DOI: 10.1002/jctb.1467

Review

Cardiac tissue engineering: effects of bioreactor flow environment on tissue constructs

Gordana Vunjak-Novakovic,[1]* Milica Radisic[2] and Bojana Obradovic[3]

[1]*Columbia University, New York, NY, USA*
[2]*University of Toronto, Toronto, Ontario, Canada*
[3]*Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia and Montenegro*

Abstract: The limited ability of cardiac muscle to regenerate after injury and the small number of organs available for transplantation motivate studies aimed at curative treatment options. Tissue engineering based on the integrated use of cells on biomaterial scaffolds in bioreactors may offer cardiac grafts suitable for surgical attachment to the myocardium or for basic research. In one of the current approaches, neonatal rat cardiomyocytes are combined with collagen sponges, gels or polyglycolic acid scaffolds (PGA). Cultivations performed in dishes, static or mixed flasks or rotating bioreactors yield constructs with a thin (100–200 μm) peripheral layer of tissue expressing markers of cardiac differentiation and able to propagate electrical signals. The non-uniform cell distribution is a result of oxygen diffusional limitations within the constructs. Cultivations with perfusion of culture medium through the construct enhance the convective-diffusive oxygen supply and yield 1–2 mm thick constructs with physiologically high and spatially uniform distribution of viable cells expressing cardiac markers. We review here a series of studies we conducted using cells seeded on three-dimensional scaffolds and cultured in several different bioreactors, to demonstrate that the bioreactor flow environment can have substantial effects on structural and functional properties of cardiac constructs.

Keywords: cardiac tissue; tissue constructs; biomaterial scaffold; bioreactor

INTRODUCTION

Cardiovascular disease is responsible for a preponderance of health problems in the developed countries, as well as in many developing countries. 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,[1] are the leading cause of death among newborns. Cardiovascular diseases result in substantial disability and loss of productivity, and largely contribute to the escalating costs of health care. About 61 million Americans (almost one-fourth of the population) live with cardiovascular diseases, such as coronary heart disease, congenital cardiovascular defects, and congestive heart failure, and 298.2 billion dollars were spent in 2001 to treat these diseases.[2] The economic impact of cardiovascular disease on the US health care system is expected to grow further as the population ages.

Once damaged, the heart is unable to regenerate. Currently, the only definitive treatment for end-stage heart failure is cardiac transplantation. However, the limited availability of organs for transplantation has led to the prolonged waiting periods that are often not survivable.[3] Newly investigated approaches to treat impaired myocardium include injection of myogenic cells[4] and tissue engineering of grafts suitable for surgical implantation.[5]

Tissue engineering combines the principles of biology, engineering and medicine to create functional, biologically based grafts capable of repairing native tissues following a congenital deformity, disease or trauma. *In vivo*, cell differentiation and tissue assembly are directed by multiple factors acting in concert and according to specific spatial and temporal sequences. *In vitro,* cell function can be modulated by the same factors known to play a role during normal embryogenesis. In light of this paradigm, biophysical regulation of cultured cells can be achieved by an integrated use of biomaterial scaffolds and bioreactors,

* Correspondence to: Gordana Vunjak-Novakovic, Columbia University, New York, NY, USA
E-mail: gv2131@columbia.edu
(*Received 19 October 2004; revised version received 26 October 2005; accepted 3 November 2005*)

