

Biomimetic approach to cardiac tissue engineering

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Here, we review an approach to tissue engineering of functional myocardium that is biomimetic in nature, as it involves the use of culture systems designed to recapitulate some aspects of the actual *in vivo* environment. To mimic the capillary network, subpopulations of neonatal rat heart cells were cultured on a highly porous elastomer scaffold with a parallel array of channels perfused with culture medium. To mimic oxygen supply by haemoglobin, the culture medium was supplemented with a perfluorocarbon (PFC) emulsion. Constructs cultivated in the presence of PFC contained higher amounts of DNA and cardiac markers and had significantly better contractile properties than control constructs cultured without PFC. To induce synchronous contractions of cultured constructs, electrical signals mimicking those in native heart were applied. Over only 8 days of cultivation, electrical stimulation induced cell alignment and coupling, markedly increased the amplitude of synchronous construct contractions and resulted in a remarkable level of ultrastructural organization. The biomimetic approach is discussed in the overall context of cardiac tissue engineering, and the possibility to engineer functional human cardiac grafts based on human stem cells.

Keywords: tissue engineering; cardiac; bioreactor; oxygen; vascularization; development

1. INTRODUCTION

Cardiovascular disease is responsible for a preponderance of health problems in developed countries. In developed countries, almost one-fourth of the population lives with coronary heart disease, congenital cardiovascular defects and congestive heart failure, and this already high incidence is likely to further increase as the population ages. Heart disease and stroke, the principal components of cardiovascular disease, are the first and the third leading cause of death in the US, accounting for nearly 40% of all deaths. Congenital heart defects, which occur in nearly 14 of every 1000 newborn children (Gillum 1994), are the most common congenital defects and the leading cause of death in the first year of life (Hoffman 1995*a,b*). Cardiovascular diseases result in substantial disability, and largely contribute to the escalating costs of health care.

Myocardium does not have the ability to regenerate after injury. Cell-based therapies have been considered as a novel and potentially curative treatment option, either by usage of cells alone (Koh *et al.* 1993; Soonpaa *et al.* 1994; Li *et al.* 1996; Taylor *et al.* 1998; Reinecke *et al.* 1999; Sakai *et al.* 1999; Condorelli *et al.* 2001; Etzion *et al.* 2001; Orlic *et al.* 2001; Muller-Ehmsen *et al.* 2002*a,b*; Roell *et al.* 2002; Menasche *et al.* 2003) or by scaffold-aided tissue engineering cardiac constructs

that can be surgically attached to the myocardium (Eschenhagen *et al.* 1997; Akins *et al.* 1999; Bursac *et al.* 1999; Carrier *et al.* 1999; Li *et al.* 1999, 2000; Fink *et al.* 2000; Leor *et al.* 2000; Papadaki *et al.* 2001; Kofidis *et al.* 2002; Shimizu *et al.* 2002; Zimmermann *et al.* 2002*a,b*, 2006; Radisic *et al.* 2003, 2004*a,b*, 2006). Transplanted cells included embryonic, foetal and neonatal cardiomyocytes (Leor *et al.* 1996; Scorsin *et al.* 1996), skeletal myoblasts (Taylor *et al.* 1998; Menasche *et al.* 2003), mesenchymal stem cells from bone marrow (Orlic *et al.* 2001) and cardiomyocytes derived from human embryonic stem cells (Kehat *et al.* 2004).

Transplantation of exogenous cells into damaged myocardium was reported to improve myocardial function and vascular supply (Orlic *et al.* 2001; Menasche *et al.* 2003; Kehat *et al.* 2004). However, the extent and mechanisms of myocardial repair are not well established, and several reports raise a cautionary note as they found no evidence of either transdifferentiation of stem cells into cardiac phenotype (Murry *et al.* 2004), or myocardial regeneration (Balsam *et al.* 2004). While the application of cells alone is being pursued for treating small-scale injuries, tissue engineering may provide functional cell-based substitutes of the native tissue to treat large defects. As compared with the transplantation of cells alone, engineered cardiac constructs have the potential advantage of *immediate functionality*. In addition, engineered tissues can serve as high-fidelity *in vitro* models for the study of normal and

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pathological cardiac functions, drug screening and effects of hypoxia (Carrier *et al.* 2002a; Bursac *et al.* 2003; Radisic *et al.* 2004a, 2005c).

Three-dimensional cardiac tissue constructs that express structural and physiological features characteristic of native cardiac muscle have been engineered using foetal or neonatal rat cardiac myocytes (CMs) cultured in collagen gels with mechanical stimulation (Eschenhagen *et al.* 1997; Fink *et al.* 2000; Zimmermann *et al.* 2000, 2002b), on collagen fibres (Akins *et al.* 1999), fibrous polyglycolic acid scaffolds (Bursac *et al.* 1999; Carrier *et al.* 1999, a,b; Papadaki *et al.* 2001) and porous collagen scaffolds (Li *et al.* 1999, 2000; Radisic *et al.* 2003). In early studies, cells were seeded onto scaffolds and cultivated in dishes (Carrier *et al.* 1999; Li *et al.* 1999; Leor *et al.* 2000; Papadaki *et al.* 2001), spinner flasks (Bursac *et al.* 1999; Carrier *et al.* 1999; Papadaki *et al.* 2001) or in rotating vessels (Akins *et al.* 1999; Carrier *et al.* 1999; Papadaki *et al.* 2001). Oxygen dissolved in medium was transported to the cells by molecular diffusion, which could support the viability of only a 100 μm thick outer layer of functional tissue but not the construct interior, which remained largely acellular (Bursac *et al.* 1999; Carrier *et al.* 1999; Zimmermann *et al.* 2000; Papadaki *et al.* 2001).

In standard culture systems (dishes, flasks and rotating vessels), cardiomyocytes did not align in parallel as in the native heart and they remained poorly differentiated (Bursac *et al.* 1999; Carrier *et al.* 1999). Application of contraction alone via cyclic mechanical stretch significantly improved the level of cell differentiation and force of contraction, but some hallmarks of cardiac differentiation were still missing (including M bands and intercalated (IC) discs; Zimmermann *et al.* 2002b). In addition, the distribution and frequency of gap junctions, critical for electrical signal propagation in the tissue, remained unclear. Notably, excitation–contraction coupling, which is crucial for the development and function of native myocardium (Severs 2000), has not been fully established. More recently, clinically sized ($6 \times 8 \times 2$ mm thick), compact cardiac constructs with physiological cell densities were engineered *in vitro* by mimicking various aspects of the *in vivo* environment in native myocardium, including oxygen supply, by perfusion of culture medium supplemented with oxygen carriers (Radisic *et al.* 2005a) and the induction of construct contractions by electrical pacing signals (Radisic *et al.* 2004a).

