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Research Article

Epithelial Cellular Growth and Morphological Response to an Ultra-Flat Substrate

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- Nanotopography
- MDCK cells
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Abstract

Nanostructures have a profound effect on cellular behavior. Nanostructures have been shown to effect cellular adhesion, proliferation and differentiation in a myriad of cell types in various ways. Cells interact with nanostructures as small as 10 nm; however there is not a clear understanding of whether cells interact with anything smaller. In this study we investigate the role that sub-10nm sized features play oncellulargrowth and morphology by comparing the growth and morphological responses of MDCK epithelial cells and NIH3T3 fibroblasts cultured on an ultra-flat/atomically flat, "nanosmooth"Silicon (Si) Wafer and a "nanorough"Glass coverslip substrate. We have found that loss of sub 10 nm features results in profound alteration to the growth of MDCK epithelial cells and alters cell morphology and actin cytoskeletal organization. Theseresults demonstrate the importance of considering nanoscale structure, even irregular structure, during device design.

INTRODUCTION

Nanotopography influences many aspects of cellular behavior. Mechanotransduction is the primary mechanism by which topography influences cells [1,2] and complicates the understanding of the role nanotopography in cellular growth and differentiation. The physical properties of the extracellular matrix play an important role in regulating many cellular processes. The fact that cells respond to physical cues in their microenvironment has been known for quite some time, as the term contact guidance was first used in the mid-20th century [3]. Recently with the advent of new and sophisticated fabrication techniques, scientists have moved from the microscale to the nanoscale and found that cells respond to nano-patterned substrates in profound ways. Nanotopography has been shown to affectcell adhesion both positively and negatively [2]. Fibroblasts cultured on 27 nm features created by polymer demixingexhibit increased initial adhesion [4]. Cell based adhesion is dependent on the size and distribution of surface topography; small 20 nm nano-islands of structure increased cell adhesion in both fibroblasts and mesenchymal stem cells,but interestingly an increased in size of the structural islands, cells became less adhesive [5]. Nanogroovesand nano scale fibers align cells [2,6-8], which in the case of myocytes increasesmyogensis [2,7-8]. In contrast, randomly oriented nanoscale features facilitates cell spreading [2,9] which in the case of osteocytes accelerates osteogensesis [2,10]. Organized pits can limit adhesion and up regulate adipogensis [2,11]. It has been show that a cell can detect a nanoscale features down to 10 nm [12].

The question still remains: what is the minimal nanoscale feature that cells respond on a nanostructure structured surface? This is important when designing a microscale or nanoscale biologically interfacing device, because nothing is known regarding the effects of nanoscale variation in the sub 10 nm realm on cell growth. To investigate the minimum feature size of a surface that influences cellular behavior, weusedMadin-Darby canine kidney (MDCK) cells and NIH3T3 fibroblasts cultured on glass cover slips and 5x5 mm Si Wafers. It is well known that tissue culture cells grow on glass and in this paper we show that standard glass coverslip that are often used in tissue culture experiment have an inherently nanostructured surface with random features in the sub 10 nm range, making it an ideal control to determine the rolethat sub 10 nm structures affect cellular behavior. By culturing cells on avirtually atomically flat Silicon Wafer, we demonstrate a differential growth and morphological responses to sub 10 nm nanostructures that is associated with cell density and cell type.

MATERIALS AND METHODS

Substrate preparation

Si Wafer was purchased from Ted Pella, Inc., product #16008; wafer was precut into 5x5 micro-meter bits. Glass substrates were Fisher Brand Microscope Cover Glass (1 oz.), 22x22 mm, 12-542-13, LOT# 050610-9. Substrates were cleaned by 10 min. wash in Acetone at 70° C, followed by 2 min.wash in methanol, then substrates were cleaned with RCA-1 cleaning procedure:

1:1:5 of ammonium hydroxide, hydrogen peroxide, deionized water.

