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REVIEW

# Cardiac tissue engineering

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*Abstract:* We hypothesized that clinically sized (1–5 mm thick),compact cardiac constructs containing physiologically high density of viable cells ($\approx 10^8$ cells/cm$^3$) can be engineered *in vitro* by using biomimetic culture systems capable of providing oxygen transport and electrical stimulation, designed to mimic those in native heart. This hypothesis was tested by culturing rat heart cells on polymer scaffolds, either with perfusion of culture medium (physiologic interstitial velocity, supplementation of perfluorocarbons), or with electrical stimulation (continuous application of biphasic pulses, 2 ms, 5 V, 1 Hz). Tissue constructs cultured without perfusion or electrical stimulation served as controls. Medium perfusion and addition of perfluorocarbons resulted in compact, thick constructs containing physiologic density of viable, electromechanically coupled cells, in contrast to control constructs which had only a $\approx$ 100 μm thick peripheral region with functionally connected cells. Electrical stimulation of cultured constructs resulted in markedly improved contractile properties, increased amounts of cardiac proteins, and remarkably well developed ultrastructure (similar to that of native heart) as compared to non-stimulated controls. We discuss here the state of the art of cardiac tissue engineering, in light of the biomimetic approach that reproduces *in vitro* some of the conditions present during normal tissue development.

*Keywords:* bioreactor, myocardium, oxygen, perfluorocarbon, electrical simulation, excitation, contraction.

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## 1. INTRODUCTION

Cardiovascular disease is responsible for a preponderance of health problems, and its impact is expected to grow further 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 developed countries, accounting for nearly 40 % of all deaths. Congential heart defects, which occur in nearly 14 of every 1000 newborn children,[1] are the most common congenital defects and the leading cause of death in the first year of life.[2,3] 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.[4]

Tissue engineering has emerged over the last decade as an interdisciplinary field with tremendous potential. Substantial progress has been made in areas of biopolymers,[5] cell-material interactions[6] and bio-mimetic culture devices.[7] Functional tissues have been developed and implanted *in vivo*, including (but not limited to) cartilage,[8] bone,[8] bladder[10] and blood vessels.[11, 12] Engineered tissues can also provide high-fidelity models for basic studies of cell function and tissue development, and responses to genetic alterations, drugs, hypoxia, and physical stimuli.[13, 14] However, fundamental and all-encompassing problems remain. One of the most important ones is mass transfer into the tissue beyond the ≈ 100 μm thick peripheral region, both during the *in vitro* cultivation and following implantation *in vivo*. This explains why tissue engineering has been most successful with tissues that are either thin (*e.g.* bladder) or have low oxygen requirements (*e.g.*, cartilage). Our current inability to vascularize and perfuse thick cell masses has hindered efforts to build many types of tissues, including, most critically, cardiac muscle.

Three-dimensional tissue constructs that express structural and physiological features characteristic of native cardiac muscle have been engineering using fetal or neonatal rat cardiac myocytes (CM) cultured in collagen gels with mechanical stimulation,[15–18] on collagen fibres,[19] fibrous polyglycolic acid scaffolds[20–24] and porous collagen scaffolds.[25–27] Cells were seeded onto scaffolds and cultivated in dishes,[21, 24, 25, 28] Oxygen dissolved in medium was transported to the cells by molecular diffusion, which provided enough oxygen for an approximately 100 μm thick outer layer of functional tissue but not for the construct interior, which remained relatively acellular.[17, 20, 21, 24] Construct cultivation in perfused cartidges merkedly improved the uniformity of cell distribution, but the overall cell density remained low due to the diffusional limitations of oxygen supply during scaffold seeding.[22, 23] To overcome this problem, we recently employed a bioreactor with interstitial flow of culture medium that provides sufficient oxygen supply during both seeding and cultivation, and thereby enables the maintenance of physiologic densities of viable cardiac myocytes.[27] In a separate recent study, we enhanced excitation-contraction coupling of cardic myocytes in cultured constructs by applying cardiac-like electrical stimulation.[14]

Key factors necessary for production of functional cardiac tissue are:

- Biodegradable and biocompatible scaffolds that provide adequate mass transfer, vascularization, and transduction of mechanical and electrical signals
- Culture systems that promote differentiated function and excitation – contraction coupling of cardiac cells
- Functional vascular networks embedded into the cardiac muscle to promote nutrient transfer, angigiogenesis and integration with the host vasculature.

We discuss here the state of the art of cardiac tissue engineering, and focus on the ”biomimetic” aproach that reproduces *in vitro* – by using cells, scaffolds and bioreactors – some of the conditions present during normal tissue development.

