared to scaffolds with CMs alone. Donor-derived vessels were functionally integrated with host coronary vasculature, as demonstrated by their incorporation of intraventricularly-injected fluorescent microspheres. Naito *et al.* used the cell population from the native rat heart, which contained both CMs and non-myocytes, to engineer the cardiac tissue with completely defined culture conditions (i.e. serum and Matrigel free) (107). It was also found that the pre-culture of fibroblasts and ECs followed by the seeding of CMs improved the functionality of microscale cardiac organoids, as compared to simultaneous tri-culture (26, 108). In a separate study, ECs were sandwiched between stacked cell sheets to form pre-vascular networks (109), which then connected to the host blood vessels upon implantation in an MI model to support further vascularization of the cell sheets (110-111).

5.2. Proangiogenic scaffolds

Vascularization can be further enhanced using appropriate geometries (112-114) and extracellular matrix components (45, 115) in biomaterials and scaffolds. Microtemplating was used to fabricate tissue engineering scaffolds consisting of interconnected pores of 30-40μm in diameter that promoted angiogenesis post-MI in rats (112). Raghavan *et al.* (113) cultured ECs within 50-200μm wide and 50-100μm high collagen-filled channels. Cells organized into tubes with lumens within 1-2 days. Branched tube formation was also achieved (113). Bettinger *et al.* (114) showed that nanofabricated substrates of 1.2μm in period and 0.6μm in depth improved alignment, elongation and migration of endothelial progenitor cells (EPCs), thus enhancing capillary tube formation. Besides micropatterning and controlling porosity, Deng *et al.* (45) showed increased vascular endothelial-cadherin expression and formation of vascular-like structures by ECs on collagen-chitosan matrix compared to collagen alone.

5.3. Incorporation of proangiogenic biomolecules

Proangiogenic growth factors can also be used for vascularization of engineered cardiac tissues. They can be delivered in various forms, including soluble factors, encapsulated in microparticles, and physically or covalently immobilized into biomaterials for tissue engineering. Saif *et al.* (116) fabricated poly (lactic-co-glycolic acid)-based microparticles to release vascular endothelial growth factor (VEGF), hepatocyte growth factor and angiopoietin-1 upon injection in murine hindlimb ischemia models. The release of multiple factors improved vasculogenic progenitor cell therapy by promoting homing and incorporation of intravenously-administered progenitors, in turn increasing microvessel density and vascular smooth muscle cells necessary for vessel stabilization. Similarly, alginate microparticles were integrated into collagen/fibronectin gels to transplant ECs with co-delivery of VEGF and monocyte chemotactic protein-1 (MCP-1) (117). VEGF improved survival of transplanted ECs and vessel formation, while MCP-1 induced mural cell recruitment and vascular stability. Incorporation of biomolecules is essential for cell-based therapeutic vascularization to overcome apoptosis and lack of recruitment of host cells.

Small molecules such as thymosin beta4 (118) or ascorbic acid (115) can also be utilized to enhance angiogenesis. Smart *et al.* (118) showed that the peptide thymosin beta4 facilitates neovascularization by resident progenitors and stabilizes vascular plexus by collateral vessel growth, thus sustaining the myocardium after ischemic damage. Martinez *et al.* supplemented myocardial grafts with ascorbic acid, which increased number of blood vessels, donor cells and ECs and improved cardiomyoblast survival at 6 days post-implantation in renal pouches of rats (115). Other biomolecules were used to induce growth factor production and in turn angiogenesis (118-119). For example, erythropoietin-induced VEGF expression in CMs was shown to stimulate myocardial endothelial proliferation and increase homing of EPCs to the myocardium in rats with heart failure (119).