Cell culture

MDCK epithelial cells and NIH3T3 cells were used. MDCK cells were cultured with HyClone DMEM/High Glucose cell media, cat#: SH30022.01, 4.00 uM L Glutamine, 4500 mg/L Glucose. NIH3T3 cells were cultured with [DMEM]. Each experiment, which was repeated at least three times, cells were cultured in a small petri dish and placed in an incubator at 37.6° C at 6% CO2. Experiments were run at 30 minutes, 2 hours, 4 hours, 1 day, and 4 days. Cells were seeded at a concentration of 2.5×10^5 cells/ml (low concentration) and 6.4×10^4 cells/ml (high concentration).

Cell imaging

Cells were fixed with 4% Paraformaldehyde, Sigma-Aldrich (P6148-1KG), stained with Hoechst 33342 at 1:3000 dilution, Phalloidin 488 at 1:1000 in 1XPBS. Imaging was done with Zeiss Observer.21 Confocal Microscope, Axio Rel. 4.8 software. Cells were mounted with Aqua Poly/Mount, Polysciences, Inc. cat#: 18606. For cell viability and island growth experiments we examined cell viability using anAcridine Orange/Ethidium Bromide procedure. We imaged the all samples using a Zeiss Axio Observer Z1, Spinning Disc Confocal Microscope. We observed was an increase in the fluorescence signal on the Si Wafer. Due to the consistent a doubling of the intensity of the signal of all samples taken on the Si Wafer, we believe this is due to the reflective nature of a polished Si Wafer despite the fact that the confocal eliminates most out of plane light,

AFM measurements of Substrate surface topography

Substrates were cleaned by standard RCA-1 protocol, placed in a cleaned Petridishes, and sealed with Para film inside level 7 cleanroom conditions prior to each experiment. For each experiment, the sealed Petri dishes were opened and placed immediately in the AFM to minimize the amount of organic contaminant during AFM imaging.

RESULTS

We investigated the limits of the size of nanoscale structures that influence cellular behavior by culturing cells on a glass cover slip and Si Wafer. We chose glass because of inherent sub 10 nm features on the surface, whereas the Si Wafer is nearly atomically flat. The glass cover slip is amorphoussilicon, with small, irregular nanostructures on the surface that are on average 5-10nm in height (Figure 1A). In contrast, the Si Wafer is crystalline silicon with a virtually nanostructure free, "nanosmooth" surface (Figure 1B). We used these two substrates to investigate the role of surface nanostructure on cell growth and cellular morphologyindependent of surface chemistry, as glass and the Si Wafer share identical surface chemistries. Both surfaces, especially the Si Wafer were thoroughly cleaned prior to all experiments. If not cleaned properly (see methods), the Si Wafer demonstrated and interest effects on cellular growth and morphology. Specifically, the nuclei as shown by Hoechst staining were significantly larger when compared to glass controls (Supplemental (Figure 1 and Table 1). We suspect this is

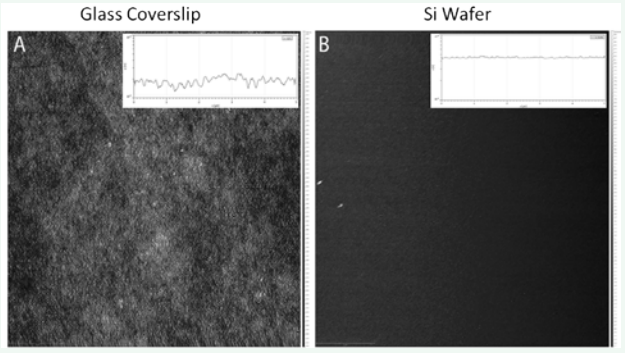

Figure 1 AFM Images of the topography of the glass and Si wafer substrate used in this study. (A) AFM of Glass coverslip, inset a graphical representation of the surface; (B) Si Wafer with image size 5x5 µm, inset a graphical representation of the surface. Profiles are filtered, log scale in order to show an easily understood sense of the topographical differences. Nano features were measured at <5 nm on glass cover slips. There was slight tip drift in x directions for (B) which did not affect results, profile was in principle the same in both x and y directions.

Table 1: MDCK cells organization into islands at a low starting concentration of cells.

