Application of Cyclic Uniaxial Stretch: New Tools for Understanding the Role of the Extracellular Matrix in Cell Biology

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Summary

In this application note, the role and importance of the structural and mechanical components of Extracellular Matrix (ECM) are investigated and compared for C2C12 and U2OS cells. Cell morphology, alignment, and cytoskeleton organization was assessed for cells cultured on NanoSurface™ chamber substrates, both in the absence (flat) and in the presence (nanopatterned) of nanotopographical features. As shown here, cells tend to align perpendicular to the direction of stretch on flat substrates (in the absence of nanotopographical features) whereas in the case of nanopatterned substrates (in the presence of nanotopographical features), cells align in the direction of nanoscale physical features and disregard the direction of stretch.

Introduction

Mammalian cells sense and use the structural, mechanical and chemical cues from their immediate environment to drive a number of processes. Cells in the body, such as fat and muscle[1, 2], cardiomyocytes (circulation)[3] and epithelial cells (respiration)[4] are constantly subjected to varying mechanical loading environments, during both normal and exercise regimes. Additionally, the increasing evidence over the past few decades has shown that the Extracellular Matrix (ECM) plays a critical role in the differentiation, development, and function of various cell types. In addition to playing a vital role in determining the 'health' of a cell or tissue, the ECM plays a critical role in directing cell differentiation. Understanding the effects of these environmental cues on the regular cellular functions such as growth, migration, differentiation and cell-matrix interactions are, therefore, critical.

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With the ethical challenges and limitations associated with *in vivo* animal models, it becomes crucial to develop *in vitro* platforms capable of mimicking structural and functional phenotypes similar to native tissue. Recently, it has been demonstrated that nanoscale features in the cell's local environment can influence regular cell function and processes such as morphology, alignment, adhesion, migration, proliferation, and cytoskeleton organization. Thus, cell biologists and bioengineers have developed new tools—primarily adapted from the microelectronics industry— to support their efforts towards understanding the role that these cues play in controlling and directing cell function and signaling. Indeed, it has become clear that factors such as size, orientation, geometry, and physicochemical properties of the cell microenvironment contribute to biological function. Furthermore, the dynamic of the mechanical loading environment of cells is another factor that influence such diverse processes, both at the cellular and molecular levels[5, 6]. Here, we investigated the combined influences of the structural (nanopatterned ECM) and mechanical (application of cyclical stimuli) cues on the morphology, alignment, and cytoskeleton organization of different cell types.