## 2. BIOMIMETIC APPROACH

The overall objective of tissue engineering is the restoration of normal tissue function. A damaged tissue should ideally be replaced by an engineered graft that can reestablish appropriate structure, composition, cell signaling and key function(s) of the native tissue. In light of this paradigm, the clinical utility of tissue engineering will likely depend on our ability to replicate the *site-specific* properties of the tissue being replaced (biochemical composition, architectural organization, function), maintain the specific *cell phenotype*, and provide integration (structural and functional) with the host tissues.

*In vivo*, the processes of cell differentiation during embryogenesis and adult tissue remodeling are directed by multiple factors acting in concert and according to specific spatial and temporal sequences (Fig. 1). Undifferentiated (stem) cells

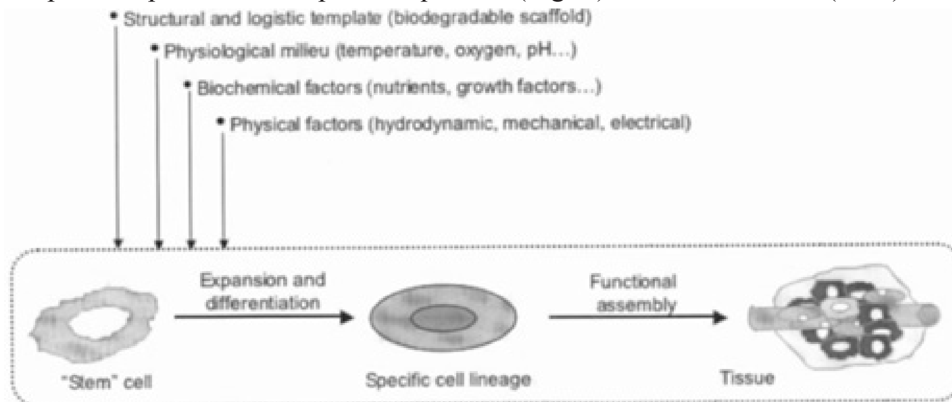


Fig. 1. Developmental paradigm. Tissue development and remodeling, *in vivo* and *in vitro* involves the proliferation and differentiation of stem/progenitor cells and their subsequent assembly into a tissue structure. Cell function and the progression of tissue assembly depend on: (a) the availability of a scaffold for cell attachment and tissue formation, (b) the maintenance of physiological conditions in cell/tissue environment, (c) supply of nutrients, oxygen, metabolites and growth factors, and (d) presence of physical regulatory factors.

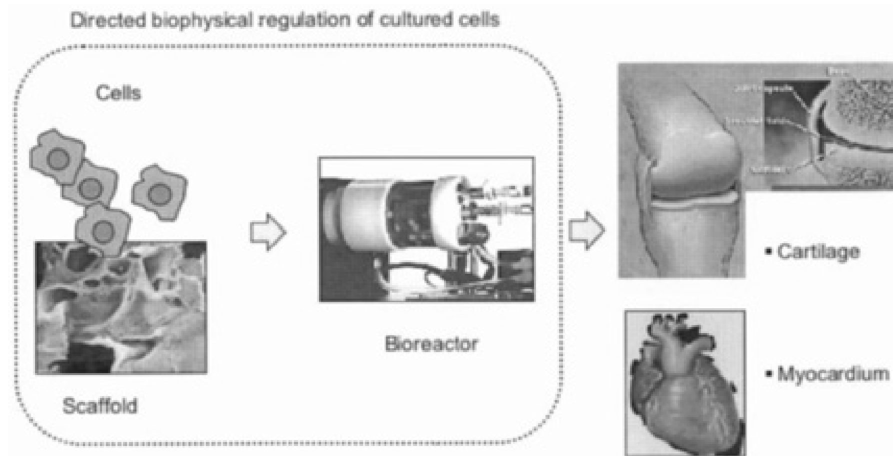


Fig. 2. Tissue engineering paradigm. The regulatory factors of cell differentiation and tissue assembly depicted in Fig. 1 can be utilized *in vitro* to engineer functional tissues by an integrated use of isolated cells, biomaterial scaffolds and bioreactors. The cells themeslves (either differentiated or progenitor/stem cells seeded onto a scaffold and cultured in a bioreactor) carry out the porcess 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.

first differentiate into specific cell lineages (bone, muscle, nerves, blood vessels) and then undergo functional asssembly into tissue structures. In most cases, tissues contain more than one cell type and vascular supply. Both phases depend on at least four gorups of factors: (a) a structural template for cell attachment and tissue formation, (b) a physiological milieu, (c) exchange of nutrients, oxygen and metabolites, and (b) physical regulatory signals.

