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Spatiotemporal control of cardiac anisotropy using dynamic nanotopographic cues

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ABSTRACT

Coordinated extracellular matrix spatiotemporal reorganization helps regulate cellular differentiation, maturation, and function *in vivo*, and is therefore vital for the correct formation, maintenance, and healing of complex anatomic structures. In order to evaluate the potential for cultured cells to respond to dynamic changes in their *in vitro* microenvironment, as they do *in vivo,* the collective behavior of primary cardiac muscle cells cultured on nanofabricated substrates with controllable anisotropic topographies was studied. A thermally induced shape memory polymer (SMP) was employed to assess the effects of a 90° transition in substrate pattern orientation on the contractile direction and structural organization of cardiomyocyte sheets. Cardiomyocyte sheets cultured on SMPs exhibited anisotropic contractions before shape transition. 48 h after heat-induced shape transition, the direction of cardiomyocyte contraction reoriented significantly and exhibited a bimodal distribution, with peaks at ~45 and −45° ($P < 0.001$). Immunocytochemical analysis highlighted the significant structural changes that the cells underwent in response to the shift in underlying topography. The presented results demonstrate that initial anisotropic nanotopographic cues do not permanently determine the organizational fate or contractile properties of cardiomyocytes in culture. Given the importance of surface cues in regulating primary and stem cell development, investigation of such tunable nanotopographies may have important implications for advancing cellular maturation and performance *in vitro,* as well as improving our understanding of cellular development in response to dynamic biophysical cues.

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1. Introduction

The human extracellular matrix (ECM) is composed of a myriad of protein polymers, signaling molecules, proteases, and growth factors that are specifically tailored, in terms of architecture and composition, to every organ in the body and have pivotal roles in the correct remodeling of each tissue [1]. The ECM forms a dynamic microenvironment that promotes temporal changes in tissue structure to help regulate key cell functions and mechanisms [2,3]. During embryogenesis, the ECM is particularly important because the spatiotemporal regulation of cell migration, reorganization, and differentiation must be finely coordinated to enable the formation of complex anatomic structures [4]. During heart development specifically, ECM signaling and structural cues play a pivotal role in promoting the development of structural and functional anisotropy, which are critical for enabling effective blood ejection from the ventricles. Given the close association between structural signaling and cardiac physiological function, *in vitro* studies of the mechanisms that underpin cardiomyocyte maturation and performance would greatly benefit from the development of culture environments that more closely recapitulate the dynamic nature of the cardiac microenvironment.

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The ability for substrate nanotopography to control cellular and multicellular form and function *in vitro* has been widely investigated [5–11]. In particular, cardiomyocytes are known to develop highly organized and aligned cardiac cell sheets when cultured on anisotropic nanogrooved substrates. In these instances, engineered nanotopographical substrates are designed to mimic the architecture of the native myocardial ECM, which is comprised of highly aligned collagen fiber bundles with an average diameter of 120–150 nm [6]. Other engineered platforms have been designed to investigate the effect of the local density of ECM nanotopographic cues on cellular function, and have shown preferential cell migration towards an optimal topographic density [8,12,13]. While these engineered substrates are able to recapitulate the static architecture of the native myocardium or variations in topography, they do little to recreate the dynamic reorganization events that are known to occur throughout the developmental process [14,15]. With recent advances in smart biomaterials, it is now possible to design and fabricate cell culture substrates with dynamic nanoscale topographies to investigate the ability of changing substrate cues to influence cellular development [5].

Shape memory polymers (SMPs), a division of smart biomaterials, are capable of dynamic shape or surface topography transitions as induced by a range of external stimuli. These stimuli include photo irradiation, electromagnetism, pH, and temperature [16–23]. SMPs have been used to demonstrate that a cell loses its cytoskeletal structure in response to degradation of the underlying substrate topography [24–26]. For example, Davis et al. demonstrated that C3H/10T1/2 mouse embryonic fibroblasts cultured on a micropatterned topography aligned with the pattern direction. However, upon shape transition to an unpatterned flat substrate the cells devolved to random alignments [26]. While studies such as these provide interesting observations of dynamic cellular responses to topographic changes, they have so far relied exclusively on microscale topographies, which do not accurately mimic native ECM cues [8]. These studies have also only interrogated alignment at the single-cell level, which neglects tissue level organization and cell–cell cross-talk. Additionally, they have not characterized cellular functionality, which is an important phenotypic indicator of cellular development. Furthermore, these studies assessed the response of motile cells to changes in surface topographies, and therefore did not investigate cellular repolarization. Dynamic regulation of functional cellular characteristics using SMP cell culture platforms on the nanoscale remains an important advancement in the field of smart biomaterials as it enables more accurate modeling of the cell's dynamic extracellular physical environment. Analysis of confluent cell sheets and direct measurement of functional performance on these novel surfaces are of particular importance for cardiac bioengineering as they are necessary for accurately recapitulating native tissue development.