Physical and covalent immobilization of growth factors can localize growth factor activity and prolong receptor/ligand signaling to promote rapid vascularization. Zhang *et al.* (120) produced a fusion protein, CBD-VEGF, consisting of VEGF and a collagen-binding domain (CBD). Once injected into rats, CBD-VEGF was bound to the

collagen-rich cardiac extracellular matrix while retaining growth factor activity, in turn increasing capillary vessel density in infarcted hearts and reducing scar size as compared to PBS and VEGF controls. Previously, we covalently immobilized VEGF and angiopoietin-1 onto collagen scaffolds to increase the proliferation of H5V endothelial cells (121) and primary rat aortic endothelial cells (122) compared to scaffolds with no growth factor or soluble factors, thus leading to tube formation. Co-immobilization of VEGF and angiopoietin-1 further led to enhanced angiogenesis in a chicken chorioallantoic membrane assay compared to immobilized VEGF or angiopoietin-1 alone (121). There was enhanced angiogenesis and patch stability for the scaffolds with immobilized VEGF compared to VEGF-free controls when the patches were implanted to replace a full right ventricular free wall defect in rat hearts (Figure 6L-Q) (123). Dvir *et al.* prevascularized a cardiac patch, which consisted of cultured cardiac cells on an alginate scaffold capable of sustained release of several incorporated prosurvival and angiogenic factors (i.e. insulin-like growth factor-1, stromal cell derived factor 1, VEGF), by heterotopic transplantation onto the omentum for 7 days (124). The vascularized cardiac patch was explanted from the omentum and subsequently transplanted onto infarcted rat hearts post-MI. The patch prevented dilatation by inducing thicker scars, and showed both structural and electrical integration into the host tissue (Figure 6A-F).

5.4. *In vivo* vascularization

Morritt *et al.* seeded neonatal rat CMs into a plastic chamber that contained Matrigel and an arteriovenous blood vessel loop, and implanted the chamber subcutaneously in the groin to attain spontaneously contracting thick three-dimensional constructs with extensive vascularization at 4 and 10 weeks (125). By co-implanting adipose-derived stem cells with rat CMs in the same vascularized chamber, larger tissue constructs and vascular volumes were achieved compared to chambers with CMs alone (Figure 6G-K) (126). In a separate study, the arteriovenous loop was created and inserted into the cell seeding chamber at Day 0 and the myoblasts were seeded into the chamber at Day 4 or Day 7 rather than Day 0 (127). The implantation of myoblasts into the chamber at Day 7, when capillary growth was well-established, led to improved survival of myoblasts compared to implanting them at Day 0. This suggests the need to seed cells on established vascular beds for tissue regeneration.

6. PERSPECTIVE

An immediate application of the engineered cardiac tissue is to utilize it as an *in vitro* model system. Cardiac tissues can be engineered using human CMs to create models of healthy and diseased myocardium, which are in turn used for drug testing or cell injection. Eschenhagen *et al.* constructed miniaturized fibrin-based engineered heart tissues that were then used as a high-throughput method for drug screening and disease modeling (128). Using such method, dose dependent effect on the contractility can be easily measured by applying different concentrations of drugs such as chromanol, quinidine and erythromycin (128).

In vivo studies have been performed by transplanting embryonic (129), fetal (130-131) and neonatal CMs (132-133, 134), skeletal myoblasts (135), bone marrow stem cells (136-139), and resident cardiac progenitors (16, 140-141). Functional improvements were found compared to cases without cell injection. However, a main limitation in the previous *in vivo* myocardial cell transplantation studies is the low cell retention rate (142). More importantly, unreasonably high numbers of animals would be required to thoroughly screen multiple parameters affecting cell transplantation (i.e. cell type, culture conditions etc) *in vivo*. Recently, we used the engineered cardiac tissue as an *in vitro* model to evaluate the integration and differentiation potential of different stem and progenitor cells in the cardiac environment (25, 143). Specifically, we injected Flk1+/PDGFR-alpha+ cardiac progenitors into the engineered cardiac tissue and showed the capability of these cells to differentiate into CMs and integrate into the host tissue (25). On the other hand, mouse ES cell-derived CMs did not integrate well with the host engineered tissue (25). The engineered cardiac tissue also allowed the characterization of activities and potential to develop teratomas of the residual undifferentiated cells when using mouse ES cell-derived populations (143).