A "biomimetic" aproach to tissue eingineering attempts to mimic *in vitro* certain aspects of the environment present *in vivo* during normal development, in order to stimulate functional assembly of the cells into specialized tissues .This generally involves the presence of reparative cells (the actual "tissue engineers"), the use of scaffolds (designed to provide a structural and logistic template for tissue development and biodegrade at a controlled rate), and bioreactors (designed to control cellular microenvironment, facilitate mass transport to the cells, and provide the necessary biochemical and physical regulatory signals) (Fig. 2). Dissociated cells are first seeded onto a three-dimensional scaffold and then cultured in a bioreactor. Together, the scaffold and bioreactor provide biophysical regulation of the cell environment.

Cardic muscle in unique in many respects, and represents a major challenge to tissue engineering. Some of the key features of native cardiac muscle, and the corresponding requirements for engineered cardiac muscle are summarized in Table I.

TABLE I. Biomimetic approach to cardiac tissue engineering: main factors of the *in vivo* myocardial environment and their *in vitro* counter parts

| | *In vivo* | *In vitro* |
|---|---|---|
| Cells | High density (≈$10^8$ cels/$cm^3$) | High density (≈$0.3x10^8$ cells/$cm^3$) |
| | Multiple cell types (cardiac myocytes, fibroblasts, endothelial cells) | Multiple cell types (cardiac myocytes, fibroblasts, endothelial cells) |
| Geometry | Capillary network (≈7 μm diameter, ≈30 μm spacing) | Parallel channel array (100–300 μm diameter, 100–300 μm spacing) |
| Mass transport | Convection: Blood flow ≈500 μm/s, Diffusion in tissue space | Convection: Medium flow ≈50–500 μm/s Diffusion in construct space |
| Oxygen carrier | Hemoglobin (arterial blood) $O_2$ dissolved in plasma 130μM $O_2$ as oxyhemoglobin 8500 μM $O_2$ total 8630 μM | PFC emulsion (5.4 vol% at 160 mmHg and 37 °C) $O_2$ dissolved in aqueous phase 220 μM $O_2$ in PFC droplets 230 μM $O_2$ total 450 μM |
| Physical signals | Excitation (by electrical signals from pacer cells); synchronous contractions. Electro-mechanical cell coupling (gap junctions; contractile apparatus) | Electrical stimulation (by suprathreshold pacing signals) of cultured constructs; synchronous contractions. Electro-mechanical cell coupling |

The cardiac myocyte is the most physically energetic cell in the body, contracting more than 3 billion times in an average human lifespan, and pumping over 7,000 liters of blood per day along 100,000 miles of blood vessels. Heart muscle contains three major subpopulations of cells – cardiac myocytes, fibroblasts and endothelial cells – at a total concentration of approximately 100 million cells/$cm^3$. Due to its extremely high cell density, and high respiration rates of the cells, cardiac muscle is highly metabolically active, consumes large amounts of oxygen, and cannot tolerate hypoxia for prolonged periods of time.[29,30] Therefore nutrients, particularly oxygen, are depleted within a relatively thin layer of viable tissue, and viable cells are concentrated mostly in the peripheral region of the construct. Natural myocardium obviates this difficulty through a rich vasculature, with average capillary-to-capillary distances in rat heart of only 17 – 19 μm, approximately the width of an individual cardiac myocyte.[31–34]

Oxygen supply to cardiac cells is provided by hemoglobin an oxygen carrier (please note that hemoglobin increases the amount of oxygen in blood by two orders of magnitude, Table I). In engineered cardiac muscle, one can mimic blood flow through the capillary network by culturing cells on scaffolds that contain an array of channels that are perfused with culture medium.[38] Synthetic oxygen carriers, such as perfluorocarbons, can be supplemented to culture medium in order to increase its oxygen-carrying capacity.

In addition, cardiac cells do not proliferate, such that any loss of the cells is permanent. Cell requirements for cardiac tissue engineering thus include the estab-

lishment and maintenance of physiologically high density of viable cells and cell co-culture.

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 cardiac myocytes and trigger release of calcium, which in turn stimulates contraction of the myofibrils. Electro-mechanical coupling of the myocytes is crucial for their contractile function.[35] To enhance the establishment of functional connections, cells cultured on scaffolds can be stimulated by electrical signals similar to those in the native heart,[14] or subjected to direct mechanical stimulation.[18] In either case, it is esential that the cells become electromechanically coupled and capable of synchronously responding to electrical pacing signals, rather than contracting spontaneously.

Therefore, a "biomimetic" approach to engineering of functonal equivalents of native myocardium should provide several physiologically relevant features:

- *Structural template* for tissue formation, in form of porous biodegradable polymer scaffold with large (≈100 μm) interconnected pores and an array of channels (180 μm in diameter)
- *Physiologically high and spatially uniform seeding density* of cardiac myocytes in scaffold pores (to obtain compact tissue and promote cell–cell interactions)
- *Endothelial cell lining of channels* to provide paracrine signals, enhance construct angiogenesis, and protect cardiac myocytes from hydrodynamic shear
- *Convective flow through scaffold channels* (to enhance mass transport of nutrients, oxygen and metabolites)
- *Control of medium composition,* pH and oxygen levels *via* gas and medium exchange, and supplementation with perfluorocarbons to increase oxygen contents
- *Electrical stimulation* at a physiologic frequency and supra-threshold amplitude (to promote excitation-contraction cell coupling and thereby enhance functional assembly of engineered tissue).

