Biomaterials 35 (2014) 3819–3828

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Robust T-tubulation and maturation of cardiomyocytes using tissue-engineered epicardial mimetics

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ARTICLE INFO

Article history:
Received 4 January 2014
Accepted 20 January 2014
Available online 6 February 2014

Keywords:
Cardiac tissue engineering
Hydrogel
Calcium
Electrophysiology
T-tubules

ABSTRACT

Complex three-dimensional (3-D) heart structure is an important determinant of cardiac electrical and mechanical function. In this study, we set to develop a versatile tissue-engineered system that can promote important aspects of cardiac functional maturation and reproduce variations in myofiber directions present in native ventricular epicardium. We cultured neonatal rat cardiomyocytes within a 3-D hydrogel environment using microfabricated elastomeric molds with hexagonal posts. By varying individual post orientations along the directions derived from diffusion tensor magnetic resonance imaging (DTMRI) maps of human ventricle, we created large (2.5 × 2.5 cm^2) 3-D cardiac tissue patches with cardiomyocyte alignment that replicated human epicardial fiber orientations. After 3 weeks of culture, the advanced structural and functional maturation of the engineered 3-D cardiac tissues compared to age-matched 2-D monolayers was evident from: 1) the presence of dense, aligned and electromechanically-coupled cardiomyocytes, quiescent fibroblasts, and interspersed capillary-like structures, 2) action potential propagation with near-adult conduction velocity and directional dependence on local cardiomyocyte orientation, and 3) robust formation of T-tubules aligned with Z-disks, co-localization of L-type Ca^{2+} channels and ryanodine receptors, and accelerated Ca^{2+} transient kinetics. This biomimetic tissue-engineered platform can enable systematic *in vitro* studies of cardiac structure–function relationships and promote the development of advanced tissue engineering strategies for cardiac repair and regeneration.

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1. Introduction

The complex anisotropic structure of the native myocardium, including spatially varying 3-D orientations of myocardial fibers [1,2], governs coordinated electrical activity and efficient pumping of the heart. Conversely, abnormalities in cardiac tissue structure caused by myocardial diseases or congenital defects can severely compromise cardiac function including induction of lethal arrhythmias [3,4]. To investigate the roles of cardiac micro- and macrostructure in action potential conduction *in vitro*, we previously combined high-resolution cell micropatterning and diffusion tensor magnetic resonance imaging (DTMRI) to create 2-D cultures (monolayers) of neonatal rat cardiomyocytes replicating realistic structure of ventricular tissue [5]. Electrophysiological studies in these cultures revealed that intrinsic variations in intramural cardiac fiber orientation underlie the spatial non-uniformity of action potential conduction and directly determine the likelihood, location, and spatiotemporal dynamics of conduction block [6,7].

While these and many other studies in 2-D cardiomyocyte cultures [8–11] have provided important insights into cardiac function and pathology, the use of 2-D cell cultures is highly limited in its ability to faithfully represent the natural 3-D microenvironment of native tissue. In particular, 2-D cultured cardiomyocytes are firmly adhered to a rigid substrate which dramatically alters their shape and mechanical loading, and in turn can adversely affect their differentiation, hypertrophy, and electromechanical function [12,13]. Furthermore, concentrations of extracellular soluble factors, oxygen content, and cellular composition (e.g. absence of microvasculature) in cultured cardiac monolayers significantly differ from those of native myocardium. The above differences from *in vivo* environment are believed to yield a relatively “immature” phenotype of 2-D cultured primary or pluripotent stem cell-derived cardiomyocytes which regardless of the culture duration never attain true rod shape, membrane T-tubules, or polarized intercellular junctions characteristic of adult tissue [14–17].

Over the last fifteen years, various 3-D cardiomyocyte culture systems have been utilized to better reproduce the native tissue microenvironment *in vitro*; however, methods to precisely control local and regional cell alignment within these engineered tissue constructs are still lacking. Furthermore, while cardiomyocytes in

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0142-9612/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2014.01.045

both 2-D and 3-D culture environments can exhibit a bi-nucleated, elongated, and striated phenotype [18–20], their ability to attain mature excitation–contraction coupling machinery, including the robust formation of T-tubules or co-localization of L-type Ca^{2+} channels and ryanodine receptors (RyR), have not been previously shown. Building on our previous work in 2-D cardiac monolayers [5], we set to develop a versatile fabrication approach to generate relatively large (2.5×2.5 cm^2) 3-D cardiac tissue patches in which cardiomyocytes are locally aligned to reproduce DTMRI-measured orientation of epicardial fibers from human ventricle. We hypothesized that compared to age-matched 2-D monolayers, this tissue-engineered cardiomimetic 3-D environment will significantly promote structural and functional maturation of neonatal rat cardiomyocytes toward the adult phenotype. To test this hypothesis, we systematically compared 2-D and 3-D cardiomyocyte cultures with respect to T-tubulation, distribution of L-type Ca^{2+} channels and RyRs, capillary formation, action potential propagation, and generation of Ca^{2+} transients.