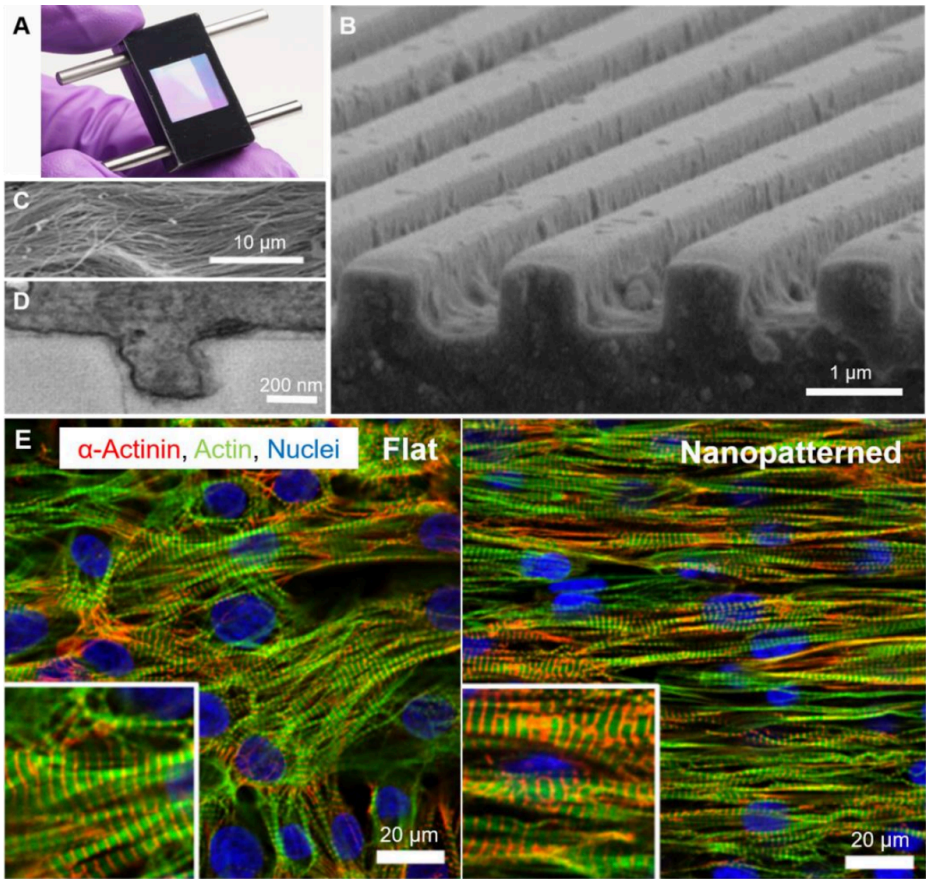

Figure 1: Structural impact of nanotopographical patterned substrates. *(A) NanoSurface™ Plates 96-well plate format features a glass bottom with nanopatterned surface topography.* ***(B)*** *Scanning Electron Microscopy image of the culture surface shows the highly-precise and accurate nanoscale features that are used to mimic the native extracellular matrix.* ***(C)*** *The aligned architecture of the underlying matrix of the native myocardium.* ***(D)*** *Small membrane invaginations in the scale of focal adhesions form on the grooved surface following cell adhesion on the substrate.* ***(E)*** *Heart cells cultured on NanoSurface Plates demonstrate enhanced structural and phenotypic development.*

Findings

Recent innovation in nanofabrication techniques coupled with the need for development of more physiologically relevant *in vitro* platforms have led to the introduction of more complex systems combining different components of the ECM. Here, we report on the delineation of the role and importance of each of the mechanical and structural cues of the ECM in directing C2C12 and U2OS cells organization. Cells were cultured on flat and nanopatterned substrates and their morphology and alignment was studies both in the presence

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and in the absence of mechanical loading environments. Unlike many of the previously reported approaches focused on controlling the structural aspect of the ECM, NanoSurface™ cell stretching chambers from Curi Bio (Seattle, USA) provides a precise nanotopography while maintaining compatibility with standard laboratory techniques (Fig.1A). The nanopatterned culture surface topography (Fig.1B) closely mimics the fibrillar structure of the ECM (Fig.1C). As adherent cells attach to the surface, membrane invaginations in the scale of a focal adhesion form on the grooved surface (Fig.1D). This structural patterning drives higherorder of the cytoskeletal and tissue structure similar to what is seen in native tissue, but is typically absent in cells grown on traditional glass and plastic cultureware (Fig.1E), promoting the development of a more physiologically relevant structural and functional phenotypes.

Figure 2: The Curi Cytostretcher. (A) *The Cytostretcher combines nanopatterning with stretchable elastomeric chambers, with a compact, fully-programmable instrument designed to survive the harsh incubator environment for weeks to months at a time.* ***(B)*** *The Cytostretcher-LV combines nanopatterning and cell stretching with high-resolution inverted optical microscopy, with optional CO_2 and temperature control.* ***(C)*** *A variety of stretching chamber formats are available; large formats designed for assays that require high levels of cells or proteins can be used, while smaller formats that support higher-throughput experimentation can be accommodated by the instrument design.*