We discuss here the key parameters of this new ‘biomimetic’ approach to cardiac tissue engineering, involving the cultivation of cells on scaffolds under conditions designed to provide developmental cues found *in vivo*. The focus is on the implementation of *in vivo*-like oxygen transport to support physiological concentrations of viable cells in clinically sized constructs, and electrical stimulation of cultured constructs to establish orderly excitation–contraction coupling.

2. REQUIREMENTS DERIVED FROM NATIVE MYOCARDIUM

(a) Key parameters

In vivo, the processes of cell differentiation during embryogenesis and adult tissue remodelling are

directed by multiple factors acting in concert and according to specific spatial and temporal sequences. Undifferentiated (stem) cells first differentiate into specific cell lineages (bone, muscle, nerves and blood vessels) and then undergo functional assembly into tissue structures. Both phases depend on at least four groups of factors: (i) a structural template for cell attachment and tissue formation, (ii) a physiological milieu, (iii) levels of nutrients, oxygen and metabolites, and (iv) physical regulatory signals (figure 1). It is thought that the cell function *in vitro* can be modulated by the same factors known to play a role during embryogenesis, by an integrated use of biomaterial scaffolds and bioreactors (figure 2).

Based on these general principles, a biomimetic approach to cardiac tissue engineering has been established, which involves the *in vitro* creation of immature but functional tissues by an integrated use of (i) populations of cardiac cells, (ii) biomaterial scaffolds that serve as a structural and logistic template for tissue development and biodegrade at a controlled rate, and (iii) bioreactors that provide environmental control and regulatory factors necessary for the cells to regenerate a functional tissue. A bioreactor is designed to perform one or more of the following functions: (i) establish spatially uniform, physiologically high cell concentration within the scaffold, (ii) maintain micro-environmental conditions via efficient mass transfer between the cells and culture medium (e.g. temperature, pH, osmolality, levels of oxygen, nutrients, metabolites and regulatory molecules), and (iii) apply physical signals relevant for cardiac development and function (e.g. interstitial flow, hydrodynamic shear and electrical stimuli). The environmental factors inherent for native myocardium *in vivo* and their *in vitro* counterparts are listed in table 1.

(b) Cell populations

Native myocardium (cardiac muscle) is a highly differentiated tissue composed of CMs and fibroblasts with a dense supporting vasculature and collagen-based extracellular matrix (ECM), and an average cell density of $1\text{--}10 \times 10^8$ cells cm^{-3} . The myocytes form a three-dimensional syncytium that enables propagation of electrical signals across specialized intracellular junctions to produce coordinated mechanical contractions that pump blood forward. Only 20–40% of the cells in the heart are CMs, but they occupy 80–90% of the heart volume (Nag 1980). Cardiac fibroblasts contribute to most of the non-myocytes in the myocardium. The main roles of cardiac fibroblasts are to secrete the components of the ECM and transmit mechanical force by the receptor-mediated connections to the ECM (Sussman *et al.* 2002). The myocardial ECM consists of a fibrillar collagen network, with predominant collagen types I and III, a basement membrane, proteoglycans, glycosaminoglycans and a variety of other bioactive molecules (Burlew & Weber 2002). The exact composition of the ECM is regulated by a crosstalk between myocytes and fibroblasts (Sussman *et al.* 2002). Recent studies demonstrated that cardiac fibroblasts propagate electrical stimuli over the distances of the order of 100 μm via gap junction communications (Gaudesius *et al.* 2003).

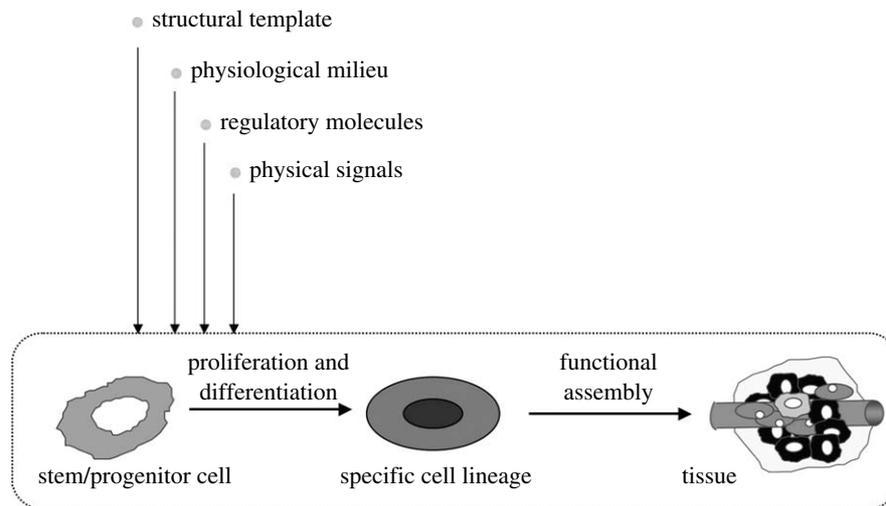


Figure 1. Developmental paradigm. Tissue development and remodelling, *in vivo* and *in vitro*, involve the proliferation and differentiation of stem/progenitor cells and their subsequent assembly into a tissue structure. Cell function and the progression of tissue assembly depend on (i) the availability of a scaffold for cell attachment and tissue formation, (ii) the maintenance of physiological conditions in cell/tissue environment, (iii) the supply of nutrients, oxygen, metabolites and growth factors, and (iv) the presence of physical regulatory factors (adapted from Radisic *et al.* 2005b).

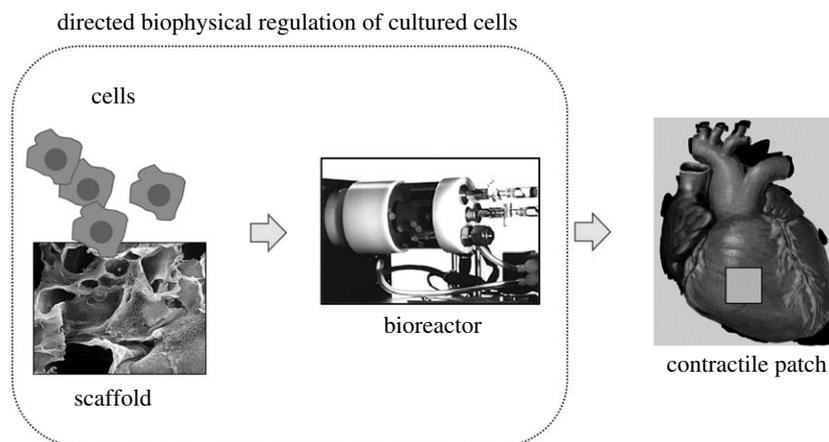


Figure 2. Tissue engineering paradigm. The regulatory factors of cell differentiation and tissue assembly shown in figure 1 can be used *in vitro* to engineer functional tissues by an integrated use of isolated cells, biomaterial scaffolds and bioreactors. The cells themselves (either differentiated or progenitor/stem cells seeded onto a scaffold and cultured in a bioreactor) carry out the process of tissue formation in response to regulatory signals. The scaffold provides a structural, mechanical and logistic template for cell attachment and tissue formation. The bioreactor provides the environmental conditions and regulatory signals (biochemical and physical) that induce, enhance or at least support the development of functional tissue constructs (adapted from Radisic *et al.* 2005b).