Substrate	Glass Cover Slip		Si Wafer	
	Day 1	Day 4	Day 1	Day 4
Average number of Islands per field	3±1 (n=8 fov)	NA, Confluent	3.55±1.5(n=9 fov)	NA, no islands
Average number of cells per Island	8.6±9.2cells/ island (N=21)	NA. near confluent (Figure 2G)	3.74±1.5 cells/island** (n=29)	NA, no islands (Figure 2H)

**P=0.025, fov – field of view

due to the presence of contaminates such as metal oxides on the wafer that are not removed with a simpler cleaning procedure like an acetone wash.

To determine whether cell density has any effect on the growth of cell on "nanorough"(glass) or "nanosmooth"(Si Wafer) surfaces we cultured both MDCK cells and NIH3T3 cells at low ($2.5\ X10^5$cells/ml)and high densities ($6.4X10^6$cells/ml) on our substrates. While NIH3T3 cells at either density showed little difference in growthon either substrate (Figure 2A,B,E,F), MDCK epithelial cells cultured at lower concentrations showed a considerable difference in growth when grown on Si Wafer as compared to Glass (Figure 2C,D,G,H). MDCK cells exhibited growth to confluence on the glass substrate when compared to the Si Wafer at the low cell concentration concentrations (Figure 2C, Day 1; 2G, Day4). Typically on the glass substrate MDCK cells plated at the lower concentration will flatted and spread on contact with the substrate and begin to divide, initially forming isolated islands of cells (Figure 2C); by Day 4 the cells will form a nearly confluent epithelial monolayer (Figure 2G). MDCK cells plated onto the Si Wafer at lower concentration deviate from this normal growth:at Day 1, MDCK cells on the Si Wafer form small islands that are comprised of few cells furthermore these islands are rounded and lack a spreading morphology (Figure 2D);after

four days of culture on the Si Wafer, the small rounded islands of MDCK cells remain as on Day 1 except fewer in number (Figure 2H).

When cultured at a higher initial concentration the cells (6.4 X10^6), the MDCK cells behaved differently with regard to growth but not morphology (Figure 3). The growthof cells (both NIH3T3 and MDCK cells) showed no significant difference when grown on either glass or the Si Wafer.

In addition to altered growth, MDCK cells exhibited an alteredcellular morphology as well. This was evident by less spreading and a more rounded appearance of the MDCK cells on Si Wafer when compared to glass controls (Figure 4, compared A to B). When plated at a low starting concentration MDCK cells initiated growth in small clusters or islands of cellsrather than as isolated, single cells.While both substrate has essentially the same number of islands at day 1, after four days of growth those on the glass substrate grew to near confluence (Table 1) while those MDCK cells on the Si Wafer substrate were lost (Table 1) resulting in virtually no cells present (Figure 2H). While the frequency of MDCK islands per field of view was the same on both the Glass and Si wafer substrates, the number of cells in