In addition to controlling the physical shape of the cell's microenvironment, the dynamic of the mechanical loading environment of cells plays an important role in directing cell and tissue organization. Cells in the body sense a variety of mechanical stimuli – such as hydrodynamic shear flow[7, 8, 9] and substratum strain[6, 10] – due to both normal and exercise regimes. It has been long known that mammalian cells sense and respond to these mechanical cues. Here, the Curi Cytostretcher-LV (Fig.2A) was used to apply cyclic uniaxial stretch to cells cultured on both flat and nanopatterned substrates. The Cytostretcher-LV is microscope compatible cell stretching device (Fig.2B) capable of providing the environmental control required for long-term live cell imaging. Furthermore, the adjustable stretch plane allows the user to maintain the focus throughout the stretch. Lastly, the device is available in a variety of formats, so that the user can mechanically condition many cultures in parallel (Fig.2C). Larger chambers offer more culture area (up to 25 cm2) while smaller chambers offer higher throughput (up to 24 wells).

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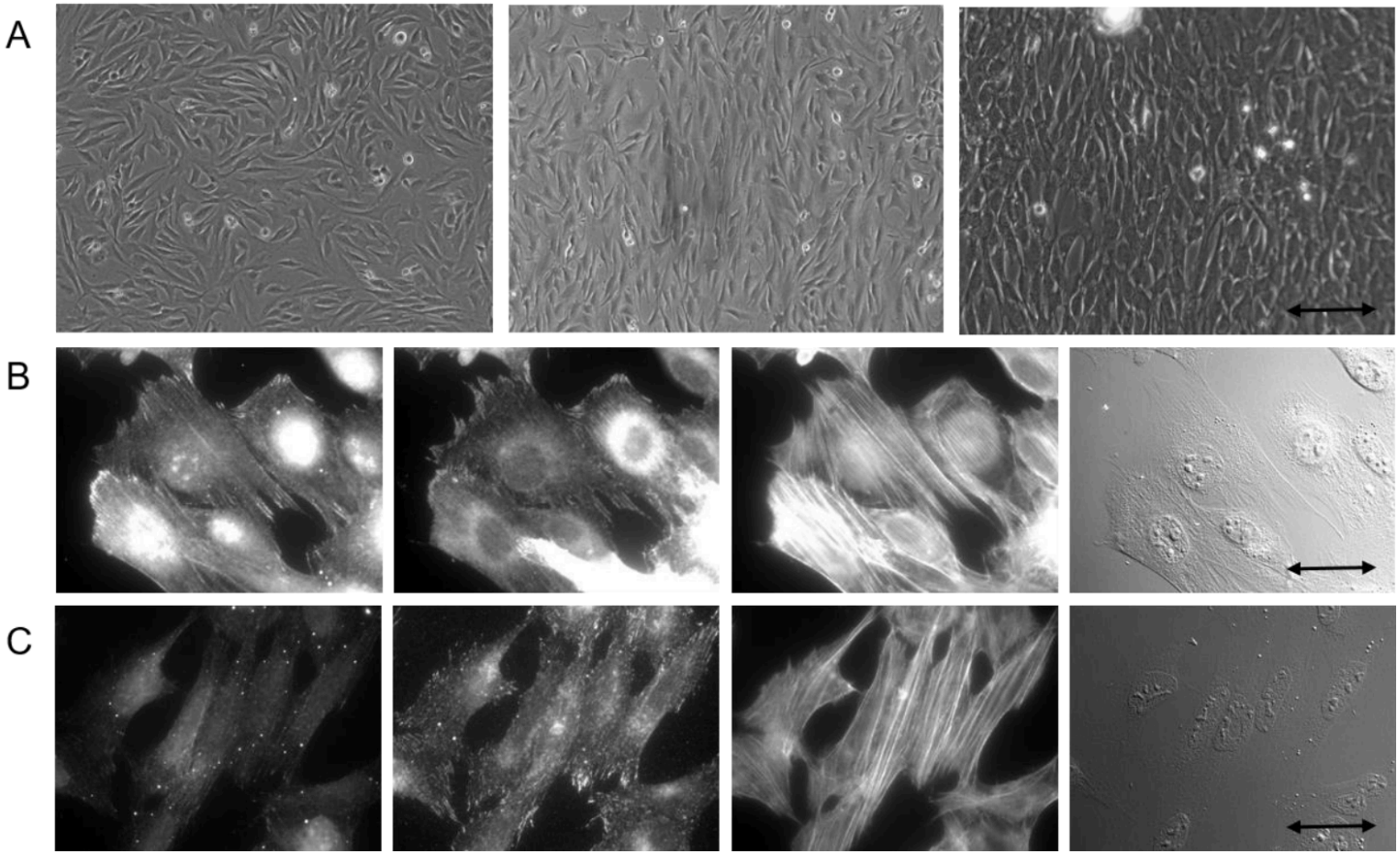