Table 1. Factors governing cardiac tissue development *in vivo* and *in vitro*.

	<i>in vivo</i>	<i>in vitro</i>
cells	high-density 5×10^8 cells cm^{-3} (Mandarim-de-Lacerda & Pereira, 2000) multiple cell types (myocytes, endothelial cells and fibroblasts)	high-density $(0.5-1) \times 10^8$ cells cm^{-3} multiple cell types
oxygen supply	blood flow	interstitial flow of culture medium
geometry	dense capillary network within the tissue (diameter $10 \mu\text{m}$, spacing $20 \mu\text{m}$)	scaffold with a parallel array of channels
oxygen carrier	haemoglobin	synthetic oxygen carrier perfluorocarbon (PFC) emulsion (Oxygent)
excitation-contraction coupling	electrical signal propagation ventricle contraction	electrical stimulation construct contraction

CMs and endothelial cells interact during embryonic development and in adult life. Myocardium secretes angiogenic factors that interact with endothelial cells. For

example, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) that are released by ischemic myocardium act on endothelial

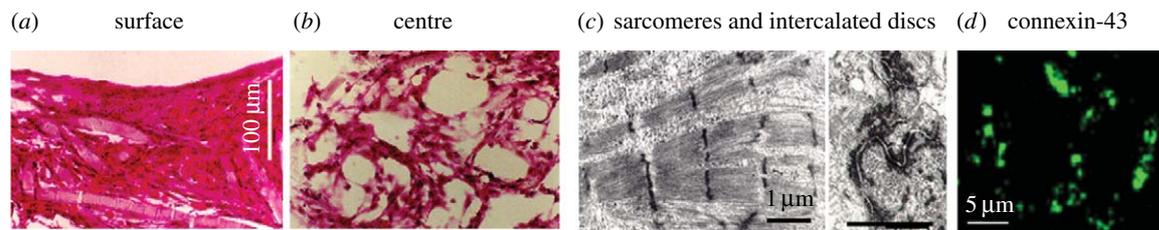


Figure 3. Cardiac constructs engineered in a conventional culture system. Constructs seeded and cultured with medium flow at construct surfaces formed (a) approximately 100 µm thick peripheral region (b) around an acellular interior. Cells in the peripheral region were electrically connected via gap junctions (d), immunostain for connexin-43 (c) exhibited cardiac-specific ultrastructural features, and propagated electrical signals over a distance of 5 mm (as recorded by a linear array of electrodes; Papadaki *et al.* 2001) (adapted from Carrier *et al.* 1999).

cells to promote local angiogenesis. Cardiac myocytes stimulate endothelial cells' production of platelet derived growth factor-beta (PDGF-β), which combines with PDGF-α to induce endothelial cells' expression of von Willebrand factor (Edelberg *et al.* 1998). Cardiac-induced PDGF stimulates endothelial cells' secretion of VEGF and the VEGF receptor Flk-1, which are critical components of angiogenesis. Conversely, endothelium secretes multiple factors that affect cardiac myocytes. Nitric oxide secreted by endothelial cells causes vasodilatation of coronary vessels, exerts direct effects on myocardium, and decreases isotonic twitch shortening isolated myocytes and enhances myocardial relaxation (Shah *et al.* 1996; MacCarthy *et al.* 2001). These effects combine to decrease myocardial oxygen requirements, principally via increases in cGMP in myocytes and inhibition of Ca²⁺ entry through L-type calcium channels (Parratt *et al.* 1997). Hypoxic endothelial cells secrete substances that reduce actin-activated cardiac myosin ATPase activity and inhibit myocardial cross-bridge cycling, thereby reducing oxygen usage (Shah *et al.* 1997).

(c) Oxygen supply

Owing to the high density and high metabolic activity of the cells, myocardium consumes large amounts of oxygen, and cannot tolerate hypoxia for prolonged periods of time (Vander *et al.* 1985; Schoen 1999). Therefore, nutrients and in particular oxygen are quickly depleted. In native myocardium, oxygen is supplied to the cells through a rich vasculature, with average capillary-to-capillary distances in rat heart of only 17–19 µm, a width of an individual CM (Steinhausen *et al.* 1978; Korecky *et al.* 1982; Rakusan & Korecky 1982; Zadeh *et al.* 1986). The solubility of oxygen in plasma is very low. However, haemoglobin increases the total oxygen content of blood by two orders of magnitude, and thereby increases the mass of tissue that can be supported in a single pass through capillary network. The average oxygen concentration in arterial blood is 130 µM and in the venous blood it is 54 µM (Fournier 1998).

(d) Excitation–contraction coupling

The CM is the most physically energetic cell in the body, contracting more than three billion times in an average human lifespan, and pumping over 7000 l of blood per day along 160 000 km of blood vessels. Morphologically, intact CMs have an elongated rod-shaped appearance. Their contractile apparatus consists of sarcomeres, high metabolic activity is supported by the

high density of mitochondria, and electrical signal propagation is provided by specialized intercellular connections, gap junctions (MacKenna *et al.* 1994; Brilla *et al.* 1995). The control of heart contractions is almost entirely self-contained, and can be attributed to the groups of specialized CMs (pacemakers), the fastest of which are located in the sinoatrial node. Contraction of the cardiac muscle is driven by the waves of electrical excitation generated by pacing cells that spread rapidly along the membranes of adjoining CMs and trigger release of calcium, which in turn stimulates contraction of the myofibrils. Electromechanical coupling of the myocytes is crucial for their synchronous response to electrical pacing signals (Severs 2000).