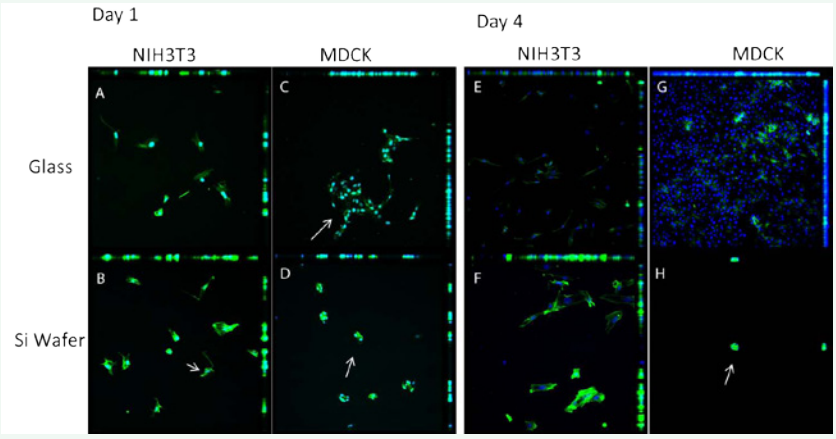

Figure 2 Growth of MDCK and NIN3T3 cells on Glass and Si Wafer with "low" concentration of cells. (A) Glass Cover Slip, NIH3T3 cells at 1 day. (B) Si Wafer substrate, NIH3T3 cells at 1 day, note spreading of cells; (C) Glass Cover Slip MDCKcells at 1 day, cells are clustered on substrate in small islands (arrow), (D) Si Wafer MDCK Cells at 1 day, cells are isolated and found in small round clusters of 3-4 cells (arrow); (E) Glass Cover Slip, NIH3T3 cells at 4 days; (F) Si Wafer, NIH3T3 cells at 4 days (G) Glass Cover Slip, MDCK cells at 4 days, cells are confluent and cover the entire surface; (H) Si Wafer, MDCK cells at 4 days, cells remain in small round cluster, fewer in number than day 1 (arrow). 10x Objective, 5 µm field of view.

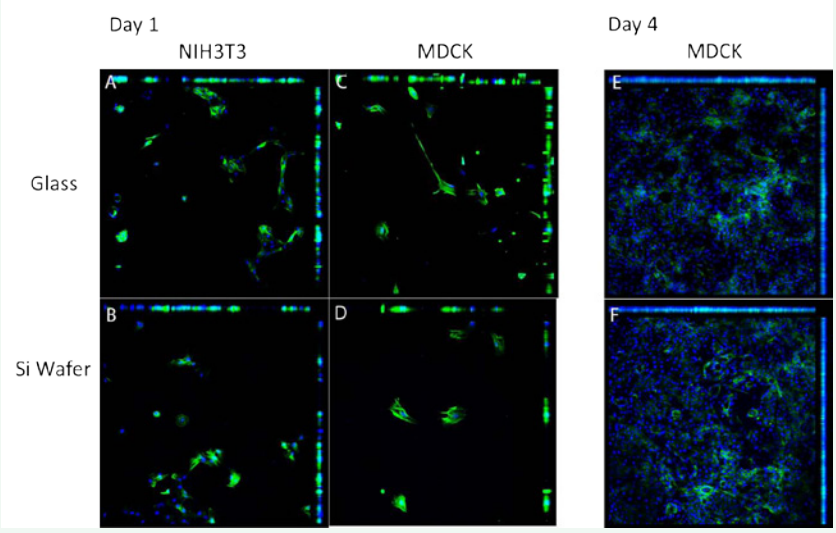

Figure 3 Growth of MDCK and NIH3T3 cells on Glass and Si Wafer with "high" concentration of cells. A) Glass Cover Slip, NIH3T3 cells at 1 day. (B) Si Wafer substrate, NIH3T3 cells at 1 day, note spreading/extension of cells; (C) Glass Cover Slip MDCK cells at 1 day, cells are clustered on substrate in small islands (arrow), (D) Si Wafer MDCK Cells at 1 day, cells are isolated and found in small round clusters of 3-4 cells (arrow); (E) Glass Cover Slip, MDCK cells at 4 days, cells are confluent and cover the entire surface; (F) Si Wafer, MDCK cells at 4 days, cells remain in small round cluster, fewer in number than day 1 (arrow). (Differences in actin can be noted, namely that cells can be seen to be less spread on Si Wafer.10x Objective, 5mm field of view.

each island varied greatly (Table 1). At day 1, MDCK islands on the Si wafer had on average 3.5 cells per island with the largest cluster observed containing eight cells; the MDCK islands on the glass substrates has a significantly higher number of cells per island with nearly nine cells per cluster (Table 1), with the largest cluster containing 22 cells.

In these experiments we noticed that the actin cytoskeleton appeared altered in both NIH3T3 fibroblasts and MDCK epithelial cells when grown on Si wafers. Actin appears brighter on the Si Wafer partially due to the reflection of the light from the mirrored surface of the SiWafer, but also due to significant changes in the cellular organization and distribution of F-actin (Figure 5). When grown on a glass substrate MDCK cells exhibit distinct stress fibers along the length of the cell (Figure 5A). When grown on "nanosmooth" Si Wafer, the f-actin organization in MDCK cells grown on the Si wafer display a rounded morphology with a large amount of cortical actin and little showed fewer cellular extension, most interestingly the extensions that are present have little actin along the leading edge (Figure 5B).