Figure 3: The morphology and alignment of C2C12 and U2OS cells cultured on flat chambers, following 10% uniaxial stretch at 1 Hz frequency. (A) *The wide-field phase contrast image of C2C12 cells under no mechanical stretch (left), and 2 hours (middle) and 6 hours (right) of stretch (Arrows show the direction of uniaxial stretch). C2C12* ***(B)*** *and U2OS* ***(C)*** *cells that were fluorescently tagged with focal adhesion proteins FHL (far-left) and paxillin (middle-left), and phalloidin staining for actin cytoskeleton (middle-right) revealed that focal adhesions form long fibrillar structures perpendicular to the axis of stretch. Cells were imaged using 60x DIC objective (right).*

To assess the relative contribution of surface patterning and mechanical stimuli on cell orientation, cells cultured on both flat and nanopatterned substrates were exposed to cyclic uniaxial stretch (10% strain, 1 Hz frequency, and up to 2 hours). When stretched on isotropic (flat substrates), C2C12s tend to align perpendicularly to the axis of stretch, as seen in wide-field phase contrast images (Fig.3A). Cells transfected with fluorescently tagged focal adhesion-associated proteins (FHL and paxillin) show that this alignment is due to the structure of the cytoskeleton, as focal adhesions form long fibrillar structures perpendicular to the axis of stretch. This is further exhibited in the actin cytoskeleton, as measured with phalloidin staining (Fig.3B). This phenomenon was also observed in other cell types (U2OS bone oseosarcoma cells; Fig.3C).

In contrast, cells grown in the presence of nanotopographical cues show structural organization closely aligned with the direction of the topography, as would be expected *in vivo* where cells are typically aligned to fibrillar structures in the extracellular matrix. Interestingly, when stretched along the axis of the topographical cues, patterned cells do not realign perpendicular to the axis of stretch (Fig.4A). This suggest that the extracellular cues dominate cellular alignment mechanisms when compared to applied mechanical strain. Imaging of the molecular components of the cytoskeleton further corroborates these observations (Fig.4B, C).