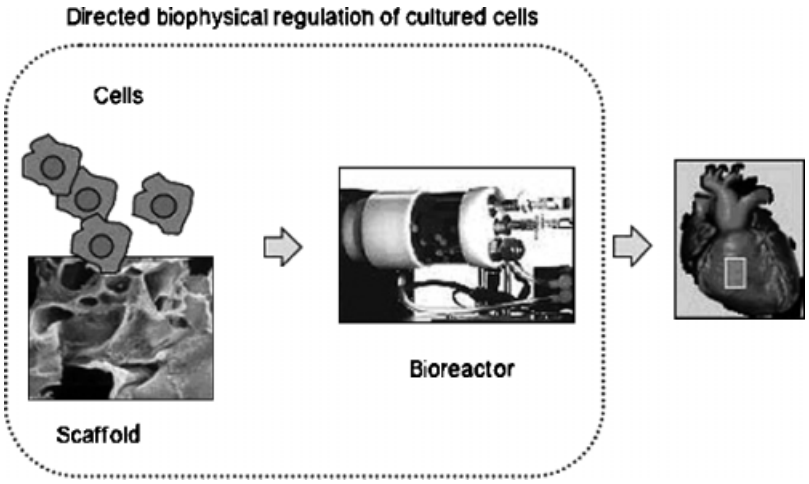

Figure 1. Tissue engineering paradigm. The regulatory factors of cell differentiation and tissue assembly *in vivo* can be utilized *in vitro* to engineer functional tissues by an integrated use of isolated cells, biomaterial scaffolds and bioreactors. The cells themselves (either differentiated or progenitor/stem 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*. Functional tissue engineering of cartilage and myocardium: bioreactor aspects. In *Scaffolding in Tissue Engineering*, Marcel Dekker, New York, pp.491–520 (2005).

as shown in Fig. 1. A 'biomimetic' approach to tissue engineering involves the *in vitro* creation of functional tissues by an integrated use of: (i) cells that can be selected, expanded, and transfected to express the genes of interest, (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 conditions necessary for tissue development.

Tissue engineering of 1–5 mm thick, functional cardiac constructs critically depends on our ability to seed the cells at a high and spatially uniform initial density, and to enhance the supply of oxygen to maintain their viability and function. Native cardiac tissue is vascularized, with capillaries spaced at 20 μm distances, an indicator of the high oxygen demand.[6,7] Engineered tissues that would resemble some properties of native myocardium could also serve as models for basic studies of cell function, tissue development, and responses to gene alterations, drugs, hypoxia, and physical stimuli.

ENGINEERED TISSUE COMPONENTS

Cells

Three-dimensional cardiac tissue constructs were successfully cultivated in dishes using a variety of scaffolds and cell sources. Fetal rat ventricular cardiac myocytes were expanded after isolation, inoculated into collagen sponges and cultivated in static dishes for up to 4 weeks.[8] The cells proliferated with time in culture and expressed multiple sarcomeres. Adult human ventricular cells were used in a similar system, although they exhibited no proliferation.[9] Fetal cardiac cells were also cultivated on alginate scaffolds in static 96-well plates. After 4 days in culture the cells formed spontaneously beating aggregates in the scaffold pores.[10] Cell seeding densities of the order of 10^8 cells cm^{-3} were achieved in the alginate scaffolds using centrifugal forces during seeding.[11] Neonatal rat cardiac myocytes formed spontaneously contracting constructs when inoculated in collagen sponges within 36 h[12] and maintained their activity for up to 12 weeks. The contractile force increased upon addition of Ca^{2+} and epinephrine.

Two-week constructs based on neonatal rat cardiomyocytes exhibited spontaneously beating areas, whereas constructs based on embryonic chick myocytes exhibited no contractions and reduced in size by 60%. Immunohistochemistry revealed the presence of a large number of nonmyocytes in constructs based on embryonic chick heart cells, while constructs based on neonatal rat cells consisted mostly of elongated cardiomyocytes.[13] Constructs based on cardiomyocytes enriched by preplating exhibited lower excitation threshold (ET), higher conduction velocity, higher maximum capture rate (MCR), and higher maximum and average amplitude.[14]

Scaffolds

The scaffolds utilized for cardiac tissue engineering include collagen fibers,[15] collagen sponges[8,9,12] and polyglycolic acid meshes.[13,14,16] The main advantage of a synthetic scaffold such as PGA is that it provides mechanical stability, while scaffolds based on natural cell polymers such as collagen enable rapid cell attachment. The scaffold-free approaches include casting the cells in collagen gels followed by mechanical stimulation[17–19] and stacking of confluent cardiac cell monolayers.[20] The main advantage of scaffold-free approaches is the higher active force generated by such tissues. However, the main disadvantage remains tailoring the shape and dimensions of the scaffold-free engineered tissues.