2. Materials and methods

A detailed description of experimental methods is provided in the Online data supplement.

2.1. Tissue mold fabrication

Elastomeric polydimethylsiloxane (PDMS) molds were designed to allow reproducible generation of cardiac tissue patches with anatomically accurate cell orientations based on the 3-D DTMRI fiber direction map of a human ventricle (kindly provided by Drs. Helm, Winslow, and McVeigh via http://www.ccbm.jhu.edu). Briefly, epicardial projections of DTMRI-measured fiber direction vectors were represented by 2 mm long, 200 μm wide hexagonally shaped quivers and printed on a transparence photomask (Fig. 1A–D). The high aspect-ratio soft

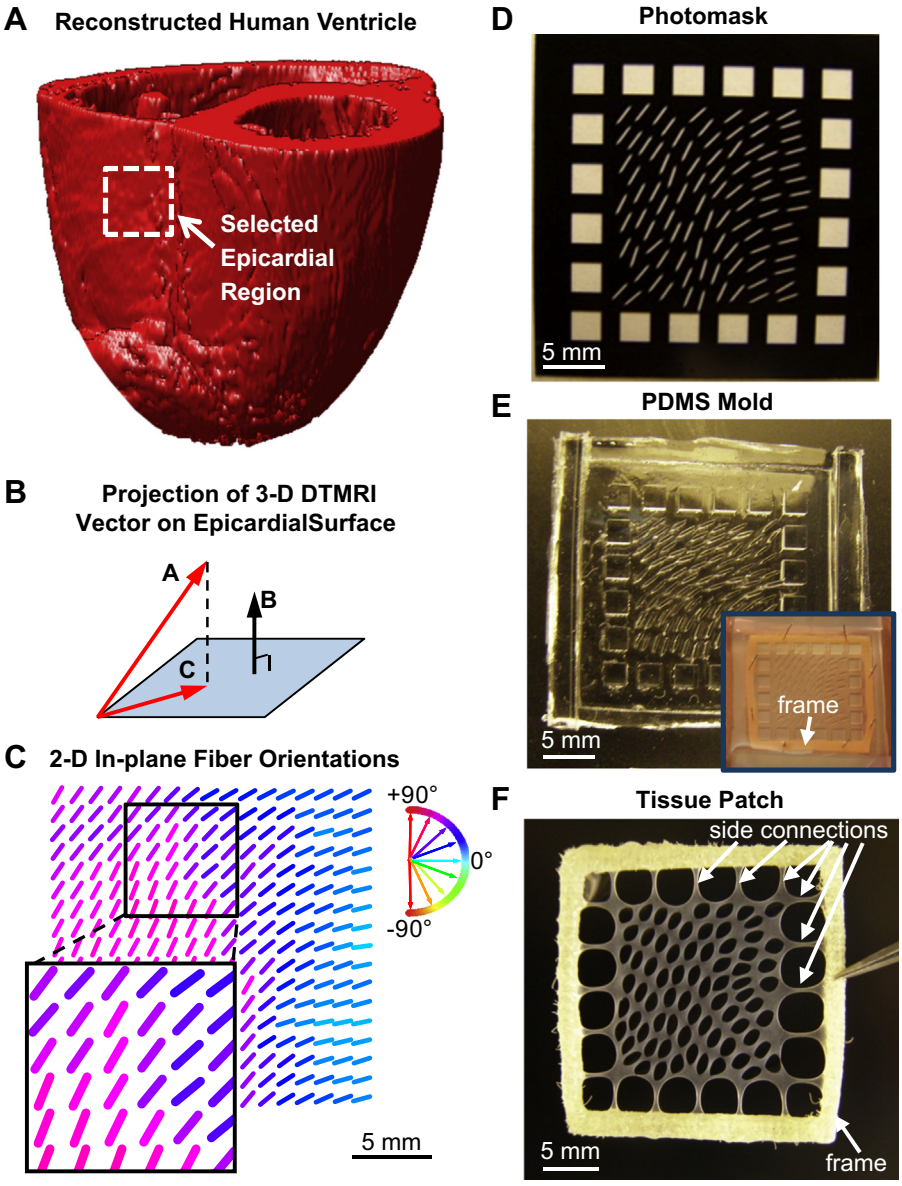

Fig. 1. Fabrication of 3-D cardiac tissue patches with DTMRI-derived human epicardial fiber orientations. A) A human ventricle reconstructed from DTMRI data by surface rendering. B) Schematic showing a vector projection **C** of a 3-D DTMRI fiber orientation vector **A** onto the epicardial surface (with surface normal vector **B**). C) Map of in-plane fiber orientation vectors from the selected epicardial region in A. D) Corresponding photomask with transparent 2-D quivers positioned along the streamlines generated from the vector map in C. E) PDMS mold with hexagonal posts corresponding to photomask quivers in D. Inset, Velcro® frame pinned within the PDMS mold. F) Resulting 3-week-old tissue patch removed from the PDMS mold by a pair of forceps. The patch is attached to the frame by thin side connections that allow easy detachment by scissors.

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