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Research Paper

Micro-composite substrates for the study of cell-matrix mechanical interactions

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ABSTRACT

The chemical and physical gradients in the native cell microenvironment induce intracellular polarization and control cell behaviors such as morphology, migration and phenotypic changes. Directed cell migration in response to substrate stiffness gradients, known as durotaxis or mechanotaxis, has drawn attention due to its significance in development, metastasis, and wound healing. We developed a microcomposite substrate (μCS) platform with a microfabricated base and collagen hydrogel top to generate physiological linear stiffness gradients without any variation in chemical or transport properties. This platform is compatible with both 2D and 3D cell culturing and can be assembled with common supplies found in most biology labs. Ligament fibroblasts (LFs) and mesenchymal stem cells (MSCs) both respond to the mechanical gradient with directed migration. Interestingly, LFs exhibit higher mechanosensitivity compared with MSCs. Polarized nonmuscle myosin IIB distribution was also found on the μCS gradient, confirming previous reports. This robust system provides an easily accessible platform to study cell mechanosensing and a more physiological microenvironment for cell studies.

1. Introduction

The in situ cell microenvironment is often nonhomogenous and anisotropic. The chemical and physical gradients in the extracellular milieu induce intracellular polarization and controls cell behaviors such as morphology, migration and phenotypic changes (Engler et al., 2006; Lo et al., 2000). Directed cell migration in response to chemical gradients, termed chemotaxis, has been widely studied in the context of development and metastasis (Cimetta et al., 2010; Wang et al., 1994). Directed cell migration in response to substrate stiffness gradients, known as durotaxis or mechanotaxis, has drawn attention recently (Lo et al., 2000; Raab et al., 2012; Tse and Engler, 2011; Wang et al., 2012). Other than the aforementioned context, durotaxis may also be involved in wound healing. For instance, the fibrin clot and platelet and myofibroblastic contractions at the wound site increase local stiffness, thus promoting cell migration toward the wound (Janmey et al., 2009). In addition to migration, substrate stiffness is known to influence cell morphology, cytoskeleton organization, proliferation and phenotypic expressions (Engler et al., 2006; Janmey et al., 2009; Klein et al., 2009; Yeung et al., 2005).

A number of approaches have been utilized to generate stiffness gradients for the study of durotaxis. Polyacrylamide crosslinking can be controlled via lithographic or concentration

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gradients to generate stiffness changes (Byfield et al., 2009; Tse and Engler, 2011). These technologies often require microfluidic devices or clean room access, which are not widely available to biologists. Moreover, changes in polyacrylamide crosslinking and density may change protein tethering behaviors, thus changing the mechanical feedback mechanisms (Trappmann et al., 2012). Another limitation of the polyacrylamide gel is that it is not compatible with 3D cell encapsulation due to its toxicity. Other polymer systems have been developed to control 3D stiffness (Khetan et al., 2013; Legant et al., 2010). However, to our knowledge, few studies have investigated polymeric systems of 3D stiffness gradients, due to the difficulty in controlling cell-polymeric interactions in 3D (Khetan et al., 2013). Hadjipanayi and coworkers generated a wedge-shaped collagen hydrogel, which was then compressed to a uniform thickness, thus resulting in a scaffold of varying density (Hadjipanayi et al., 2009). While cells are maintained in a 3D state in this system, they are subjected to inhomogeneous compression and increases in collagen and ligand density.

Alternatively, studies have utilized hydrogel thickness to modulate the effective surface stiffness (Leong et al., 2010). Mesenchymal stem cells (MSCs) on the thin collagen gels exhibit similar behaviors as those grown on hard substrates and the cells cultured on thick gels behaved similarly as those grown on soft substrates (Feng et al., 2013; Leong et al., 2010). Taking advantage of this approach, we generated a micro-composite substrate (μCS) with gradients by controlling collagen hydrogel thickness with precise microfabricated topography, without changes in collagen density. This approach is completely cell compatible and provides both 2D and 3D capabilities. Moreover, the homogeneous gel density ensures that, in the 3D configuration, cells of the same distance to the gel surface are in the same nutrient/waste environment as well as mechanical conditions. The resulting stiffness gradient is linear within the field of view using a standard 10× objective. Furthermore, the same concept can be applied to a variety of materials available in most life science labs to generate substrates of different stiffness or stiffness gradients.