Most recently, gels (Matrigel) were combined with scaffolds (collagen sponge) to achieve rapid cell inoculation and attachment along with the possibility of tailoring tissue shape and dimensions through the use of scaffolds.[21]

Bioreactors

Bioreactor cultivation of cardiac tissue constructs has been done routinely in static flasks, mixed flasks, rotating vessels and perfused cartridges[13,14,16] (Fig. 2). The hydrodynamic environment of a bioreactor is important as it can affect cell function in at least two ways: via associated effects on mass transport between the tissue and culture medium, and by direct physical stimulation of the cells.

In static flasks (Fig. 2(A)), the cultured tissues are fixed in place and exposed to static medium, such that the mass transport between the tissue and culture medium is governed by molecular diffusion, and there is no hydrodynamic shear acting at the cells.

In mixed flasks (Fig. 2(A)) tissues are fixed in place and exposed to well mixed medium. The flow conditions in spinner flasks are turbulent, with the associated hydrodynamic shear that was below the level causing cell death or damage, but sufficient to affect the function of cells at construct surfaces.[13] Mass transport between the tissue and culture medium is enhanced by convection, whereas the transport within the tissue remains governed by molecular diffusion, as in static flasks.

In rotating vessels, cultured tissues are dynamically suspended in the rotating flow without external fixation. The flow conditions were characterized as dynamic and laminar, with tissue constructs settling in a tumble-slide regime associated with fluctuations in fluid pressure, velocity and shear.[13] Mass transport between the tissue and culture medium is enhanced by dynamic laminar convection, a flow regime that is stimulatory to the cells, whereas the transport within the tissue remains governed by molecular diffusion. Two types of rotating bioreactors: a high aspect ratio vessel (HARV) and a slow turning lateral vessel (STLV, shown in Fig. 2B) were utilized for cardiac tissue engineering.[13,16] The main difference is that the HARV has a larger gas exchange membrane and therefore better oxygen supply to the constructs.[13,16,22]

In perfused cartridges, constructs are cultivated with direct perfusion of culture medium through the cultured tissue, in order to achieve convective-diffusive oxygen transport throughout the construct volume. Constructs were perfused at interstitial velocities comparable to those of blood flow in native tissues.