## 3. PHYSIOLOGICAL CELL DENSITY

The technique of seeding that was specifically developed for cardiac tissue engineering involves (a) rapid inoculation of cardiac cells into collagen sponges using Matrigel® as a cell delivery vehicle, and (b) 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.[27] Uniderectional flow of culture medium was maintained for the duration of cultiva-

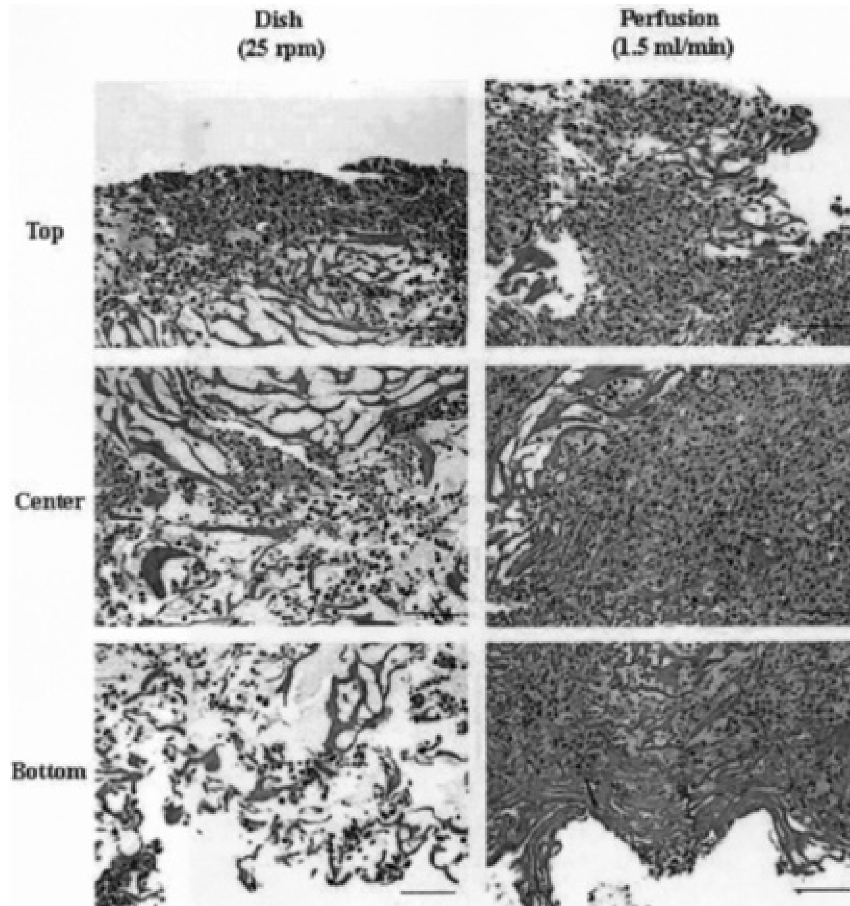


Fig. 3. Effects of perfusion during seeding and cultivation on cardiac cell distribution. Cross-sections of constructs inoculated with 12 million cells and then transferred for a period of 4.5 h either into dishes (25 rpm, left) or into perfused cartidges (1.5 ml/min, right). The top, center and bottom areas of a 650 μm wide strip extending from one construct surface to the other are shown. Scale bar 100 μm (with permission from Radisic *et al. Biotech. Bioeng.* **82** (2003) 403 – 414 (Fig. 4)).

tion. 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, center and bottom areas of a 0.65 μm wide strip extending from one construct surface to the other are shown in Fig. 3. Constructs seeded in dishes had most cells located in the ≈ 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 (Fig. 3). Clearly, medium perfusion during seeding was key for engineering thick constructs with high densities of viable cells, presumably due to enhanced transport of oxygen within the construct.

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 (81.6 ± 3.7 %) was not significantly different than the viability of the freshly isolated cells (83.8 ± 2.0) and it was markedly higher than the cell viability in dishgrown constructs (47.4 ± 7.8 %).[38] Consistently, the mole ratio of lactate produced to glucose consumed (L/G) was ≈ 1 for perfused constructs, indicating aerobic cell metabolism. In dishes, L/G increased progressively from 1 to ≈ 2, indicating a transition to anaerobic cell metabolism. Cell damage, assessed by monitoring the activity of lactate dehydrogenase (LDH) 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 phase, whereas the cells from dish-grown constructs appeared unable to complete the cell cycle and accumulated in the G2/M phase. Cells expressing cardiac-specific differentiation markers (sarcomeric α-actin, sarcomeric tropomyosin, cardic tropolin I) were present throughout the perfused constructs, and only within a ≈ 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 construcst contracted synchronously, had lower excitation thresholds 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.

