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Valve interstitial cell shape modulates cell contractility independent of cell phenotype

Ishita Tandon [1], Atefeh Razavi [1], Prashanth Ravishankar, Addison Walker, Nasya M. Sturdivant, Ngoc Thien Lam, Jeffrey C. Wolchok, Kartik Balachandran *

Department of Biomedical Engineering, University of Arkansas, Fayetteville, AR 72701, USA

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ABSTRACT

Valve interstitial cells are dispersed throughout the heart valve and play an important role in maintaining its integrity, function, and phenotype. While prior studies have detailed the role of external mechanical and biological factors in the function of the interstitial cell, the role of cell shape in regulating contractile function, in the context of normal and diseased phenotypes, is not well understood. Thus, the aim of this study was to elucidate the link between cell shape, phenotype, and acute functional contractile output. Valve interstitial cell monolayers with defined cellular shapes were engineered via constraining cells to micropatterned protein lines (10, 20, 40, 60 or 80 μm wide). Samples were cultured in either normal or osteogenic medium. Cellular shape and architecture were quantified via fluorescent imaging techniques. Cellular contractility was quantified using a valve thin film assay and phenotype analyzed via western blotting, zymography, and qRT-PCR. In all pattern widths, cells were highly aligned, with maximum cell and nuclear elongation occurring for the 10 μm pattern width. Cellular contractility was highest for the most elongated cells, but was also increased in cells on the widest pattern (80 μm) that also had increased CX43 expression, suggesting a role for both elongated shape and increased cell-cell contact in regulating contractility. Cells cultured in osteogenic medium had greater expression of smooth muscle markers and correspondingly increased contractile stress responses. Cell phenotype did not significantly correlate with altered cell shape, suggesting that cellular shape plays a significant role in the regulation of valve contractile function independent of phenotype.

1. Introduction

Cardiac valves maintain their integrity and function by a complex interplay between the tissue, cells, and surrounding hemodynamic and mechanical environment (Chester and Taylor, 2007). Valve interstitial cells (VICs) are dispersed within the valve leaflets and are crucial in regulating valve matrix composition, function, and disease progression (Taylor et al., 2003). VICs exist as a heterogeneous spectra of phenotypes (Chester and Taylor, 2007; Liu et al., 2007; Taylor et al., 2003). During valve disease, pathogenic stimuli results in activated and osteoblastic phenotypes, potentiating remodeling or further disease progression, respectively (Liu et al., 2007). Another class of smooth-muscle like VICs that appear to be involved in mediating an active contractile response within the tissue (El-Hamamsy et al., 2009; Taylor et al., 2003). Most prior studies have focused on the role of the VIC phenotype in modulating valve function and disease progression. These studies have reported the role of chemical mediators like bone morphogenic proteins, nitric oxide, and the notch pathway in regulating transition to an osteoblastic VIC (Chen et al., 2015; Farrar et al., 2015; Li et al., 2013; Walker et al., 2004; Xu and Gotlieb, 2013). Others have reported the role of mechanical stimulation in potentiating changes in the VIC phenotype (Balachandran et al., 2011, 2009, 2010; Chen et al., 2015; Gould et al., 2013; Hutcheson et al., 2012; Ku et al., 2006; Stephens et al., 2011; Sucosky et al., 2009). An added recent paradigm are the implications of VIC phenotype on its contractile function (Balachandran et al., 2012; El-Hamamsy et al., 2009; Merryman et al., 2006). Relatively few studies discuss the role of VIC shape in regulating its contractile function or biological phenotype.

Cellular shape, enforced by constraining the boundary conditions of cell spreading, potentiates altered cellular function in other cell types. For instance, capillary endothelial cells cultured on single large circles (20 μm) had a significantly greater apoptosis than when cultured on multiple smaller circles (Chen et al., 1997). Rate of proliferation also depended on geometry of the cell

* Correspondence to: Kartik Balachandran, Ph.D., Department of Biomedical Engineering, University of Arkansas, 122 John A. White Jr. Engineering Hall, Fayetteville, AR 72701, USA. Fax: +1 479 575 4346.
E-mail address: kbalacha@uark.edu (K. Balachandran).
[1] Both authors contributed equally.

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monolayer (Nelson et al., 2005). It was also reported that nuclear shape was regulated by the tension in actomyosin fibers that expanded as cells became more elongated, suggesting an overall balance of intracellular forces (Versaevel et al., 2012). Several recent papers have extended these structural alterations in cells to pro-inflammatory phenotype (McWhorter et al., 2013), cell differentiation (McBeath et al., 2004), and cell motility (Parker et al., 2002). In the context of VICs, it has been acknowledged that VICs alter their shape in response to externally applied mechanical load (Sacks et al., 2009), and that applied load can affect phenotype. In light of these earlier studies, it is therefore important to understand how VICs alter their function and phenotype in response to changes in cell shape and architecture.

We hypothesized that cellular shape directly affects acute contractile function in VICs. To test this hypothesis, we utilized a protein microcontact printing technique (Balachandran et al., 2011) to engineer VIC monolayers constrained with control over cellular shape. VIC contractility was quantified using a valve thin film cantilever assay, and phenotype analyzed via western blots, gelatin zymography, and qRT-PCR. We report that contractility, normalized per cell, was maximum for an elongated cell shape, but also increased in the wider patterns that had increased cell-cell contact as evidenced by increased expression of connexin-43. Cells cultured in osteogenic medium had increased cellular contractility. Cell shape did not have any effect on cell phenotype. Taken as a whole, our results suggest that cell shape plays a significant role in moderating VIC function, and that phenotype measurements alone may not accurately indicate VIC function.

