Microfabricated Nanogrooves to Promote in Vitro Muscle Fiber Differentiation and Maturation

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Primary CNF Tools Used: Plasma-Therm Unaxis DRIE, Oxford Cobra etcher, Heidelberg DWL 2000 Mask Writer, GCA AS200 i-line Stepper, Karl Suss MA6 Contact Aligner, Anatech SCE-110-RF Resist Stripper, P-7 Profilometer, MVD-100; Oxford 81 etcher; Unaxis 770 Deep Silicon Etcher; BLE150 Hotplate; EcoClean Asher; PDMS spin coater

Abstract:

Muscular dystrophy encompasses a group of devastating diseases affecting skeletal muscle in young children and resulting in reduced mobility and premature death. Gaining a better understanding of the underlying disease mechanism is crucial for developing effective therapies, which are currently lacking. One challenge in studying the pathogenesis of muscular dystrophies is that common in vitro models based on the differentiation of skeletal muscle stem cells (myoblasts) do not match the form and function of skeletal muscle tissue in vivo, including the highly aligned and organized muscle fibers. Culturing myoblasts on nanostructured surfaces that provide physical cues for the differentiating muscle cells has been shown previously to promote the formation of mature and highly aligned muscle fibers. Here, we demonstrate the use of microfabricated silicon wafers that serve as molds to generate polydimethylsiloxane (PDMS) membranes with ~700 nm wide and ~1300 nm deep nanogrooves. These PDMS substrates, when coated with Matrigel extracellular matrix, enable the differentiation of primary mouse myoblasts into mature and well-aligned skeletal muscle fibers, which we are now using as a model system to study Emery-Dreifuss muscular dystrophy and other muscle diseases caused by mutations in the LMNA gene.

Summary of Research:

Mutations in the LMNA gene, which encodes the nuclear envelope proteins lamin A and C (lamin A/C), cause Emery-Dreifuss muscular dystrophy, congenital muscular dystrophy, and dilated cardiomyopathy. Although lamin A/C is expressed in nearly every tissue, most LMNA mutations primarily affect striated muscle, i.e., skeletal and cardiac muscle. Both the molecular mechanism underlying the muscle-specific defects and the pathobiology of the disease remain incompletely understood, presenting a major obstacle in the development of effective therapies. While animal models for these diseases are available, they are limited in their ability to allow detailed cell and molecular level observations of the disease progression, which is required to identify pathogenic mechanisms. In vitro models, on the other hand, are well suited for imaging but do not capture the structure and function of mature muscle tissue, limiting their usefulness. To overcome this challenge, we developed PDMS-based in vitro cell culture substrates with nanostructured ridges that are ~700 nm wide, ~1300 nm tall, and separated by ~700 nm wide gaps, to resemble in vivo tissue architecture and promote muscle cell differentiation and alignment.

We first fabricate silicon wafers to serve as molds to cast the nanostructured PDMS substrates (Figure 1). Oir620-7i photoresist is spin-coated onto a 4-inch wafer and exposed to UV light using the GCA AS200 i-line stepper to imprint the pattern into the photoresist layer. After developing the photoresists using the 726 MIF, we perform a silicon etch with the Unaxis 770 Deep Silicon Etcher, removing silicon in the regions not covered by photoresist. After etching, the remaining photoresist is stripped, and a hydrophobic Teflon coating is applied to the wafer to prevent PDMS from sticking, thus making the wafer suitable as a mold to cast thin sheets of PDMS containing the desired nanostructures. We confirmed the desired dimensions of the ridges on the silicon wafer using the Zygo Optical Profiler and scanning electron microscopy (Figure 2). Subsequently, PDMS is spin-

coated onto the silicon wafer and cured by baking for >2 hours at 60°C. The PDMS sheets are then removed from the wafer and coated with Matrigel, an extracellular matrix molecule that supports muscle cells in vivo. Primary mouse myoblasts are cultured on the nanostructured PDMS substrate and induced to differentiate into muscle fibers, starting one day after plating. We use an Airyscan LSM900 confocal microscope to observe the cells and assess their alignment and maturity based on their sarcomeric structures. Figure 3 shows a representative image of wild-type muscle cells differentiated into >400 µm long muscle fibers with the characteristic striated sarcomere patterning and high alignment between muscle fibers.