3. TISSUE ENGINEERING

(a) Model system

In a tissue engineering model system we previously established, differentiated heart cells are used in conjunction with a biodegradable scaffold (designed to serve as a structural and logistic template for tissue development) and a bioreactor (designed to enable environmental control and support cell differentiation and functional assembly into an engineered tissue; figure 2). Cells were isolated from 1- to 2-day-old neonatal rat heart ventricles, separated into non-myocyte and myocyte fractions, and inoculated into scaffolds using a gel (Matrigel) as a cell delivery vehicle (Radisic *et al.* 2003). Scaffolds need to be made of biocompatible materials (preferentially those already approved for clinical use) and degrade with time (such that the final tissue graft does not contain any foreign material). We used scaffolds made either of natural polymers (e.g. collagen in form of a sponge; Radisic *et al.* 2004b) or synthetic polymers (e.g. polyglycolic acid in form of a fibrous mesh; Carrier *et al.* 1999) or polyglycerol sebacate in form of a sponge (Radisic *et al.* 2004b). In all cases, scaffolds had high void volumes (greater than 90%) and contained large interconnected pores.

Scaffold structure determines the transport of nutrients, metabolites and regulatory molecules to and from the cells, whereas the scaffold chemistry has an important role in cell attachment and differentiation. Mechanical properties of the scaffold should ideally match those of the native tissue, to provide mechanical integrity of the forming tissue and support an *in vivo*-like mechanotransduction between the cells and their environment.

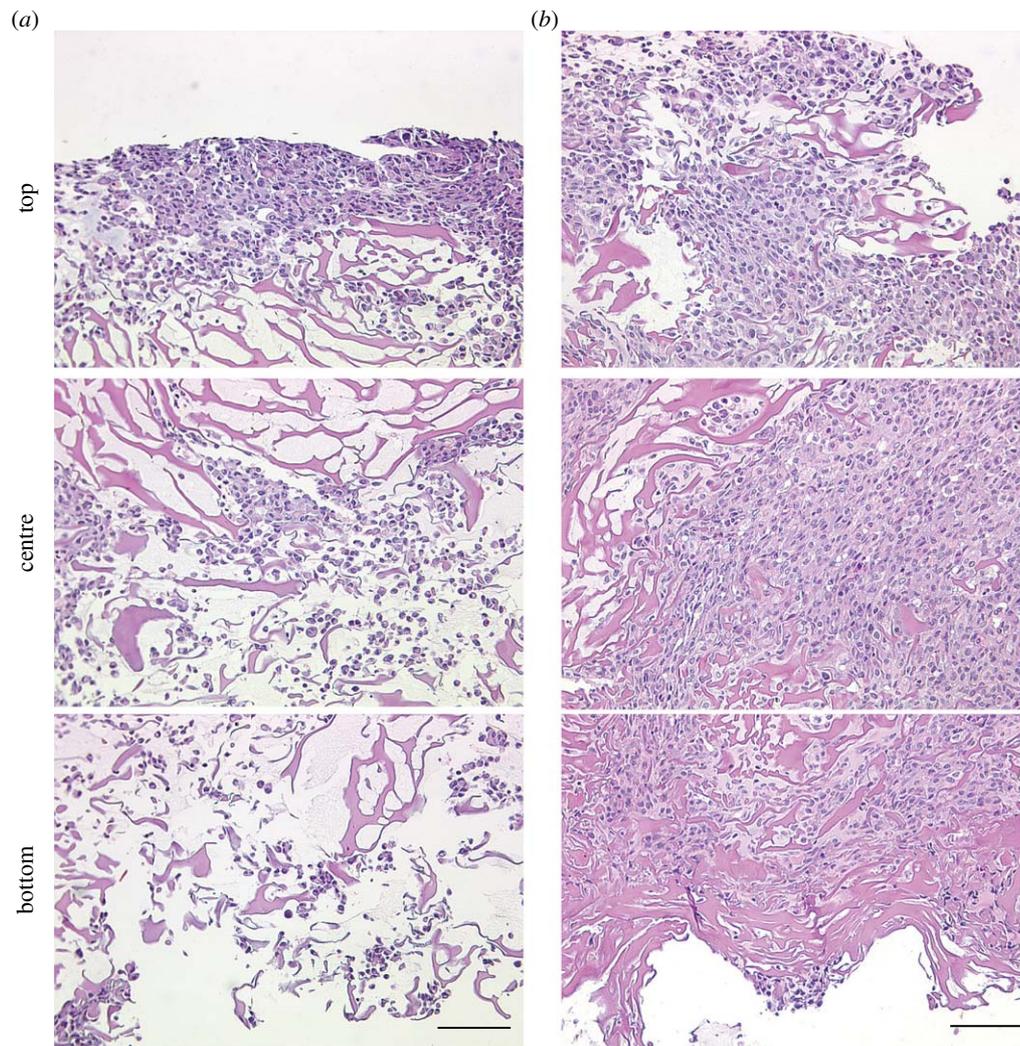


Figure 4. Effects of perfusion during seeding and cultivation on cardiac cell distribution. Cross-sections of constructs inoculated with 12 million cells and then transferred for a period of 4.5 h either into (a) dishes (25 r.p.m.) or into (b) perfused cartridges (1.5 ml min^{-1}). The top, centre and bottom areas of a $650 \mu\text{m}$ wide strip extending from one construct surface to the other are shown. Scale bar, $100 \mu\text{m}$ (adapted from Radisic *et al.* 2003).

(b) Diffusional limitations of conventional culture systems

The culture vessels most frequently used for cardiac tissue engineering include: Petri dishes (static or mixed), flasks (static or magnetically stirred), rotating vessels and perfused cartridges. Flasks contain constructs that are fixed in place and cultured either statically or with magnetic stirring, with gas exchange through loosened side-arm caps. Rotating vessels contain constructs that are freely suspended in culture medium between two concentric cylinders the inner of which serves as a gas exchange membrane, by adjusting the rate of vessel rotation (Carrier *et al.* 1999). Perfused cartridges are designed to provide interstitial fluid flow through the construct, with gas exchange in an external fluid loop (Carrier *et al.* 2002b).

Constructs cultured in bioreactors providing convective flow of culture medium at construct surfaces (Bursac *et al.* 1999; Carrier *et al.* 1999) contained only a $100 \mu\text{m}$ thick peripheral region (figure 3a) around a largely acellular interior (figure 3b), a result of diffusional constraints of oxygen supply (Carrier *et al.* 1999, 2002b).

In the outer tissue layer, cells were electrically connected via the gap junctional protein connexin-43 (Cx-43; figure 3d), exhibited cardiac-specific ultrastructural features (figure 3c), and propagated electrical signals over millimetre distances. Engineered constructs exhibited synchronous contractility, and could be captured and paced at a range of frequencies (20–300 b.p.m.; Bursac *et al.* 1999; Papadaki *et al.* 2001).