Although interstitial medium flow enabled engineering of compact tissue that had physiologic density of viable aerobically metabolizing cells, most cells were round and mononucleated. This was likely due to the exposure of cardiac myocytes 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 cardiac myocytes. This motiviated the design of scaffolds with arrays of channels that provide a separate compartment for medium flow.

## 4. OXYGEN SUPPLY

To test the feasibility of using channeled scaffolds, cardiac constructs were first engineering using a channeled collagen scaffold (Ultrafoam™, 1 cm in diameter x 3 mm thisk) seeded with neonatal cardiac myocytes and cultivated in perfusion at 0.5 ml/min for 10 days. The channel maintained its initial diameter and was surrounded with a 300 μm thick tissue layer. However, collagen is not optimal for cardiac tissue engineering due to its poor structural integrity. We thus explored the use of an elastomer, poly(glycerol sebacate), PGS,[5] pretreated with neonatal rat cardiofibroblasts for 3 days in orbitally mixed dishes, followed by addition of rat cardiomyocytes and perfusion cultivation.[38] After only 3 days of culture, cells on

scaffolds formed constructs that contracted synchronously in response to electrical stimulation. The scaffold pores remained open and the pressure drop measured ascross the construct was as low as 0.1 kPa/mm.

PGS is obtained by condensation of glycerol and sebacic acid, and formed by salt-leaching into a three-dimensional network with a desired pore size (*e.g.*, ≈ 100 μm), porosity (> 95%) and thickness (1 – 5 mm). The cross-linked and hydrogen bonds contirbute to its unique elastic properties. PGS degrades by hydrolysis of its ester bond into glycerol (likely adsorbed in the body) and sebacic acid (secreted by urine either directly or metabolized into carboxylic acids). *In vivo* (5 weeks of subcutaneous implantation), PGS scaffold was biocompatible and lost its mass lineary (to ≈ 20 % of initial over 5 weaks), such that its shape and structural integrity were well maintained. In contrast, poly-lactide-glycolide (PLG) had abrupt loss of weight after an initial lag phase.[18] *In vitro* (in buffer, at 37 ºC) PGS degraded more slowly (to ≈ 80 % of initial over 8 weeks if unseeded, to 65 % of initial if seeded with hepatocytes). The mechanical properties of PGS resemble vulcanized rubber: PGS is highly elastic and capable of up to 400 % elongation before it yields.

To mimic the capillary network, neonatal rat heart cells were cultured on PGS scaffolds with a parallel array of channels made using a laser cutting/engraving sysem (Fig. 4C) and perfused with culture medium. To mimic oxygen supply by hemoglobin, culture medium was supplemented by 5.4 %v/v PFC emulsion (17 vol% Oxygent™, kindly donated by Alliance Pharmaceuticals Corp. (San Diego CA)); constructs perfused with unsupplemented culture medium served as controls. Construcs were subjected to unidirectional medium flow at a flow rate 0.1 ml/min provided by a multi-channel peristaltic pump (Isma Tec) (Fig. 4A).

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 (Fig 4B). 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 a higher oxygen concentrations in the medium. Due to the small size of PFC particles, the passive diffusion of dissolved oxygen from the PFC phase into the aqueous phase was very fast, and estimated not to be a rate-limiting step in this system.[38] For comparison, in un-supplemented culture medium, oxygen was depleted faster since there was no oxygen carrier phase that acts as a reservoir.[37]

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 mmHg *vs*. 45 mm Hg between the construct inlet and outlet at the flow rate of 0.1 ml/min). 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 to 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.