Having established this experimental platform, we are now using it to compare LMNA mutant and wild- type muscle cells and determine the molecular mechanism responsible for the muscle defects. In addition to promoting muscle differentiation and alignment, the flexible PDMS substrate also enables us to apply mechanical strain to the muscle fibers, allowing us to test the hypothesis that the LMNA mutant muscle cells are more sensitive to mechanical stress, which could explain the muscle-specific defects of many laminopathies.

Taken together, this application illustrates new uses of the available nanofabrication technologies to create improved in vitro models to study normal muscle differentiation and muscle disease.

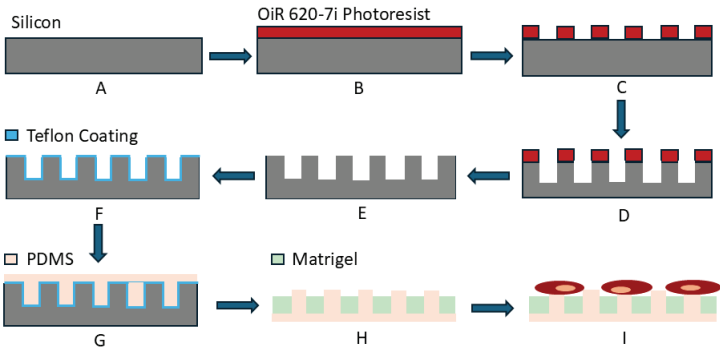

Figure 1: Nanofabrication process for nanostructured surfaces. A 4-inch n-type silicon wafer (A) is spin coated with OiR620-7i photoresist to 0.72 µm thickness (B), followed by exposure and development of the photoresist using the AutoStep 200 DSW i-line Stepper to create the desired patterns (C), which are then etched 1.3 µm deep into the silicon wafer using the Unaxis 770 system (D). After stripping the photoresist (E), a thin Teflon coating is applied (F) to prevent PDMS from sticking when cast onto the wafer (G). After removal of the PDMS substrate from the wafer, extracellular matrix, Matrigel, is applied to the wafer (H). Finally, primary muscle stem cells (myoblasts) are cultured on the substrate (I) and induced to differentiate into skeletal muscle fibers.

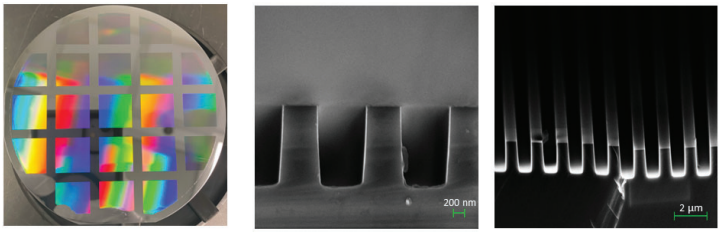

Figure 2: Characterization of the nanostructured silicon wafer. Photograph of the final wafer, with arrays of 1 cm × 1 cm squares containing the nanostructured ridges visible (left). Scanning electron microscope (SEM) images of the cross-section of the wafer are shown at high (center, scale bar = 200 nm) and low resolution (right, scale bar = 2 µm). The width of each ridge is around 660 nm, and the depth is 1.3 µm. The SEM images were taken on the Zeiss Ultra SEM.

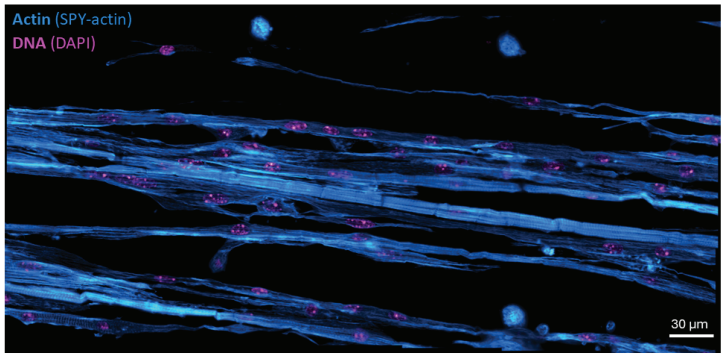

Figure 3: Differentiated skeletal muscle fibers on nanostructured substrate. Fluorescence microscopy image of primary mouse myoblast differentiated into muscle fibers and stained for DNA (DAPI) and actin (SPY-actin 555) to visualize sarcomeres, the contractile units of striated muscle.