In an attempt to enhance mass transport between culture medium and cells within cultured constructs, we later developed a bioreactor with interstitial medium flow (Carrier *et al.* 2002b). Cells were seeded onto scaffolds in tissue culture dishes and subsequently cultured with medium perfusion (interstitial flow through the construct). Hence, the transport of oxygen from the medium to the cells occurred via diffusion during cell seeding, and by a combination of diffusion and convection during cultivation. During cultivation, the flow of medium redistributed the cells evenly across the entire volume of the construct, but the cell density remained low due to the limitations in oxygen transport during cell seeding.

(c) *Perfusion during seeding enabled physiological cell density*

To provide oxygen supply to the cells at levels necessary to maintain their viability, we developed a technique of seeding that involves (i) rapid cell inoculation into collagen sponges using Matrigel as a cell delivery vehicle and (ii) transfer of inoculated scaffolds into perfused cartridges with immediate establishment of the interstitial flow of culture medium. Forward–reverse flow was used for the initial period of 1.5–4.5 h in order to further increase the rate and spatial uniformity of cell seeding (Radisic *et al.* 2003). Unidirectional flow of culture medium was maintained for the duration of cultivation. In this system, cells were ‘locked’ into the scaffold during a short (10 min) gelation period, and supplied with oxygen at all times during culture.

Cell distributions in the top, centre and bottom areas of a 650 μm wide strip extending from one construct surface to the other are shown in figure 4. Constructs seeded in dishes had most cells located within a 100 μm thick layer at the top surface, and only a small number of cells penetrated the entire construct depth. Constructs seeded in perfusion had high and spatially uniform cell density throughout the perfused volume of the construct. Clearly, medium perfusion during seeding was a key for engineering thick constructs with high densities of viable cells, due to enhanced transport of oxygen within the construct.

(d) *Perfusion during cultivation enabled cell viability and aerobic metabolism*

Throughout the cultivation, the number of live cells in perfused constructs was significantly higher than in dish-grown constructs. Importantly, the final cell viability in perfused constructs cultured for 8 days ($81.6 \pm 3.7\%$) was indistinguishable from the viability of the freshly isolated cells ($83.8 \pm 2.0\%$) and it was markedly higher than the cell viability in dish-grown constructs ($47.4 \pm 7.8\%$; Radisic *et al.* 2004b). Consistently, the molar ratio of lactate produced to glucose consumed (L/G) was approximately 1 for perfused constructs, indicating aerobic cell metabolism. In contrast, in orbitally mixed dishes, L/G increased progressively from 1 to approximately 2, indicating a transition to anaerobic cell metabolism. Cell damage, assessed by monitoring the activity of lactate dehydrogenase in culture medium, was at all time points significantly lower in perfusion than in dish cultures.

Perfused constructs and native ventricles had more cells in the S-phase than in the G2/M-phases, whereas the cells from dish-grown constructs appeared unable to complete the cell cycle and accumulated in the G2/M-phases. Cells expressing cardiac-specific differentiation markers (sarcomeric α -actin, sarcomeric tropomyosin and cardiac troponin I) were present throughout the perfused constructs, and only within an approximately 100 μm thick surface layer in dish-grown constructs.

Spontaneous contractions were observed in some constructs early in culture and ceased after approximately 5 days of cultivation, indicating the maturation of engineered tissue. In response to electrical stimulation, perfused constructs contracted synchronously, with lower excitation thresholds (ETs) and recovered their

baseline function levels following treatment with a gap junction blocker; dish-grown constructs exhibited arrhythmic contractile patterns and failed to recover their baseline levels (Radisic *et al.* 2004b). However, most cells were round and mononucleated, a situation that was probably due to the exposure of CMs to hydrodynamic shear, in contrast to the native heart muscle where blood is confined within the capillary bed and therefore not in direct contact with CMs. This motivated the design of scaffolds with arrays of channels that provide a separate compartment for medium flow.

(e) *In vivo-like oxygen supply: medium perfusion, channelled scaffolds and oxygen carriers*

To mimic the capillary network, neonatal rat heart cells were cultured on an elastomer scaffold with an array of parallel channels and perfused with culture medium (figure 5a). Constructs were subjected to unidirectional medium flow at a flow rate of 0.1 ml min⁻¹. To mimic oxygen supply by haemoglobin, culture medium was supplemented with 5.4% (v/v) PFC emulsion (Oxygent, Alliance Pharmaceuticals, San Diego, CA); constructs perfused with unsupplemented culture medium served as controls (figure 5b).

The elastomer, poly(glycerol sebacate) (PGS) was obtained by condensation of glycerol and sebacic acid, and formed by salt leaching into a three-dimensional network of large interconnected approximately 100 μm pores, high void volume (greater than 95%) and thicknesses of 1–5 mm (Wang *et al.* 2002). The cross-links and hydrogen bonds contribute to its unique elastic properties resembling vulcanized rubber and being capable of up to 400% elongation before it yields. PGS degrades by hydrolysis of its ester bond into glycerol (probably adsorbed in the body) and sebacic acid (secreted by urine either directly or metabolized into carboxylic acids). *In vivo* (subcutaneous implantation), PGS scaffold was biocompatible and lost its mass linearly (to approx. 20% of initial over 5 weeks), such that its shape and structural integrity were well maintained.

An array of parallel channels with diameter of approximately 350 μm and wall-to-wall spacing of approximately 220 μm was made using a laser beam (figure 5c). The scaffold was first pretreated with neonatal rat cardiofibroblasts for 3 days and then with rat cardiomyocytes, and cultured with perfusion of culture medium (Radisic *et al.* 2005a). After only 3 days *in vitro*, the subpopulations of cells on scaffolds formed constructs that contracted synchronously in response to electrical stimulation. The scaffold pores remained open (figure 5d,e) and the pressure drop measured across the construct was as low as 0.1 kPa mm⁻¹ construct thickness.