This versatile and simple platform was validated using MSCs and ligament fibroblasts (LFs). LFs have been shown to express smooth muscle-actin, exhibit phenotypes of contractile myofibroblasts and migrate at wound sites (Murray et al., 2002; Murray and Spector, 1999; Unterhauser et al., 2004). In addition to linear gradients, we also generated patterns to produce a radial gradient to study more complex mechanical cues that may occur during mesenchymal condensation or scar formation (Ghosh et al., 2009; Mammoto and Ingber, 2010; Tse and Engler, 2011). 3D capability of our system was demonstrated by the durotactic behavior of MSCs and the mechanisms behind durotaxis in 2D and 3D were explored.

2. Materials and methods

2.1. Micro-composite substrate (μCS)

The composite substrate was prepared with collagen gel and PDMS (polydimethylsiloxane, Sylgard 184, Dow Corning, USA) microstructure. The PDMS microstructure was fabricated by soft lithography. Briefly, SU-8 2025 photoresist (MicroChem, USA) was spin-coated on a silicon wafer, exposed to UV light through a mask, and developed to form a master with feature heights of 60 μm. PDMS elastomer was prepared with 10% cross-linking agent in PDMS solution and casted on the master at 70 °C for 2 h. Collagen gel (BD, USA, rat tail type I, 1.5 mg/mL) was neutralized according to the manufacturer's protocol and immobilized on the PDMS microstructure by treating the PDMS surface with air plasma for 10 min (Harrick Plasma, USA) and incubated overnight at 37 °C. Plasma is believed to activate PDMS surfaces by changing its hydrophobicity, thus promoting protein immobilization (Chang et al., 2007). Collagen gel height was controlled with silicon spacers (100 and 400 μm thick) and cover glass. The 'window' in the spacers, in which the collagen gel periphery is defined, is 1 cm × 1 cm in size. In control studies, uniform thickness substrates were made by binding collagen gels of specified thickness to smooth PDMS substrates. Fig. 1A illustrates the design of the composite substrates.

2.2. Mechanical measurements

Effective modulus at the surface of composite substrate was determined by nano-indentation analysis using an atomic force microscope (AFM, AsylumResearch MFP-3D-BIO, USA) equipped with a Molecular Force Probe 3D controller. Soft silicon nitride cantilevers (TR400PB, Olympus, USA) with a pyramidal tip (nominated spring constant of 0.02 N/m) were calibrated by the thermal fluctuation method in PBS. The cantilever descended toward the collagen gel at a velocity of 1.98 μm/s to achieve an indentation depth of 1 μm. Force–distance curves were collected and analyzed according to the Hertz model (Rotsch et al., 1999; Rotsch and Radmacher, 2000). The gel was modeled with a Poisson ratio of 0.5 (Leong et al., 2010). Effective moduli across the substrates (parallel to gradient) were obtained every 100 μm. 20 repeated force curves on 10 locations obtained from three samples were used to calculate the average effective modulus for each type of substrate.

2.3. Cell culture

Ligament fibroblasts (LFs) were harvested from anterior cruciate ligaments (ACLs) of young porcine knees via enzymatic digestion. Ligament sections were diced and incubated in 0.2% collagenase solution (Invitrogen, USA) on a shaker overnight. After digestion, the mixture was passed through a 100 μm cell strainer and the isolated LFs were maintained without passaging. Human bone marrow-derived mesenchymal stem cells (MSCs) were kindly provided by Dr. Shih-Chieh Hung at National Yang-Ming University (Tsai et al., 2010). For all studies, MSCs were used at passages 11–20. Unless otherwise noted, cells were seeded at 7000 cell/cm^2 for LFs and 5000 cell/cm^2 for MSCs.

2.4. Cell displacement monitoring

For migration studies, cells were cultured overnight in the composite substrate and fitted into a modified migration chamber as previously described (Chao et al., 2000; Tandon

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