Tension Development and Nuclear Eccentricity in Topographically Controlled Cardiac Syncytium

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Abstract. The goal of this study was to use topographic control by microfabricated scaffolds with 3-dimensional surfaces to induce active tension development and enhanced contractility in engineered cardiac syncytium (a high density cardiac cell structure with reconstituted cell-to-cell connections and synchronized tissue-like behavior). Deeply microgrooved (feature height 50μm) elastic scaffolds were designed using polydimethylsiloxane molding, and neonatal rat cardiomycocytes were grown on them to confluence. Engineered cardiac cell constructs on the topographically modified (T) scaffolds showed higher order of intra and intercellular organization (fiber-like structures) compared to those grown on various flat surfaces (F), and developed self-organized persistent electrical and mechanical activity. These structural and functional changes were accompanied by a statistically significant ($p < 0.001$) increase in nuclear eccentricity (mean ± S.E.: 0.79 ± 0.01 $n = 137$ in T vs. 0.64 ± 0.01 $n = 863$ in F), and a preferential nuclear orientation, deviating from the axis of the grooves at a shallow angle. The orientation of the nuclei correlated well with the actin fiber arrangement in the T-samples, as well as with the direction of maximum displacement. Topography-induced nuclear deformation, a sign of tension development, implies further structural changes in transcription and cell signaling. In conclusion, we demonstrate topographic control of electromechanics in engineered cardiac syncytium, without external mechanical or electrical stimulation. These findings suggest a possibility to use controlled microenvironments in the design of biological autonomous force generators with reconstituted excitable tissue.

Key Words. topography, cardiomycocytes, microfabrication, cardiac tissue engineering

Introduction

Engineering functional cardiac tissue \textit{in vitro} is an ambitious actively pursued endeavor (Zimmermann et al., 2002; Fink et al., 2000). Along with the obvious clinical benefit of the eventual final product (tissue repair for the human heart), a few potential \textit{in vitro} applications seem of no lesser importance and interest. One of them is the use of successfully engineered cardiac tissue for drug development—target screening and validation of potential pharmacological compounds (Nave et al., 2002). Another interesting application of engineered excitable and contractile tissue (such as cardiac muscle) stems from its potential (Kakugo et al., 2002) to be used as a biological machine for performing useful work with high efficiency. Achieving the desired functionality through minimum external intervention, relying on the self-organizing properties of biological matter, is a laudable, yet insufficiently explored approach.

Previous research has demonstrated the feasibility of perturbing cell function and survival by controlling the cellular microenvironment (Chen et al., 1997). In the context of cardiac tissue, cell patterning and guidance have been applied by manipulating the extracellular matrix (ECM) proteins: micropatterns of desired shape and size (Rohr et al., 1991) or oriented gels (Simpson et al., 1994) invoked reciprocal structural and functional cellular responses. Most of these studies dealt with 2-dimensional preparations. Topographic control within a true 3-dimensional setting is expected to promote higher order of organization. Several groups (Clark et al., 1990), including one (Deutsch et al., 2000) dealing with primary cardiac cells at low density, have pointed out the ability of the cells to make use of topographic features for out-of-plane support. Our goal in this study was to engineer cardiac syncytium on microfabricated topographically complex scaffolds. We anticipated that the 3-dimensional landscape, combined with such a well-connected network of excitable contractile cells would bring about unique system behavior, including the promotion of organized electrical and mechanical activity. We characterize several aspects of the topography-induced structural and functional changes, focusing on nuclear...
deformation as a consequence of the increased level of organization and enhanced inherent mechanical activity, and with important implications for re-programming the cells.

This work has been presented before in an abstract form (Entcheva and Bien, 2002).

Methods

Scaffolds and topography
Several different scaffold materials were used in this study. As flat rigid surfaces for cell growth we used glass and polyvinylchloride (both from VWR, West Chesler, PA), polymethylsiloxane (PDMS) and cellulose-acetate (CA) surfaces. PDMS scaffolds (Sylgard 184 from Dow Corning, Midland, MI) were prepared in the usual ratio of 1:10 of curing agent to elastomer and baked for 2 hours at 60 °C. CA membranes were prepared from cellulose acetate, triethyl citrate and acetone (all from Aldrich, St. Louis, MD) and let form thin membranes over 4–5 hours. To investigate the effects of topography on cellular growth and function, PDMS scaffolds were constructed by molding from premanufactured micro-grooved masters. The resultant scaffold surface had regularly spaced features of uniform height with trapezoidal grooves and triangular ridges. The features, explored here, are significantly larger than those examined in previous studies (Deutsch et al., 2000). Cross sections of scaffolds were imaged under conventional microscopy to confirm topographical features (Figure 1A).

Cardiac syncytium
Cardiomycocytes were cultured from neonatal rat hearts as described elsewhere (Entcheva et al., 2000). Briefly, cardiomyocytes were isolated from the ventricles of 3-day old rats by enzymatic digestion with trypsin and collagenase. Cells were plated at high density (0.9 × 10⁶ cells per ml) onto fibronectin-coated scaffolds to allow the reconstitution of cell-to-cell contacts and the establishment of a functional syncytium. Electrical cell-to-cell connections were confirmed by gap junctional (Connexin 43) staining (Bien et al., 2002) and assessment of multicellular behavior. The engineered cell constructs (Figure 1B) were maintained at 37 °C with 5% CO₂ in medium 199 supplemented with 2% fetal bovine serum.

Electromechanical measurements
Electromechanical measurements were performed at day 3–6 after culturing, under perfusion with oxygenated Tyrode’s solution at 32–35 °C. Intracellular calcium levels were determined using a ratiometric fluorescence measurement technique, as described previously (Bien et al., 2002). Cells were stained with 10 μM of the calcium-sensitive dye Fura-2 (Molecular Probes, Eugene, OR) at room temperature for 20 minutes, and unincorporated stain was washed out for another 20 minutes. Samples were then imaged using an inverted fluorescence microscope (Nikon 20 × Fluor objective, NA 0.75) with excitation at 365 nm and 380 nm, and the fluorescence intensity determined through a 510 nm band-pass filter using a photo-multiplier detector (IonOptix, Milton, MA). Cell length was tracked simultaneously at long wavelengths using fast (250 Hz) imaging with a CCD camera and software video-trackers of cell edges along the axis of deformation. Movies of multicellular deformation were recorded using a Nikon Coolpix 950 camera attached to the eyepiece of the microscope.

Fluorescent labeling and confocal microscopy
At day 7 of culture cardiomyocytes were fixed in 3.7% formaldehyde and permeabilized with 0.02% Triton-X 100. Cells were co-stained with phalloidin-Alexa 488 (Molecular Probes) for F-actin and with TOTO-3 (Molecular Probes) for nuclei. Samples were then mounted on a glass slide with VectaShield (Vector Laboratories, Burlingame, CA) and imaged with a confocal scanning laser microscope BioRad Radiance 2000 with a 60 × objective (N.A. 1.4), excited simultaneously at 488 nm for actin and 637 nm for TOTO-3. Emission at 535 nm was mapped to the green channel and 680 nm to the blue channel in a 24-bit RGB format containing 1,024 × 1,024 pixels representing 195 μm × 195 μm area. Only the blue channel, reflecting TOTO-3 staining, was used for nuclear analysis as an intensity scale.

Automated analysis of nuclear morphology
We developed and applied an automated technique for nuclear morphology analysis using the confocal images. After thresholding a grayscale nuclear image using Otsu’s method (Otsu, 1979), median spatial filtering