As the medium flowed through the channel array, oxygen was depleted from the aqueous phase of the culture medium by diffusion into the construct space where it was used for cell respiration (figure 5b). Depletion of oxygen in the aqueous phase acted as a driving force for the diffusion of dissolved oxygen from the PFC particles, thereby contributing to the maintenance of higher oxygen concentrations in the medium. Owing to the small size of PFC particles, the passive diffusion of dissolved oxygen from the PFC

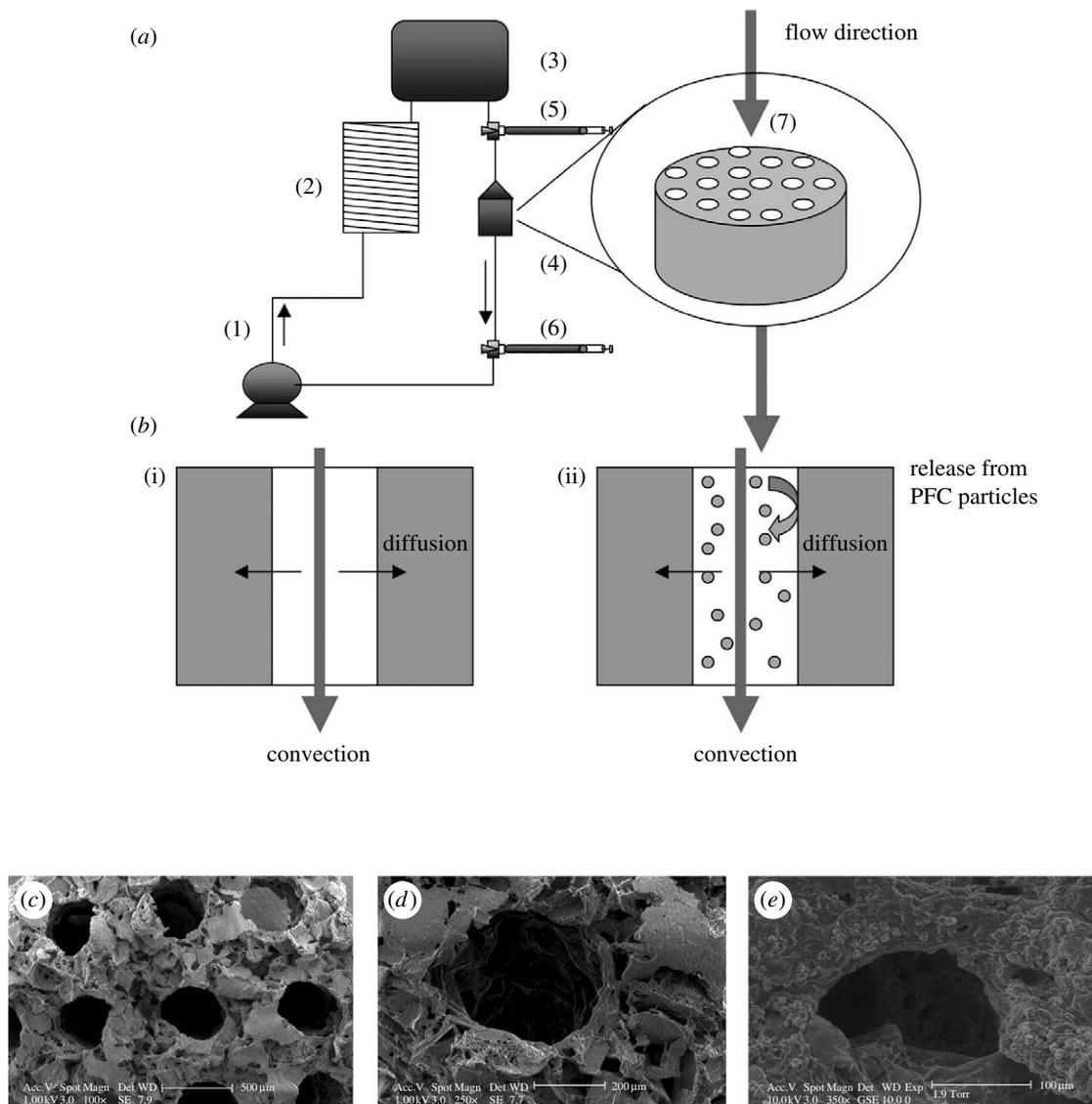


Figure 5. Oxygen supply: medium perfusion, channelled scaffolds and oxygen carriers. (a) Perfusion loop. Channelled biorubber scaffolds (7) preconditioned with cardiac fibroblasts were seeded with CMs and placed into perfusion cartridges (4) between two debubbling syringes (5,6). Medium flow (0.1 ml min^{-1}) was provided by a multi-channel peristaltic pump (1) and gas exchange was provided by a coil of thin silicone tubing (2). Loops were placed in the $37^\circ\text{C}/5\% \text{ CO}_2$ incubator vertically. (b) Modes of oxygen transport in the channelled construct perfused with culture medium include convection through the channel lumen and diffusion into the tissue space surrounding each channel. In regular culture medium ((i) control group), oxygen dissolved in the aqueous phase during gas exchange in the external loop is transported into the tissue phase and consumed by the cells. In culture medium supplemented with 10% PFC emulsion ((ii) PFC group), oxygen is replenished within the tissue construct by the release of oxygen from the PFC particles into the culture medium phase. Scanning electron micrographs of biorubber scaffold with (c) a parallel channel array and (d) of a single channel are shown at the beginning and (e) after 3 days of cultivation (adapted from Radisic *et al.* (2005a)).

phase into the aqueous phase was very fast, and estimated not to be a rate-limiting step in this system. For comparison, in non-supplemented culture medium, oxygen was depleted faster since there was no oxygen carrier phase that acted as a reservoir (Radisic *et al.* 2004a,b). In PFC-supplemented medium, the decrease in the partial pressure of oxygen in the aqueous phase was only 50% of that in control medium (28 versus 45 mmHg between the construct inlet and outlet at a flow rate of 0.1 ml min^{-1}). Consistently, constructs cultivated in the presence of PFC had higher amounts of DNA, troponin I and Cx-43, and significantly better contractile properties as compared with control constructs. In both groups, cells were present at the channel surfaces as well as within

constructs. Improved construct properties were correlated with the enhanced supply of oxygen to the cells within constructs.

(f) **Mathematical model of oxygen transport**

In order to rationalize experimental data for oxygen transport and consumption in engineered cardiac constructs with an array of channels, we developed a mathematical model of oxygen distribution in cardiac constructs similar to the Krogh cylinder model. Concentration profiles of oxygen and cells within the constructs were obtained by numerical simulation of the diffusive–convective oxygen transport and its usage by the cells (Radisic *et al.* 2004a,b). The model was used to define scaffold geometry and flow conditions

necessary to cultivate cardiac constructs with clinically relevant thicknesses (5 mm).

Oxygen profiles were modelled in a channel array consisting of channels 100 μm in diameter and 100 μm wall-to-wall spacing at a physiologically high cell density of 1×10^8 cells cm^{-3} . At a flow rate of 0.05 cm s^{-1} , oxygen concentration increased significantly in both tissue space and channel lumen with the increase in circulating PFC emulsion from 0 to 6%. Although the oxygen concentration in the tissue space increased considerably with the increase of circulating PFC concentration, we had to increase the flow rate, keeping the shear stress in the physiological range approximately 1 dyn cm^{-2} , in order to provide enough oxygen for the entire 0.5 cm thick construct. At the best conditions (0.135 cm s^{-1} and 6% PFC), oxygen is not depleted at any point in the scaffold and the minimum concentration of $33 \mu\text{M}$ is approximately five times above the K_m .