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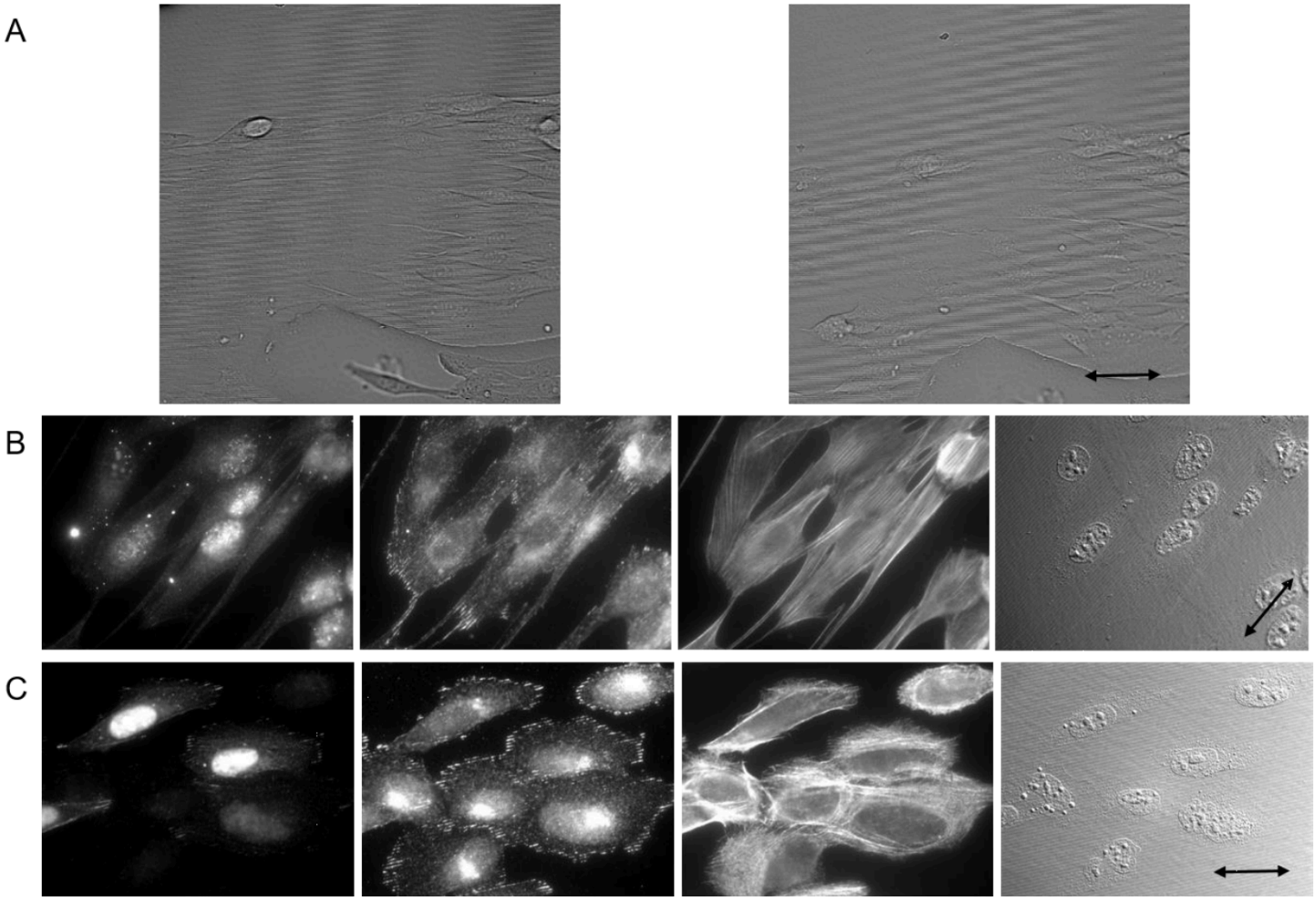

Figure 4: The morphology and alignment of C2C12 and U2OS cells cultured on nanopatterned chambers, following 10% uniaxial stretch at 1 Hz frequency. (A) *The wide-field phase contrast image of C2C12 cells under no mechanical stretch (left), and 2 hours (right) of uniaxial stretch highlight the directionality of the nanopatterns (arrows show the direction of stretch and nanotopography on the substrate). C2C12* ***(B)*** *and U2OS* ***(C)*** *cells that were fluorescently tagged with focal adhesion proteins of FHL (far-left), paxillin (middle-left), and phalloidin staining for actin cytoskeleton (middle-right) revealed that focal adhesions do not realign perpendicular to the axis of stretch in the presence of nanotopographical features. (Cells were also imaged using 60x DIC objective). Cells were imaged using 60x DIC objective (right).*

Conclusion

The structural and mechanical environment of the cell play critical roles in regulating both structural and functional phenotypes. Recreating these cues *in vitro* is critical for translating results obtained in the laboratory to *in vivo* observations. Here, we demonstrated that both structural and mechanical cues from the ECM can modulate cell morphology. While stretching in the absence of structural cues can drive differential orientation of cells in culture, the presence of physical mechanical cues circumvent the stretch-modulated realignment of these cells in culture. The combination of these cues, as recreated in the Curi Cytostretcher-LV, represents a powerful tool for understanding the roles that these stimuli play in physiological function.

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