2. Methods

2.1. Valve interstitial cell isolation and culture

Fresh porcine hearts (3–6 months old) were obtained from Cockrum's Custom Meat Processing (Rudy, AR) and transported to our laboratory in cold Dulbecco's Phosphate Buffered Saline (dPBS; Gibco, Carlsbad, CA) supplemented with 1% antibiotic/antimycotic solution. Hearts were immediately dissected aseptically. Left, right, and non-coronary aortic valve leaflets were pooled and washed in Hank's Balanced Salt Solution (HBSS; Gibco). Cells were isolated using collagenase digestion as described in previous protocols (Butcher and Nerem, 2004; Butcher et al., 2004; Gould and Butcher, 2010). Cells from passages 2–6 were used in all subsequent experiments.

2.2. Cell shape model

2.2.1. Photolithography

Photolithographic transparency masks were designed in AutoCAD (Autodesk Inc., San Rafael CA) and fabricated by CAD/Art Services Inc (Bandon, OR). The design of the masks comprised of a regular array of line patterns with five different widths (10, 20, 40, 60, and 80 μm) spaced 40 μm apart. Silicon wafers (Wafer World, West Palm Beach, FL) were then spin coated with Su-8 2005 negative photoresist (MicroChem Corp., Westborough, MA) and exposed to ultraviolet light through the photomask and developed using standard photolithographic protocols (Kane et al., 1999; Qin et al., 2010).

2.2.2. Soft lithography and cell culture

Fibronectin (Corning; 100 μg/mL) was incubated on a polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning) stamped with aforementioned microscale raised patterns for 1 h at room temperature and gently blown dry. The stamp was then placed in contact with Ultraviolet-Ozone (Novascan, Ames, IA) treated PDMS-coated 25 mm no. 1 coverslips for 10 minutes. Coverslips were then blocked for 1 min with 1% Pluronics F-127 (Sigma-Aldrich, St. Louis, MO) to prevent cell attachment onto the areas without fibronectin patterns (Fig. 1A). Fibronectin was chosen for its ease of creating high-fidelity and uniform microscale cell boundaries (Balachandran et al., 2011). Additionally, others have demonstrated that fibronectin did not significantly alter VIC phenotype compared to collagen substrates (Latif et al., 2015). Fibronectin pattern uniformity was also characterized using image analysis techniques familiar to our group (Supplementary Fig. S3) (Balachandran et al., 2011). The coverslips were washed three times with dPBS and VICs were seeded at 100,000 cells per cm^2 coverslip area in normal medium as before or in pro-osteogenic medium (normal culture medium supplemented with 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid) (Balachandran et al., 2010) and cultured at 37 °C, 5% CO_2. Cells were constrained to the fibronectin patterned portion of the substrate and spontaneously formed confluent monolayers of differing pattern widths within those boundaries (Fig. 1B).

2.3. Characterization of cellular shape and architecture

After 48 h of culture, cells were fixed with 4% PFA/1% Triton X-100 and fluorescently labeled with 1:200 Alexa Fluor 488 phalloidin (Life Technologies, Carlsbad, CA), and 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Life Technologies). Samples were mounted onto a glass slide and imaged using a custom-built resonant-scanning multiphoton microscopy platform with a 40X, 0.8 NA water immersion objective (Nikon, Japan) and a MaiTai ultrafast Ti:Sapphire tunable laser source (Spectra-Physics, Santa Clara, CA). The laser excitation source was tuned to 750 nm (DAPI-stained samples) or 960 nm (Alexa-Fluor 488-stained samples) and images

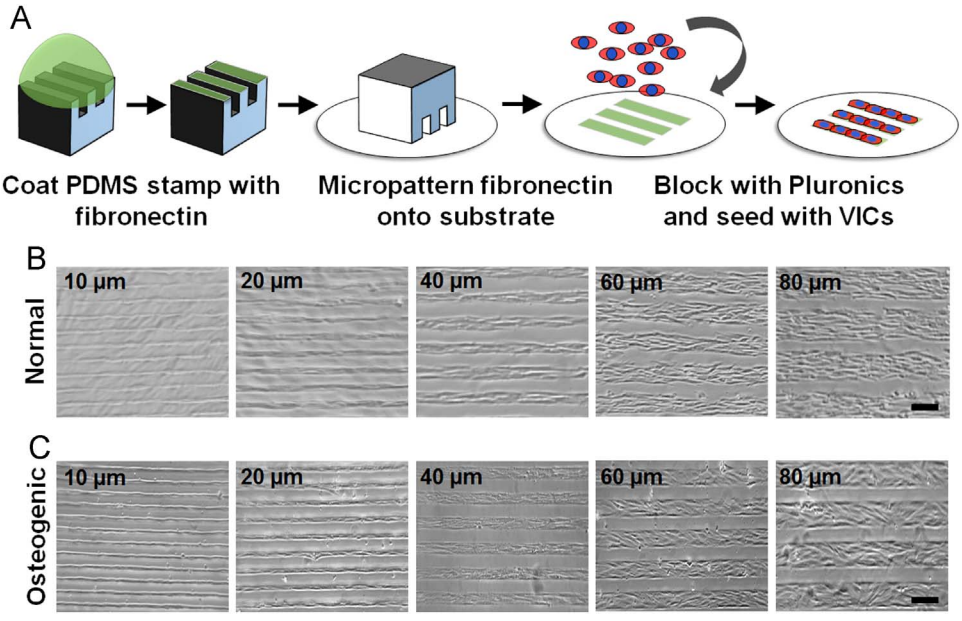

Fig. 1. *In vitro* model for controlling VIC shape. (A) Schematic representation of microcontact printing protocol. Phase contrast microscope images showing formation of VIC micro-tissues in (B) normal and (C) osteogenic media after 48 h of cell culture. (Scale bar = 80 μm).

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