(g) Excitation–contraction coupling: electrical stimulation

In native heart, mechanical stretch is induced by electrical signals, and the orderly coupling between electrical pacing signals and macroscopic contractions is crucial for the development and function of native myocardium (Severs 2000). We hypothesized that applying electrical signals designed to induce synchronous construct contractions would enhance cell differentiation and functional assembly of engineered tissue via physiologically relevant mechanisms. To test this hypothesis, cardiac constructs prepared by seeding collagen sponges ($6 \times 8 \times 1.5 \text{ mm}$) with neonatal rat ventricular cells (5×10^6) were stimulated using supra-threshold square biphasic pulses (2 ms duration, 1 Hz, 5 V/cm). The stimulation was initiated after 1–5 days of scaffold seeding (3-day period was optimal) and applied for up to 8 days. Over only 8 days *in vitro*, electrical field stimulation induced cell alignment and coupling, increased the amplitude of synchronous construct contractions by a factor of 7 and resulted in an *in vivo*-like ultrastructural organization. Development of conductive and contractile properties of cardiac constructs was concurrent, with strong dependence on the initiation and duration of electrical stimulation.

The application of electrical stimulation during construct cultivation markedly enhanced the contractile behaviour. After 8 days of culture, the amplitude of contractions was sevenfold higher in stimulated than in non-stimulated constructs (figure 6a), a result of the progressive increase with the duration of culture. The excitation threshold (ET; the minimum voltage at which the entire construct was observed to beat) decreased (figure 6b) and the maximum capture rate (MCR; the maximum pacing frequency for synchronous construct contractions) increased (figure 6c) both with time and due to electrical stimulation, suggesting functional coupling of the cells. The shape, amplitude (approx. 100 mV) and duration (approx. 200 ms) of the electrical activity recorded for cells in constructs stimulated during culture were similar to action potentials reported for constructs that were mechanically stimulated during culture (Zimmermann *et al.* 2002a,b). Notably, improved contractile properties of electrically stimulated constructs were not reflected in

differences in cell density, cell damage or cell metabolism, but correlated instead with cell differentiation.

After 8 days, stimulated constructs demonstrated a remarkable level of ultrastructural differentiation, comparable in several respects with that of native myocardium. Myofibres aligned in the direction of the electrical field lines, possibly in an attempt to decrease the apparent ET in response to pacing (Tung *et al.* 1991). In contrast, cells in non-stimulated constructs stayed round and expressed relatively low levels of cardiac markers. After 8 days, stimulated constructs exhibited a markedly higher density of Cx-43 than either early (3 days) or non-stimulated constructs. Stimulated constructs and neonatal ventricles contained abundant mitochondria positioned between myofibrils, in contrast to non-stimulated constructs containing mitochondria scattered around the cytoplasm, and substantially larger amounts of glycogen.

Electrical stimulation induced the development of long well-aligned registers of sarcomeres that closely resembled those in native myocardium (figure 6d,e, representing a hallmark of maturing cardiomyocytes; Severs 2000). The volume fraction of sarcomeres in stimulated 8-day constructs was indistinguishable from that measured for neonatal ventricles (Olivetti *et al.* 1980); in contrast, non-stimulated constructs contained only scattered and poorly organized sarcomeres. In stimulated constructs, IC discs were positioned between aligned Z lines and were as frequent as in neonatal ventricles; gap junctions were also better developed and more frequent.

On a molecular level, electrical stimulation elevated the levels of all measured cardiac proteins and enhanced the expression of the corresponding genes, without causing pathological cell hypertrophy (Di Nardo *et al.* 1993). With time in culture, the ratio of mature and immature forms of myosin heavy chain (α -MHC and β -MHC, respectively) decreased in non-stimulated and increased in stimulated constructs, suggesting that the maturation of cardiomyocytes depended both on culture duration and electrical stimulation.

These studies suggest that electrical stimulation of construct contractions during cultivation progressively enhanced the excitation–contraction coupling and improved the molecular, cellular and functional properties of engineered myocardium.

(h) Summary and ongoing developments

The overall goal of cardiac tissue engineering is to direct the cells to establish the physiological structure and function of the tissue being replaced across different hierarchical scales. To engineer myocardium, biophysical regulation of the cells needs to recapitulate multiple signals present in the native heart. The biomimetic approach to tissue engineering reviewed here focused on two specific aspects that are critical for the development and function of native myocardium: convective–diffusive oxygen transport and excitation–contraction coupling. To mimic the capillary network, culture medium was perfused through a channelled elastomer scaffold containing subpopulations of neonatal rat heart cells seeded at a physiological density. In addition, to mimic oxygen supply by haemoglobin, the culture medium was supplemented with a synthetic