As seen in the encouraging results summarized in this review, the field of cardiac tissue engineering is rapidly advancing due to the design of novel scaffolds and bioreactors, new analytical techniques and the advances in stem cell biology. For example, bioreactor systems are now capable of simultaneous application of perfusion, electrical stimulation and mechanical stimulation to enhance function of the engineered cardiac tissues. Various vascularization strategies have been developed and can now be further used in cardiac tissue engineering to improve the viability of thick tissues. Specifically, while vascular-like structures have been formed from endothelial cells using microfabrication techniques, the resulting structures have not yet been used as a vascular bed for cardiac tissue engineering. New analytical techniques can allow the real time monitoring of cell and tissue function to gain insight into the complex mechanisms of cardiogenesis and myocardial repair. More importantly, the ability to generate CMs from iPSC aided the engineering of autologous cardiac patches with clinically relevant size (~0.5cm in thickness) and cell density (~10^8 cells/mL).

However, there are many key challenges to overcome before cardiac tissue engineering can be clinically relevant (Section 3). One such challenge is to develop bioreactors for large scale production of iPSC-derived CMs. Also, non-human components used during cultivation of these cells as well as the tissue engineering process, such as serum and Matrigel, must be replaced prior to transplantation in humans. In future studies, we anticipate the generation of different subtypes of human CMs (i.e. pacemaking-, atrial-, ventricular-, or Purkinje-like phenotypes) such that different heart diseases can be treated. Mechanical and electrical stimulation can be used