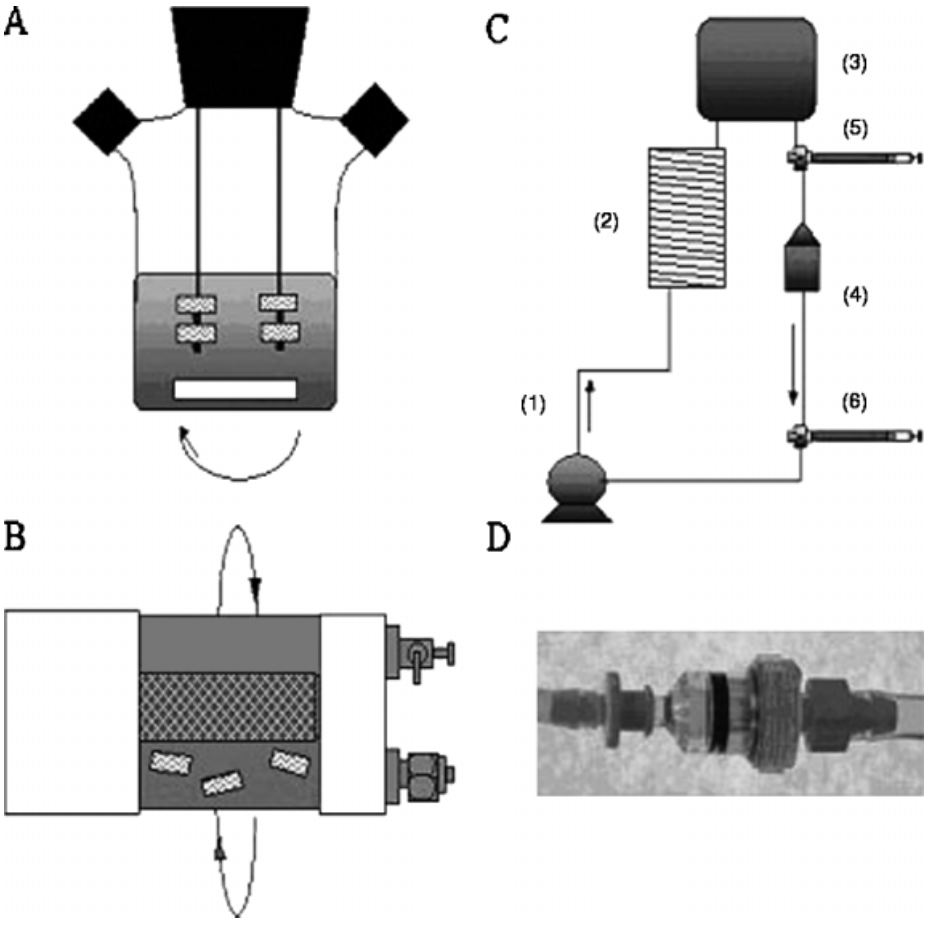

Figure 2. Schematics of the representative bioreactors for cardiac tissue engineering A: Static or mixed flasks with constructs suspended on needles. B: Rotating bioreactor with the tissue constructs suspended in the rotation flow. C: Perfusion loop consisting of a (1) peristaltic pump (2) gas exchanger (3) medium reservoir (4) perfusion cartridge (5,6) de-bubbling syringes. D: Perfusion cartridge (1.5 mL volume, 10 mm in diameter) with a tissue construct placed between two stainless steel screens and two silicone gaskets, with interstitial flow of culture medium .

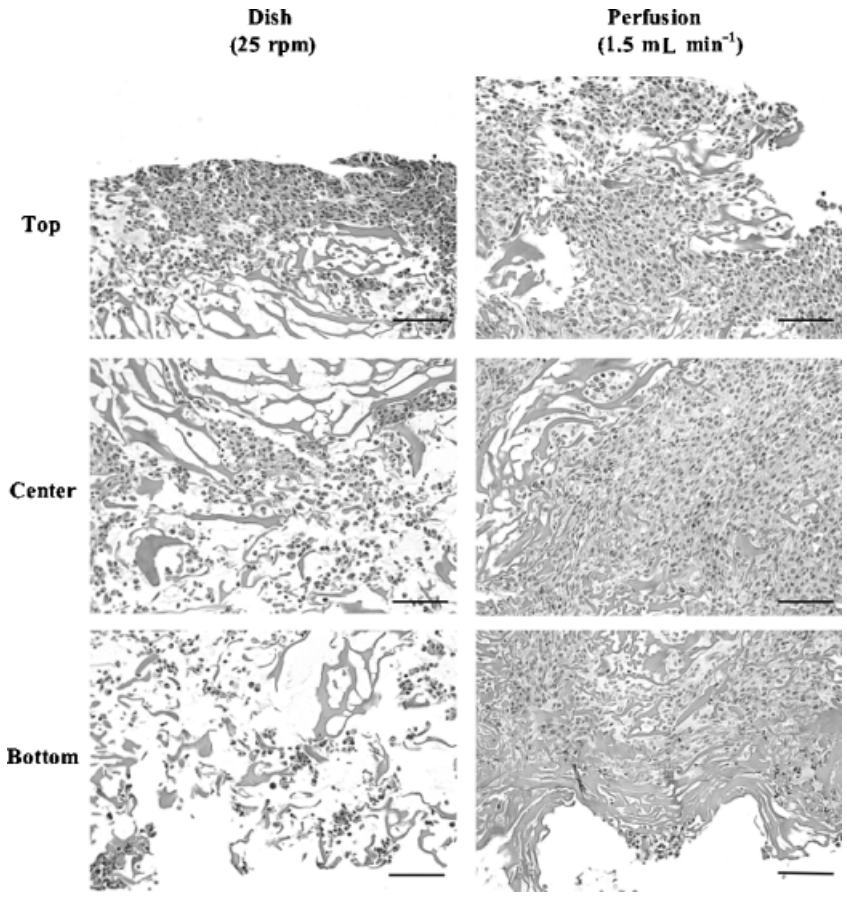

Figure 3. Effects of perfusion on cardiac cell distribution. Cross-sections of constructs inoculated with 12 million cells and transferred either into dishes (25 rpm, left) or into perfused cartridges (1.5 mL min^{-1}, right). The top, center and bottom areas of a 650 μm wide strip extending from one construct surface to the other are shown. Scale bar: 100 μm. Reproduced from Radisic *et al.*[25], with permission.