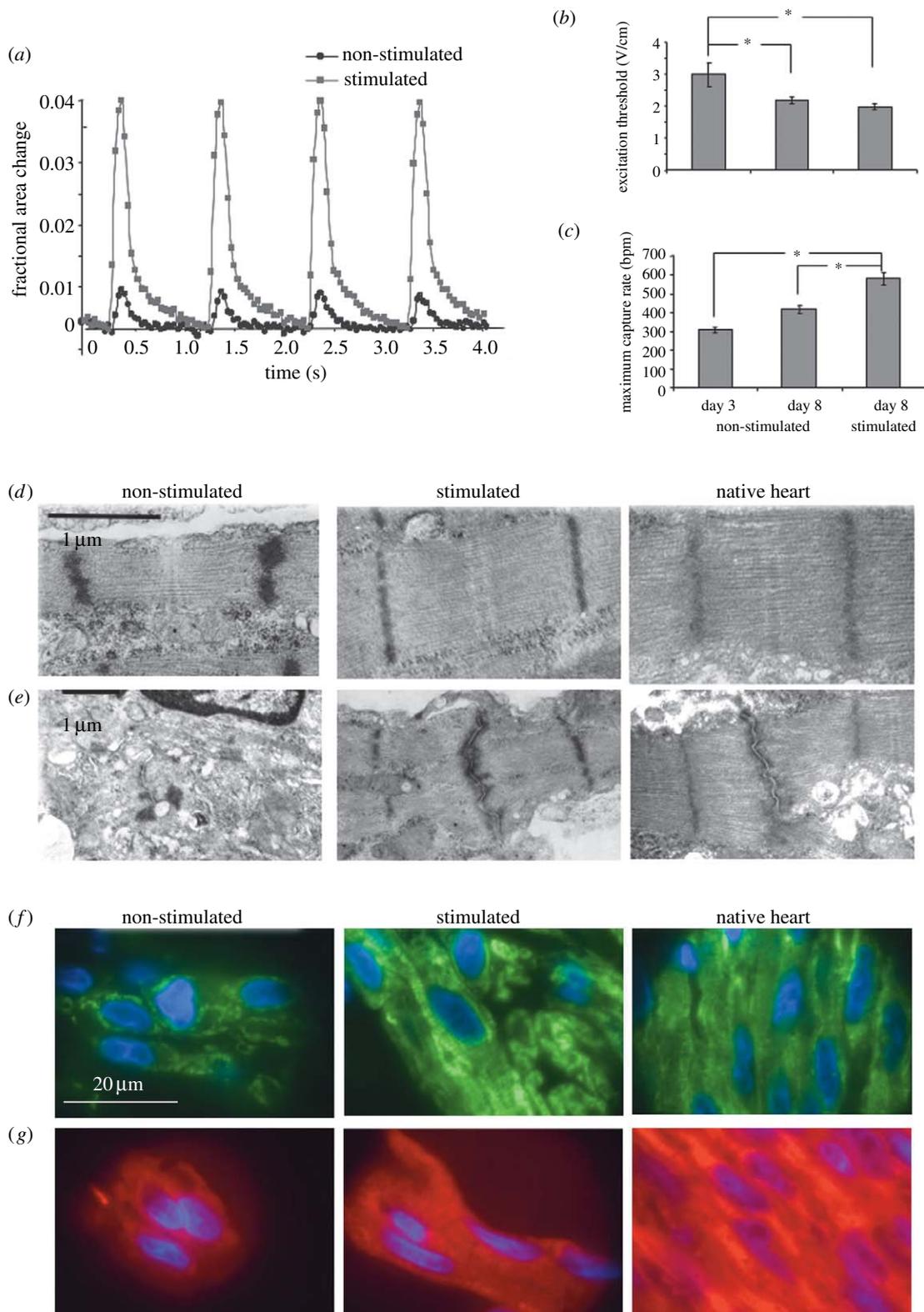


Figure 6. Effects of electrical stimulation on functional assembly of engineered cardiac constructs. (a) Contraction amplitude of constructs cultured for a total of 8 days, shown as a fractional change in the construct size. Electrical stimulation increased the amplitude of contractions by a factor of 7. (b) ET decreased and (c) MCR increased significantly both with time in culture and due to electrical stimulation. (*) denotes statistically significant differences ($p < 0.05$; Tukey's *post hoc* test with one-way ANOVA, $n = 5-10$ samples per group and time point). (d) The structure of sarcomeres and (e) gap junctions observed in micrographs of stimulated constructs after 8 days of cultivation were remarkably similar to neonatal rat ventricles and markedly better developed than in control (non-stimulated) constructs. Representative sections of constructs stained for (f) Cx-43 (green) and (g) β -MHC (red), cell nuclei are shown in blue (adapted from Radisic *et al.* (2004a)).

oxygen carrier (PFC emulsion). To promote functional assembly and electromechanical cell coupling, synchronous contractions of cultured constructs were induced

by applying electrical signals designed to mimic those in native heart. Each of these two aspects substantially enhanced the structural and functional properties of

engineered myocardium based on neonatal rat cells, over only 8 days of *in vitro* cultivation.

One major challenge is to extend the application of the same methods and principles to the cultivation of functional cardiac grafts based on human cells. Various cell sources are currently under consideration, including primitive cells from the adult heart that are capable of dividing and developing into mature heart and vascular cells (Beltrami *et al.* 2003; Oh *et al.* 2003; Messina *et al.* 2004). A recent study revealed relatively unspecialized cells that can both divide while maintaining their own population and give rise to functional heart cells (Laugwitz *et al.* 2005). These cells were demonstrated to populate the embryonic and postnatal heart and were successfully cultured *in vitro*, thus representing a potential source of cells for cardiac tissue engineering. It remains to be determined if the progenitors, regardless of their marker, can be isolated from adult human biopsies and if sufficient numbers of cardiomyocytes can be generated *in vitro*. Human embryonic stem cells represent another potential source of cardiac cells (Thomson *et al.* 1998). Notably, it was demonstrated that this differentiating system is not limited to the generation of isolated cardiac cells, but can result in a functional cardiac syncytium with a stable pacemaker activity and electrical propagation (Kehat *et al.* 2004).

Cardiac myocytes can be obtained at potentially unlimited quantities and high purity from embryonic stem cells (Klug *et al.* 1996). However, nuclear transfer is required to make them autologous and the presence of undifferentiated cells may lead to teratomas upon implantation (Laflamme & Murry 2005). Recent studies report cultivation and implantation of cardiac grafts based on mouse embryonic stem cells (Guo *et al.* 2006; Caspi *et al.* 2007).

Another challenge is to further advance the capabilities of our *in vitro* culture systems. Cultivation with direct perfusion of culture medium containing PFC enabled the maintenance of physiological density of viable differentiated cells in millimetres thick constructs. Electrical field stimulation induced cell alignment and coupling, increased the amplitude of synchronous contractions and enhanced the ultrastructural organization of engineered myocardium. However, our most advanced existing bioreactors can provide *either* local micro-environmental control of oxygen and pH (via medium perfusion), *or* the application of physical stimuli (via electrical stimulation). A system providing *both factors simultaneously*, would have a major impact on advancing our methods of cardiac tissue engineering, by subjecting the cultured cells to *multiple signals* present in the native heart. The combined application of the *in vivo*-like oxygen supply and electrical stimulation is expected to further enhance and/or accelerate cell differentiation and functional assembly. An additional advantage of this system is that it could serve as a high-fidelity model for rigorous studies of cardiac development and function. One of the key requirements for the success of the implantation strategies is electrical coupling between the graft and host myocardium (Eschenhagen *et al.* 2006). Recent studies with engineered heart tissue (Zimmermann *et al.* 2006) and cell sheets (Furuta *et al.* 2006) demonstrated such coupling, thus indicating

that functional integration between the graft and host tissue, without arrhythmias, is possible. Although these studies demonstrated feasibility of implantation of contractile cardiac patches, more studies are necessary to correlate main *in vitro* parameters to the *in vivo* outcome. Questions yet to be answered through *in vivo* studies include: how long should the constructs be cultivated before implantation, how differentiated should the cardiomyocytes be, and what is the optimal composition of the biomaterial scaffold?

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