In this study we investigated the role of dynamic anisotropic nanogrooves in regulating the cytoskeletal and focal adhesion alignment as well as contractile function of primary cardiac muscle cells in monolayers. A thermally induced poly(ε-caprolactone) (PCL) SMP with anisotropic nanotopography was engineered to transition its orientation by 90° when exposed to a change in temperature (from here on termed shape transition). The nanoscale cues employed in these experiments were designed to recapitulate the size and orientation of collagen fiber bundles present within the native ECM of human myocardial tissue [6]. The transition temperature of the PCL substrate was tuned to 33 °C, which enabled shape transition to occur at a biologically suitable temperature [27]. Using this platform, the effect of temporal changes in anisotropic nanotopography on the collective contraction orientation of primary cardiac muscle cells was assessed.

2. Materials and methods

2.1. Fabrication of cross-linked PCL films

The PCL films were prepared by cross-linking tetra-branched PCL with acrylate end-groups in the presence of linear PCL telechelic diacrylates according to a previously reported protocol [27,28]. The average degrees of polymerization of each branch for two- and four-branch PCL were 18 and 10, respectively. The equimolar amounts of PCL macromonomers were then dissolved at 45 weight % in xylene containing 2-fold molar excess benzoyl peroxide (BPO; Sigma–Aldrich, St. Louis, MO, USA) to the end-group of macromonomers. To fabricate a PCL film with permanent topography, the macromonomer solution was dispensed onto a silicon master (400 nm_500 nm_150 nm, ridge width_groove width_-depth) with a 0.1 mm thick Teflon spacer and bound by two glass slides (Fig. 1Aand Bi.). Thermal polymerization was carried out at 80 °C for 4 h (Fig. 1Bii) and the PCL film was then lifted off (Fig. 1Biii.). The thermal and mechanical properties of the cross-linked materials made from 1:1 2b20/4b10 macromonomer solution have been characterized previously [27].

2.2. Fabrication of shape memory polymer with orthogonal nanogrooves

For the study of dynamic contractile orientation, a temporary pattern, which was orthogonal to the permanent pattern, was developed. Permanent patterns were used as a control. As seen in Fig. 1Biv, the temporary pattern was heated for 2 min at 80 °C and then placed onto a silicon master mold, with the patterns of the film and mold offset by 90°. Then, the silicon master and polymer were heated again for 2 min at 80 °C, and then bound together with a pressure of 0.1 MPa (Fig. 1Bv.). The system was then cooled for 1 h at 4 °C, and the polymer was then lifted off (Fig. 1Bvi.).

2.3. Atomic force microscopy (AFM) of shape memory polymer nanotopography

The surface morphologies of cross-linked PCL films were observed by AFM (SPM-9500J3, Shimadzu Co., Kyoto, Japan) with non-contact mode using a Si_3N_4 cantilever (spring constant; 42 N/m, Nano World, Neuchâtel, Switzerland). The sample temperature was controlled using a thermo controller. The cross-linked PCL films were heated and equilibrated at 32 °C and 37 °C for 1 h, and AFM measurement was performed to obtain surface topography and height images.

2.4. Real-time topographical observation of shape transition using digital holographic microscopy

The time-dependent surface shape-memory transition of cross-linked PCL films was also observed by holographic microscopy (Lyncée tec R2100, Switzerland). Time-dependent observation in response to temperature change was conducted on a microwarm plate (KM-3, Kitazato Supply CO., Ltd, Sizuoka, Japan). The setting temperature of the microwarm plate was increased from 36.5 to 38.0 °C in air, and continuous topographical transition at the surface was observed.

2.5. Cardiomyocyte cell culture on PCL SMP substrates

Before cell culture, PCL films were sterilized by incubating for 20 min in ethanol at 4 °C, plasma treating (100 W, 0.05 Torr, and 5 min), and UV sterilizing for 1 h. The films were then incubated overnight with a fibronectin surface treatment at a concentration of