Medium flow is generally laminar, and can be either steady or pulsatile. Mass transport is enhanced by interstitial flow of medium in conjunction with external gas and medium exchange.[23–25]

EFFECTS OF BIOREACTOR CONDITIONS ON ENGINEERED TISSUE PROPERTIES

Mixed flasks

Mixing had significant effects on the metabolism and cellularity of neonatal rat cardiomyocyte/PGA constructs.[13] Constructs cultivated in mixed flasks (90 or 50 rpm) had significantly higher cellularity index (~20 μg DNA per construct) and metabolic activity using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay (~150 MTT units mg^{-1} DNA) compared with the constructs cultivated in static flasks (~5 μg DNA per construct and ~50 MTT units mg^{-1} DNA, respectively). The aerobicity of the cell culture was evaluated by the molar ratio of lactate produced and glucose consumed (L/G; mol mol^{-1}), a parameter that has the value of approximately 1 for completely aerobic cell metabolism and the value of approximately 2 for completely anaerobic metabolism. Mixing maintained the medium's gas and pH levels within the physiological range, yielding a more aerobic glucose metabolism (L/G ~ 1.5) in mixed flasks than in static flasks (L/G > 2).[13]

After 1 week of culture, constructs seeded in mixed flasks with $8 \cdot 10^6$ neonatal rat cardiac myocytes contained a peripheral tissue-like region (50–70 μm thick) in which cells stained positive for tropomyosin and organized in multiple layers in a 3-D configuration.[13] Electrophysiological studies conducted using a linear array of extracellular electrodes showed that the peripheral layer of the constructs exhibited relatively homogeneous electrical properties and sustained macroscopically continuous impulse propagation on a centimeter-size scale.[14] However, the interior of the construct remained largely acellular and a fibrous capsule on the outer surface formed as a result of turbulent flow conditions.

Rotating vessels

Cultivation of cardiac myocytes in HARVs on laminin-coated PGA in low serum medium yielded formation of a layer up to 160 μm thick of peripheral cardiac tissue with electrophysiological properties that were better than for constructs cultivated in spinner flasks and comparable to native ventricles.[16] The expression levels of cardiac proteins connexin-43, creatin kinase-MM and sarcomeric myosin heavy chain were lower in HARV-cultivated constructs than in neonatal rat ventricles but higher than in the

spinner-flask cultivated constructs.[16] Even with the laminar flow conditions present in rotating vessels the cell distribution remained non-uniform due to the oxygen diffusional limitations.

Perfused cartridges

Physiologic densities of viable cardiac myocytes were achieved by rapid cell inoculation into a porous scaffold using Matrigel® followed by the establishment of the perfusion of culture medium through the construct immediately after gel hardening (15 min).[21] Non-perfused constructs had most cells located in the 100–200 μm thick layer at the top surface, and only a small number of cells penetrated the entire construct depth (Fig. 3, left). Constructs seeded in perfusion exhibited high and spatially uniform cell density throughout the construct volume (Fig. 3, right).

To provide convective-diffusive oxygen supply to the cells, interstitial medium flow was maintained for 7 days during cultivation; constructs seeded and cultivated in dishes served as a control. Throughout the cultivation, the number of live cells in perfused constructs was significantly higher than in dish-grown constructs.[25] Notably, the number of live cells in dish-grown constructs decreased rapidly during the first day of culture and continued to decrease between days 1 and 7. In contrast, live cell numbers in perfused constructs were constant during day 1, and decreased only slowly with time in culture.

Cell viability was significantly higher in perfused than in dish-grown constructs at all time points. Importantly, the final cell viability in perfused constructs ($81.6 \pm 3.7\%$) was not significantly different from the viability of the freshly isolated cells (83.8 ± 2.0), and it was markedly higher than the cell viability in dish-grown constructs ($47.4 \pm 7.8\%$). The molar ratio of lactate produced to glucose consumed (L/G) was ~1 for perfused constructs throughout the duration of culture, indicating aerobic cell metabolism. In dishes, L/G increased progressively from 1 to 2, indicating a transient to anaerobic cell metabolism. Cell damage was assessed from measured levels of lactate dehydrogenase (LDH) in culture medium. At all time points, the levels of LDH were significantly lower in perfusion than in dish cultures, indicating that medium perfusion reduced cell damage.