DISCUSSION

In this study we examined the role that nanoscale surface topology (or the lack thereof) plays in cellular growth and morphology. MDCK cells behaved differentlyon a nanostructuredsubstrate (i.e. glass) with inherent nanostructures in the sub 10 nm rangeswhen compared to a nearly atomically flat substrate (i.e. Si Wafer). MDCK cells do not grow on these surfaces at lower cell concentrations, cells form small round clumps or islands which slowly deteriorates over time (Figure 2,Table 1), instead of dividing and forming a confluent sheet. The cell/substrate effect is an early event in the establishment of an epithelialas shown by the differences in the numbers of cells within each MDCK island initiated on a Si Wafer. Whether these differences reflect altered growth of the cells seeded onto this surface or alterations to the cell-cell

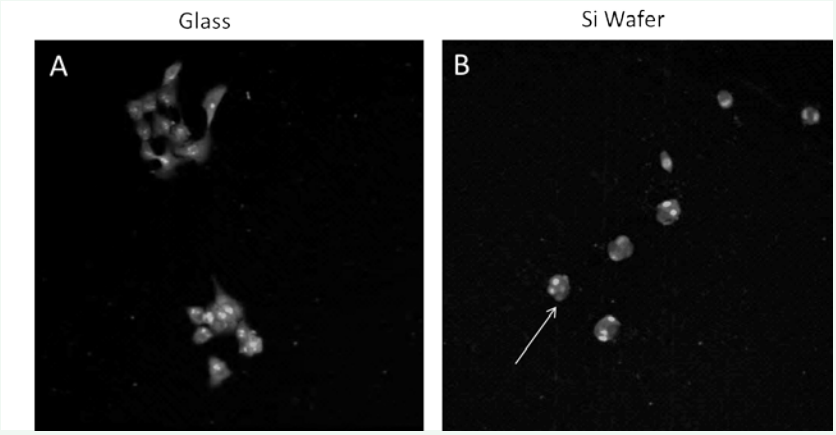

Figure 4 Preferential "island" growth of epithelial cell. Images of live MDCK on substrate after one day growth labeled with Acridine Orange.(A) Glass Cover Slip, MDCK Cells show more cells per island than those grown on Si Wafer substrate and have a more spread morphology. (B) After one day growth on Si Wafer, MDCK cells have formed small islands, with a rounded morphology (arrow). 10x Objective, 5 mm field of view.

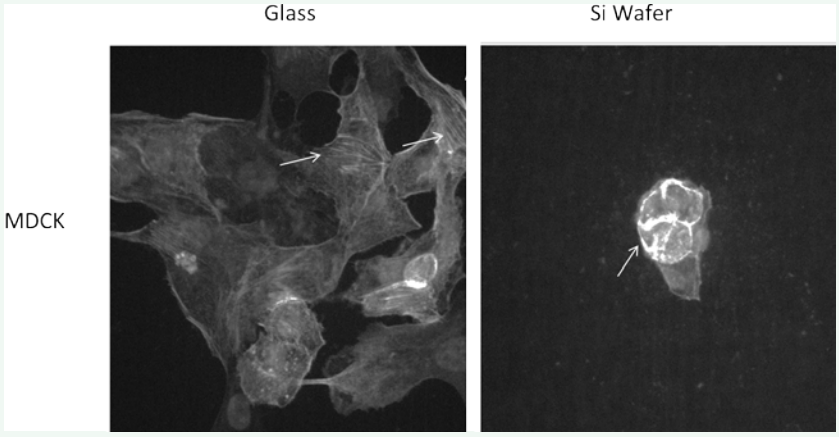

Figure 5 Morphology and F-actin localization in cells grown on Glass and Si Wafer plated under "low" cell concentration. F Actin labeled with Alexa488Phalloidin, (A) Glass Cover Slip, MDCK cells at 1 day, note the present of stress fibers of f-actin along the axis of the cells (arrow); (B) MDCK cells grown on Si Wafer, note the round appearance, cortical accumulation of actin, and the lack of any f-actin in the cellular extension (arrow). 40x Objective.