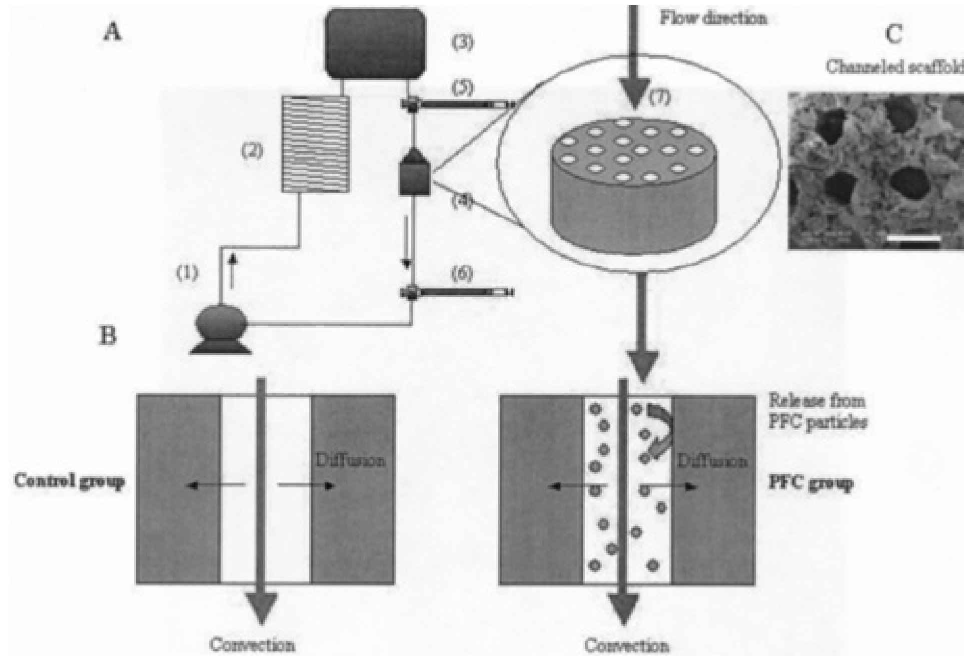


Fig. 4. Channeled scaffolds and oxygen carriers. (A). *Perfusion loop*. Channeled biorubber scaffolds (7) preconditioned with cardiac fibroblasts were seeded with cardiac myocytes and placed into perfusion cartridges (4) between two syringes (5,6). Medium flow (0.1 ml/min) was provided by a multi-channel peristaltic pump (1) and gas exchange was provided by a coil of thin silicone tubing (3 m long) (2). (B) *Modes of oxygen transport* in the channeled construct perfused with culture medium include convection through the channel lumen and diffusion into the construct space surrounding each channel. In regular culture medium (control group, shown on left) 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 by 5.4 vol% perfluorocarbon (PFC) emulsion (PFC group, shown on right), oxygen is replenished within the tissue construct by the release of oxygen from the PFC particle into the culture medium phase, by a process governed by Henry's law.[38] (C) Scanning electron micrograph of a channeled PGS scaffold. Scale bar 500 μm.

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 utilization by the cells. The model was used to evaluate the effects of medium perfusion rate, oxygen carrier and scaffold geometry on viable cell density.[38]

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 modeled in a channel array consisting of channels 100 μm in diameter and 100 μm wall-to-wall spacing at physiologically high cell density $1 \times 10^8$ cells/cm$^3$. At

0.049 cm/s oxygen concentration increased significantly in both tissue space and channel lumen with the increase in circulating PFC emulsion from 0 to 6.4 vol%. Although the oxygen concentration in the tissue space with physiological cell density increased considerably with the increase of circulating PFC concentration from 0–6.4 %, we had to increase the flow rate, keeping the shear stress in the physiological range ≈ 1 dyn/cm$^2$, in order to provide enough oxygen for the entire 0.5 cm thick construct. At our best conditions (0.135 cm/s and 6.4 % PFC) oxygen is not depleted at any point in the scaffold and the minimum concentration of 33 μ, M is approximately five times above the $K_m$ (oxygen concentration at which consumption rate in the tissue space is maintained at a maximum level).

## 5. EXCITATION–CONTRACTION COUPLING

*Mechanical stimulation*. One significant approach to cardiac tissue engineering, established by Eschenhagen and colleagues[15–18] involves the cultivation of neonatal rat heart cells in collagen gel or Matrigel, in the presence of growth factors. The cultured tissues are subjected to sustained mechanical strain. Under these conditions, cardiomyocytes and non-myocytes from 3D cardiac organoids, consisting of a well organized and highly differentiated cardiac muscle syncytium, that exhibited contractile and electrophysiologicl properties of working myocardium. First implantation expreriments in healthy rats showed survival, strong vascularization, and signs of terminal differntiation of cardiac tissue grafts.

In one set-up, neonatal rat ventricular myocytes were resuspended in a gel consisting of collagen I and Matrigel in culture medium[16] and cast into wells containing Velcro coated silicione tubes. After 4 days in culture a strip of biconcave tissue was formed fixed at each side to a piece of silicone tubing. Unidirectional and cyclic stretch at the frequency of 1.5 Hz and the strain rate of up to 20 % was employed for 6 days. Stretched constructs exhibited improved morphology and organization of cardiac myocytes, higher RNA/DNA and protein/cell raitos, and higher force of contraction.[16]

In an improved set-up, neonatal rat cardiac cells were suspended in the collagen/Matrigel mix and cast into circular molds.[18] After 7 days of static culture, the strips of cardiac tissue were placed around two rods of a custom made mechanical stretcher and subjected to either unidirectional or cyclic stretch. Histology and immunohistochemistry revealed formation of intensively interconnected, longitudinally oriented cardiac muscle bundles with morphological features resembling adult rather than immature native tissue. Primitive capillary structures were also detected. Cardiomyocytes exhibited well developed ultrastructural features: sarcomeres arranged in myofibrils, with well developed, Z, I, A H and M bands, specialized cell-cell junctions, T tubules as well as well developed basement membrane. Contractile properties were similar to those measured for native tissue, with high ratio of twich to resting tension and strong β-adrenegenic response. Action potentials characteristic of rat ventricular myocytes were recorded.