Spontaneous contractions were observed in some constructs early in culture (dish-grown constructs 2–3 days after seeding), and ceased after approximately 5 days of cultivation, indicating the maturation of engineered tissue. In response to electrical stimulation (e.g. at 5V and 60 beats per minute [bpm]), all constructs were reproducibly induced to contract synchronously. However, in perfused constructs the contraction frequency was constant, whereas in dish-grown constructs the contraction frequency spontaneously increased every 1–2 min and the contraction pattern appeared arrhythmic. Overall, medium perfusion during cell seeding and construct cultivation markedly improved the contractile behavior of engineered cardiac constructs.

After 7 days of culture, the overall tissue architecture appeared markedly better for perfused than dish-grown constructs. The 100–200 μm thick peripheral layers of constructs from both groups consisted of tightly packed cells expressing cardiac differentiation markers, in contrast to construct interiors which were markedly different. Medium perfusion maintained high and spatially uniform cell density throughout the construct volume (except in the outer-edge regions shielded from fluid flow), whereas molecular diffusion in the interiors of dish-grown constructs supported only a low density of scattered cells. Sarcomeric α-actin, cardiac troponin I and sarcomeric tropomyosin were present throughout the perfused construct volume, and only on the periphery of dish-grown constructs.[25]

SUMMARY

The restoration of normal cardiac function can potentially be achieved by functional tissue constructs grown *in vitro* by using differentiated or progenitor cells, biomaterial scaffolds and bioreactors. Engineered tissues can also serve as high-fidelity models for basic studies of cells and tissues, in response to gene alterations, drugs, hypoxia, or physical stimuli. In spite of significant achievements in cardiac tissue engineering, major challenges with respect to cell sourcing remain. Future efforts will most likely focus on (1) exploring an appropriate human cell source (e.g. differentiation of bone marrow stem cells), (2) co-culture of major cell types found in the native myocardium (e.g. fibroblasts, myocytes and endothelial cells), and (3) development of second generation bioreactors that can provide an array of physical and biochemical stimuli found in the native heart (e.g. combination of perfusion with electrical and mechanical stimulation).

REFERENCES

1 Gillum RF, Epidemiology of congenital heart disease in the United States. *Am Heart J* **127**:919–927 (1994).
2 Lysaght MJ and Reyes J, The growth of tissue engineering. *Tissue Eng* **7**:485–493 (2001).
3 Evans RW, Economic impact of mechanical cardiac assistance. *Prog Cardiovasc Dis* **43**:81–94 (2000).
4 Soonpaa MH, Koh GY, Klug MG and Field LJ, Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. *Science* **264**:98–101 (1994).
5 Zimmermann WH, Didie M, Wasmeier GH, Nixdorff U, Hess A, Melnychenko I, Boy O, Neuhuber WL, Weyand M and Eschenhagen T, Cardiac grafting of engineered heart tissue in syngenic rats. *Circulation* **106**:I151–I157 (2002).
6 Korecky B, Hai CM and Rakusan K, Functional capillary density in normal and transplanted rat hearts. *Can J Physiol Pharm* **60**:23–32 (1982).
7 Rakusan K and Korecky B, The effect of growth and aging on functional capillary supply of the rat heart. *Growth* **46**:275–281 (1982).