and cell-substrate interaction (or some combination of the two) remains to be tested; nevertheless, these observations suggests a requirement for a level of cooperative interactions among the independent cells during the reestablishments of an epithelium from singly dissociated cells. All epithelial cells including MDCK cells require intercellular junctions, which could mean that without an appropriate amount of surface energy there may not exist enough cell-surface interaction to stabilize the cytoskeletal elements of these cells, leading to the limited cell growth on the Si Wafer observed. Our results demonstrate that below a certain threshold, epithelial cells cannot overcome the lack of physical/mechanical contacts on a featureless, ultra-flat surface. This is further demonstrated by the abnormal actin cytoskeleton in these cells particularly the lack of f-actin in leading/spreading cellular extension such as lamellipodia. Our observation that the alteration to growth and morphology is ameliorated by an increase in the number of cells suggestion that the cell-cell contacts, perhaps in a mechanical force generating manner may play as significant role in the organization and reformation of an epithelium. Although we observe a subtle change in the organization of actin in the mesenchymal NIH3T3 cells, we observe no alteration to attachment of these cells to the substrate or to the growth of these cells on either substrate. Therefore, this may be a unique feature of a cellular epithelium. Previous work has shown that alteration to the mechanical stimulation ultimately results in changes to gene expression and that apart from the surface substrate, this mechanical stimulation involves both intra and extra cellular processes [1,13-15]. The role that these different mechanisms play in the disruption of epithelial – i.e. whether it is due to alteration or regulation of the cytoskeleton in these cells or due to the alteration in the formation of a function and structurally stable extracellular matrix -- remains to be tested. Nevertheless, the effect that topographical features in the order of sub 5 nm are paramount for device design because it showsthe role that nanoscale features effect epithelial cell behavior and such effects may unintentionally create an environment where by cells could promote some diseased state.

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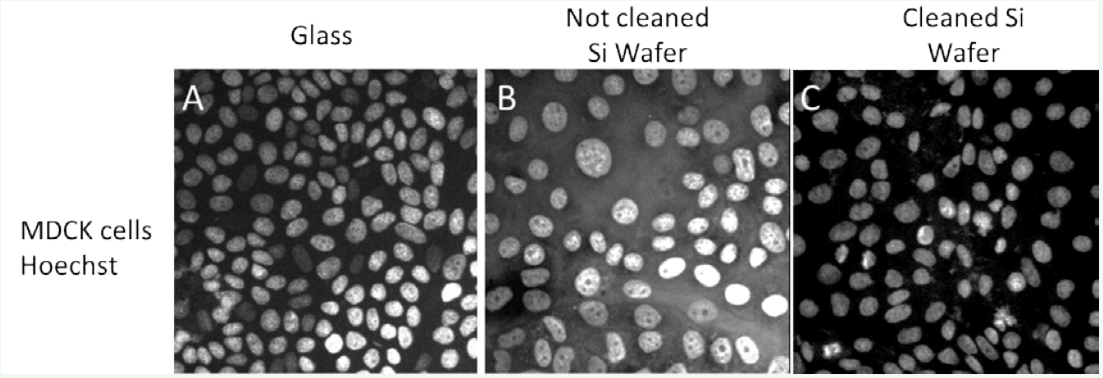

Supplemental Figure 1: Enlarged nuclei of cells grown on unprepared Si Wafer Surfaces. MDCK cells seeded onto the dish at the higher concentration and cultured for 4 days on (A) Glass, (B) Si Wafer that have not been prepared, and (C) Si Wafers that have cleaned (see methods). The nuclei have been labeled with Hoechst stain. Note the increase in the size of the nuclei in B when compared to A and C. Note the lower density of cells on the Si wafer when compared to Glass.

Supplemental Table 1: Length of the nuclei in MDCK cells grown (4 days) prepared and unprepared surfaces.

Substrate	n	Longest dimension of the nucleus (µm)
Glass	17	12.2 ± 1.9*
Not cleaned Si Wafer	22	16 ± 2.6
Cleaned Si Wafer	16	12.5 ± 2.5**

*P=5.85E-6 when Not cleaned Si Wafer compared to Glass
**P=0.000129 when Not cleaned Si Wafer compared to cleaned Si Wafer