*Electrical simulation*. Although the application of cyclic mechanical stretch alone substantially improved cell differentiation and force of contraction, the distribution of gap junctions remained unclear, and some hallmarks of cardiac development are missing, most critacally the M bands and IC discs.[18] In native heart, mechanical stretch is induced by electrical signals. The orderly coupling between electrical pacing signals and macroscopic contractions is crucial for the development and function of native myocardium.[35] In a recent study, 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 mechanism. To test this hypothesis, cardiac constructs prepared by seeding collagen sponges ($6 \times 8 \times 1.5$ mm) with neonatal rat ventricular cells ($5 \times 10^6$) were stimulated using supra-threshold square biphasic pulses (2 ms duration, 1 Hz, 5 V). The stimulation was initiated after 1 – 5 days of scaffold sedding (3 day period was optimal) and applied for up to 8 days.

Over only 8 days *in vitro*, electrical fild stimulation induced cell alignment and coupling, increased the amplitude of synchronous construct contractions by a factor of 7 and resulted in a remarkable level of ultrastructural organization. Development of conductive and contractile properties of cardiac constructs was concurrent, with strong dependence on the initation and duration of electrical stimulation.

The application of electrical stimulation during construct cultivation markedly enhanced the contractive behaviour. After 8 days of culture, the amplitude of contractions was 7-fold higher in stimulated than non-stimulated constructs (Fig. 5a), 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 (Fig. 5B) and the maximum capture rate (MCR the maximum pacing frequency for synchronous construct contractions), increased (Fig. 5C) both with time and due to electrical stimulation, suggesting functional coupling of the cells. The shape, amplitude (≈100 mV), and duration (≈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 stimulted during culture.[18]

On a molecular level, electrical stimulation elevated the levels of all measured cardiac proteins and enhanced the expression of the corresponding gens, without causing pathological cell hypertrophy.[39] With time in culture, the ratio of mature and immature form 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.

After 8 days, stimulated constructs demonstrated a remarkable level of ultrastructurl differentiation, comparable in several respects to that of native myocardium. Cells in stimulted constructs were aligned, elongated, and contained centrally positioned elongated nuclei, in contrast to round cells in non-stimulated con-