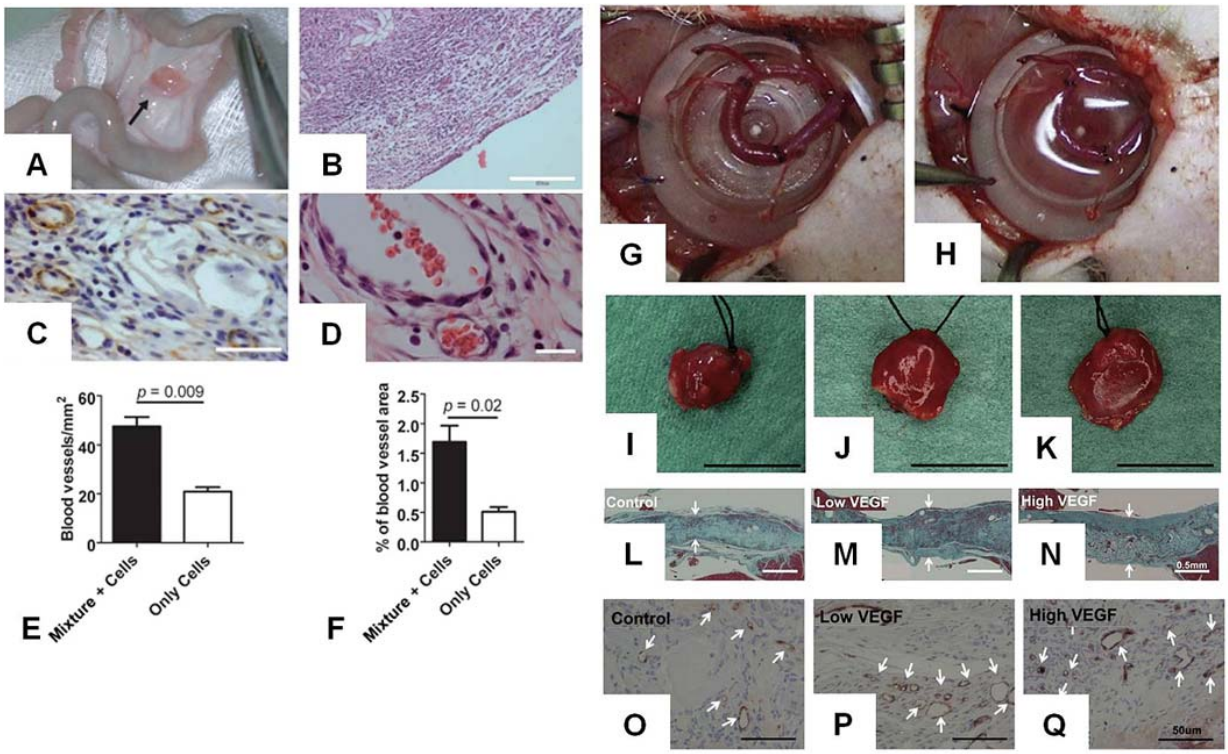

Figure 6. Vascularization of engineered cardiac tissues. (A-F) A cardiac patch consisting of cardiomyocytes seeded in an alginate scaffold capable of sustained release of IGF-1, SDF-1 and VEGF was implanted into the omentum for prevascularization (A), after which the patch was extracted and implanted into the infarcted heart (124). Hematoxylin and eosin staining showed extensive tissue ingrowth into the scaffold (B). There were mature and functional blood vessels as indicated by anti-smooth muscle actin immunostaining (C), and red blood cell content (D). Analysis showed increased blood vessel density (E) and % blood vessel area (F) in the group with a mixture of survival and angiogenic factors within the scaffold. Reproduced with permission from (124). (G-K) An arteriovenous loop was created and placed into a plastic chamber in the rat groin (G), with cells seeded in Matrigel around the arteriovenous loop (H) (126). After 6 weeks of cultivation, tissues generated from cardiomyocytes only (I), co-implantation of adipose-derived stem cells and cardiomyocytes (J), and adipose-derived stem cells only (K) were harvested, showing that the incorporation of adipose-derived stem cells led to larger tissue constructs due to their contribution to vascularization. Reproduced with permission from (126). (L-Q) Collagen scaffolds with covalently immobilized VEGF were implanted to replace full right ventricular free wall defects in rats (123). Reproduced with permission from (123). (L-N) Masson's trichrome staining at Day 28 post-implantation showing higher patch thickness for patches with low dose VEGF (M, ~14ng VEGF) and high dose VEGF (N, ~97ng VEGF), compared to VEGF-free control patch (L). Patch thickness indicated by two arrows. (O-Q) Increased CD31-positive vascular structures (arrows) for patch with high dose VEGF (Q), compared to VEGF-free control patch (O) and patch with low dose VEGF (P).

during cultivation to determine whether or not these external stimuli modulate the level of differentiation of iPSC-derived CMs. It is also necessary to determine the level of maturation that is required for these cells to create a cardiac patch that is viable, functional and easily integrated with the host tissue. Many studies in this field were conducted with the assumption that patches exhibiting properties of adult ventricular myocardium in terms of contractile force, propagation velocity and cell morphology will enable improved cardiac function upon implantation. However, this has not been proven *in vivo*.

Another challenge is to determine the required level of myocardial regeneration for sufficient level of survival and attenuation of heart failure. All previous *in vivo* studies as described in this review have shown improvements compared to untreated cases, but the current strategies cannot achieve complete myocardial regeneration. Moreover, there cannot be a direct comparison between the current strategies due to the differences in cell sources, biomaterials, animal models and experimental time points. To better assess the long-term effects of the treatments post-MI, future *in vivo* studies should be performed for longer time periods.

The engineered myocardium will be a useful *in vitro* model to study the interactions among different cell populations within the heart during embryonic development and to uncover the mechanisms by which transplanted cells promote cardiac repair. With the availability of stem cell-derived human CMs, the engineered heart tissue will become an increasingly important model system for *in vitro* drug screening. To develop high throughput systems for drug screening and developmental studies, the

miniaturization of the engineered cardiac tissues, as well as the design of microfluidic devices and Micro-Electro-Mechanical Systems (BioMEMS) will be crucial.

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