8 Li R-K, Jia ZQ, Weisel RD, Mickle DAG, Choi A and Yau TM, Survival and function of bioengineered cardiac grafts. *Circulation* **100**:II63–II69 (1999).
9 Li R-K, Yau TM, Weisel RD, Mickle DAG, Sakai T, Choi A and Jia Z-Q, Construction of a bioengineered cardiac graft. *J Thorac Cardiovasc Surg* **119**:368–375 (2000).
10 Leor J, Aboulafia-Etzion S, Dar A, Shapiro L, Barbash IM, Battler A, Granot Y and Cohen S, Bioengineered cardiac grafts: a new approach to repair the infarcted myocardium? *Circulation* **102**:III56–III61 (2000).
11 Dar A, Shachar M, Leor J and Cohen S, Cardiac tissue engineering optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds. *Biotechnol Bioeng* **80**:305–312 (2002).
12 Kofidis T, Akhyari P, Boublik J, Theodorou P, Martin U, Ruhparwar A, Fischer S, Eschenhagen T, Kubis HP, Kraft T, Leyh R and Haverich A, *In vitro* engineering of heart muscle: artificial myocardial tissue. *J Thorac Cardiovasc Surg* **124**:63–69 (2002).
13 Carrier RL, Papadaki M, Rupnick M, Schoen FJ, Bursac N, Langer R, Freed LE and Vunjak-Novakovic G, Cardiac tissue engineering: cell seeding, cultivation parameters and tissue construct characterization. *Biotechnol Bioeng* **64**:580–589 (1999).
14 Bursac N, Papadaki M, Cohen RJ, Schoen FJ, Eisenberg SR, Carrier R, Vunjak-Novakovic G and Freed LE, Cardiac muscle tissue engineering: toward an *in vitro* model for electrophysiological studies. *Am J Physiol Heart Circ Physiol* **277**:H433–H444 (1999).
15 Akins RE, Boyce RA, Madonna ML, Schroedl NA, Gonda SR, McLaughlin TA and Hartzell CR, Cardiac organogenesis *in vitro*:reestablishment of three-dimensional tissue architecture by dissociated neonatal rat ventricular cells. *Tissue Eng* **5**:103–118 (1999).
16 Papadaki M, Bursac N, Langer R, Merok J, Vunjak-Novakovic G and Freed LE, Tissue engineering of functional cardiac muscle: molecular, structural and electrophysiological studies. *Am J Physiol Heart Circ Physiol* **280**:H168–H178 (2001).
17 Eschenhagen T, Fink C, Remmers U, Scholz H, Wattchow J, Woil J, Zimmermann W, Dohmen HH, Schafer H, Bishopric N, Wakatsuki T and Elson E, Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart model system. *FASEB J* **11**:683–694 (1997).
18 Fink C, Ergun S, Kralisch D, Remmers U, Weil J and Eschenhagen T, Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. *FASEB J* **14**:669–679 (2000).
19 Zimmermann WH, Fink C, Kralish D, Remmers U, Weil J and Eschenhagen T, Three-dimensional engineered heart tissue from neonatal rat cardiac myocytes. *Biotechnol Bioeng* **68**:106–114 (2000).
20 Shimizu T, Yamato M, Isoi Y, Akutsu T, Setomaru T, Abe K, Kikuchi A, Umezu M and Okano T, Fabrication of pulsatile cardiac tissue grafts using a novel 3-dimensional cell sheet manipulation technique and temperature-responsive cell culture surfaces. *Circ Res* **90**:e40–e48 (2002).
21 Radisic M, Euloth M, Yang L, Langer R, Freed LE and Vunjak-Novakovic G, High density seeding of myocyte cells for tissue engineering. *Biotechnol Bioeng* **82**:403–414 (2003).
22 Freed LE and Vunjak-Novakovic G, Tissue engineering bioreactors, in *Principles of Tissue Engineering*, ed by Lanza RP, Langer R and Vacanti J. Academic Press, San Diego, pp 143–156 (2000).
23 Carrier RL, Rupnick M, Langer R, Schoen FJ, Freed LE and Vunjak-Novakovic G, Perfusion improves tissue architecture of engineered cardiac muscle. *Tissue Eng* **8**:175–188 (2002).
24 Carrier RL, Rupnick M, Langer R, Schoen FJ, Freed LE and Vunjak-Novakovic G, Effects of oxygen on engineered cardiac muscle. *Biotechnol Bioeng* **78**:617–625 (2002).
25 Radisic M, Yang L, Boublik J, Cohen RJ, Langer R, Freed LE and Vunjak-Novakovic G, Medium perfusion enables engineering of compact and contractile cardiac tissue. *Am J Physiol Heart Circ Physiol* **286**:H507–H516 (2004).