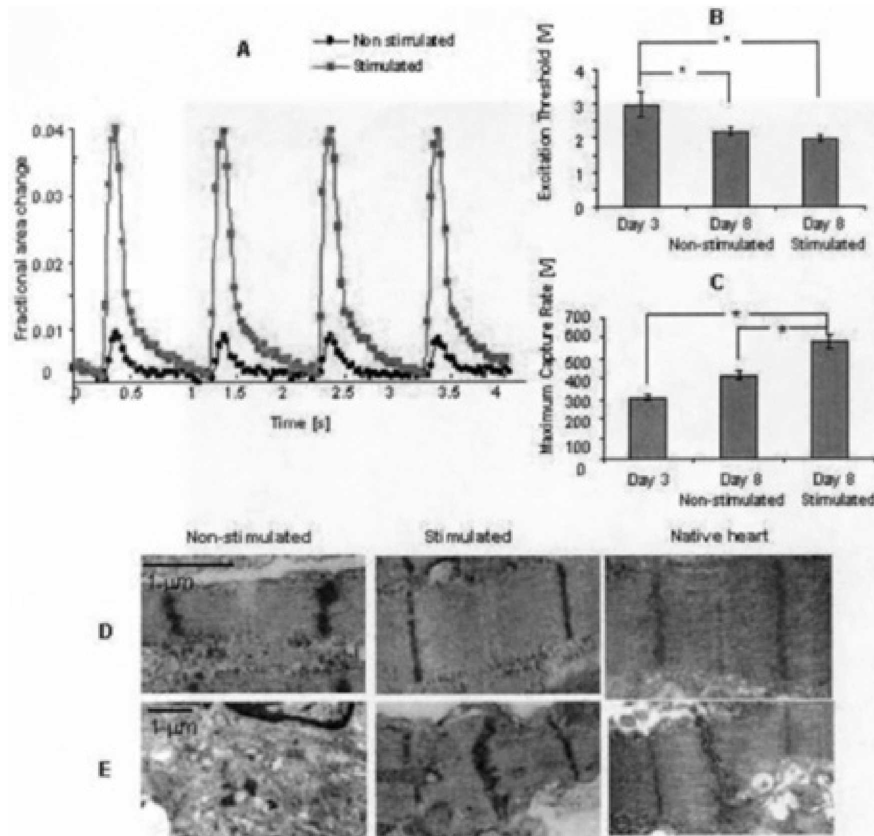


Fig. 5. Effects of electrical stimulation on funcitonal assembly of engineered cardiac constructs. (A) Contraction amplitude of constructs cultured for a total of 8 days, shown as a fractional change in the construct size. Electrical stimulation increased the amplitude of contractions by a factor of seven. (B) Excitation threshold (ET) decreased and (C) Maximum cupture rate (MCR) increased significantly both with time in culture and due to electrical stimulation. (*) Denotes statistically significant differenses ($P < 0.05$; 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.

structs that had a high nucleus-to-cytoplasm ratio. 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 sarcomers that closely resembled those in native myocardium (Fig. 5D) representing a hallmark of maturing cardiomyocytes.[35] The volume fraction of sarcomeres in stimulated 8-day constructs was indistinguishable from that measured for neonatal ventricles,[40] in contrast, non-stimulated constructs contained only scattered and poorly organized

sarcomers. In stimulated constructs intercalated discs were positioned between aligned Z lines (Fig. 5E) and were as frequent as in neonatal ventricles; gap junctions were also substantially better developed and more frequent.

Myofibers aligned in the direction of the electrical field lines, possibly in an attempt to decrease the apparent ET in response to pacing.[41] In contrast, cells is non-stimulated constructs stayed round and expressed relatively low levels of cardiac markers. After 8 days, stimulated constructs exhibited markedly higher density of Cx-43 than either early (3-day) or non-stimulted constructs. Notably, improved contractile properties of electrically stimulated constructs were not reflected in any apparent differences in construct cellularity, cell damage or cell metabolism, but correlated instead with cell differentiation. Stimulted constructs and neonatal ventricles expressed high levels of cardiac Tn-I, sarcomeric α-actin, α-MHC and β-MHC, and contained elongated cells aligned in parallel. In contrast, cells in non-stimulated constructs stayed round and expressed relatively low levels of cardiac markers. Cross striations charcteristic for mature cardiac myocytes were detected in stimulated constructs and native ventricles, but not in non-stimulated constructs.

These studies suggest that electrical stimulation of construct contractions during cultivation progressively enhanced the excitation-contraction coupling and improved the properties of engineered myocardium at the cellular, ultrastructural and tissue levels.

## SUMMARY

The paradigm discussed in this chapter is that immature but functional cardiac tissue constructs can be grown *in vitro* using culture systems based on scaffolds and bioreactors designed to recapitulate some of the factors present during normal tissue development. These constructs are expected to have the ability to regenerate the molecular, structural and functional properties of a compromised native tissue. Engineered tissues can also serve as high-fidelity models for basic studies of cells and tissues. Key factors that determine the biophysical regulation of cell function and tissue assembly include: (a) physiologically high density of heart cells, (b) scaffolds that are tailored to structurally and mechanically support cell attachment, coupling and mechanotransduction, (c) bioreactors designed to control cellular microenvironment, (d) application of biochemical and physical regulatory signals, and (e) establishment of a vascular network than can facilitate integration into the host.

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И З В О Д

## ИНЖЕЊЕРСТВО СТВАРАЊА СРЧАНОГ ТКИВА

МИЛИЦА РАДИШИЋ и ГОРДАНА ВУЊАК-НОВАКОВИЋ

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Срчано ткиво има компактну изузетно сложену структуру са високом густином ћелија (≈100 милиона по $cm^3$) које су функционално повезане и куцају синхронизовано у одговору на електричне сигнале. Ефикасан транспорт кисеоника и електрична стимулација су пресудни за нормалан развој и функцију срчаног мишића. Наша хипотеза је да су иста та два фактора пресудна и за култивацију срчаног ткива *in vitro*. Ћелије изоловане из срчаног ткива пацова су гајене на полимерној матрици (порозни колаген; синтетички порозни еластомер са мрежом канала) у два различита система: (1) са перфузијом медијума кроз мрежу канала (сурогат капилара) уз додатак перфлуорокарбона (сурогат хемоглобина), и (2) са електричном стимулацијом (сурогат нормалне срчане активности). Перфузија и електрична стимулација су значајно побољшале састав и структуру ткива, јачину и синхронизацију контракција у одговору на електричне сигнале. Током само 8 дана култивације, добијено је ткиво које има низ особина нормалног срчаног мишића, а подржава тезу да системи за култивацију ткива треба да имитирају, у највећој могућој мери, услове који постоје током нормалног развоја.

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