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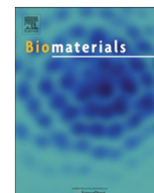

Actions of the anti-cancer drug suberoylanilide hydroxamic acid (SAHA) on human breast cancer cytoarchitecture in silicon microstructures

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Micro- and nanotechnologies are increasingly being applied in cancer research. Here we report the effects of an experimental breast cancer agent, SAHA, on the cytoarchitecture and adherence of MDA-MB-231 metastatic human breast cancer cells on flat silicon surfaces and in three dimensional (3-D) isotropic silicon microstructures. The 3-D silicon microstructure were fabricated using a single mask and single etch step process to yield arrays of star- and circular-shaped microchambers 151–168 μm in diameter and 53–68 μm deep. There was a marked expansion of the microtubule network, an increase in mean cell area and mean cell length in response to SAHA. SAHA also decreased the nuclear-to-cytoplasmic area (N/C). Atomic force microscopy (AFM) showed there was no change in cellular elasticity over the nuclear region in response to SAHA. The alterations in cytoarchitecture produced by SAHA were associated with changes in the mode of adhesion of the cells in silicon microstructures. In contrast to control cells which conformed to the microstructures, SAHA caused cells to stretch and attach to the microstructures through actin-rich cell extensions. We conclude that isotropically etched silicon microstructures comprise microenvironments that discriminate metastatic mammary cancer cells in which cytoskeletal elements reorganized in response to the anti-cancer agent SAHA.

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1. Introduction

Applications of micro- and nanotechnology in cancer research aim to improve detection, diagnosis and treatment of this devastating disease. Research topics range from nanoparticle-based therapeutics and imaging, robotic-based tumor detection, to the design of microdevices to perform fundamental studies of tumor cell biology [1]. In this regard, a number of laboratories including our own are engaged in the fabrication of microdevices to analyze human mammary cancer cell biology [2–6] and drug sensitivity [7].

A large body of work has established that the material composition of microdevices merits careful consideration because the cell substrate plays an important role in the behavior of mammary cells [8–11]. For example, implantation of cells into soft microenvironments composed of extracellular matrix protein gels most closely simulates an *in vivo* condition, and in these three dimensional (3-D) cultures, normal mammary cells adopt spherical and duct-like structures while transformed cells express a less malignant phenotype [12]. In comparison, mammary cell growth in traditional two-dimensional (2-D) cell culture on stiff surfaces such as plastic or glass promotes formation of focal adhesion complexes, cell spreading and cell motility [11]. The effect of modulating the surface stiffness and integrin binding sites on mammary cells grown in 2-D cell culture systems has also been investigated using uniformly protein coated surfaces, micro-patterning of peptides, floating collagen gels, polyacrylamide gels of varying cross-linking density, and PDMS [11,13,14]. Collectively, these investigations confirm the exquisite sensitivity of mammary cells to the nature of their contacts with the growth surface.

Silicon is a stiff material suitable for adhesion of mammary cells [2,5,6]. Furthermore silicon microdevices can be fabricated with growth surfaces containing a wide-variety of precise arrayed features including arrays of microgrooves [15] and pillars and wells [16]. We recently reported development of 3-D silicon microstructures using a single mask, single etch step fabrication process [2,5,6]; the fabrication method relies on the application of reactive ion etching (RIE) lag to etch silicon to different depths to create precise isotropic topographical patterns with features ranging in size from 10 to 200 μm. These structures impose upon cells

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a variety of topological challenges to adhesion, spreading, and cell motility.

In this work, we used the MDA-MB-231 cell culture model of highly metastatic human breast cancer [17] which is also representative of "triple-negative" breast tumors which are refractory to many standard cancer therapies [18]. The effect of SAHA, a new anti-cancer drug also known as Vorinostat, on cell attachment to flat silicon substrates and to 3-D silicon microarrays was investigated. SAHA is the first drug of its type to receive FDA approval for clinical use and represents a class of agents of increasing therapeutic importance [19,20]. Unlike many conventional cytotoxic chemotherapy agents which target DNA to kill cancer cells, SAHA inhibits a family of enzymes referred to as "histone deacetylases" (HDAC). The HDAC enzymes are known to increase levels of acetylation of many proteins, and in particular, HDAC6 targets beta-actin, alpha- and beta-tubulin and additional actin binding proteins comprising the cytoskeleton [21–23]. Our work is the first to address the use of microdevices to study this emerging class of anti-cancer agents.

2. Materials and methods

2.1. Silicon device fabrication

The fabrication process of 3-D microstructures is similar to our previous work [2,5] which relies on the applicability of RIE lag and its dependence on geometrical patterns of the photomask layout to etch silicon to different depths [2,6]. Briefly, the etching process starts by depositing of plasma enhanced chemical vapor deposition (PECVD) oxide layer on a silicon wafer. After patterning with S1813 photoresist, the oxide layer is etched using deep reactive ion etching (DRIE) process. After oxide etching, silicon is etched using DRIE SF_6 plasma to form arrays composed of star- and circular-shaped microchambers. After removal of photoresist and oxide layer, the wafer is diced into 1 cm^2 chips. Fig. 1 shows the photo image of the fabricated devices and the SEM images of the resulting microstructures. The dimensions of the etched cavities vary between 60 and 70 μm in depth and between 150 and 170 μm in diameter. These cavities contain internal microstructures consisting of either 1) circular-shaped microchambers with smooth side-walls and concentric circular rings deep within the cavity or 2) star-shaped microchambers having scalloped edges that process into the depth of the cavity as shallow tracts. The chips were sterilized in 95% ethanol then air-dried prior to use in cell culture.

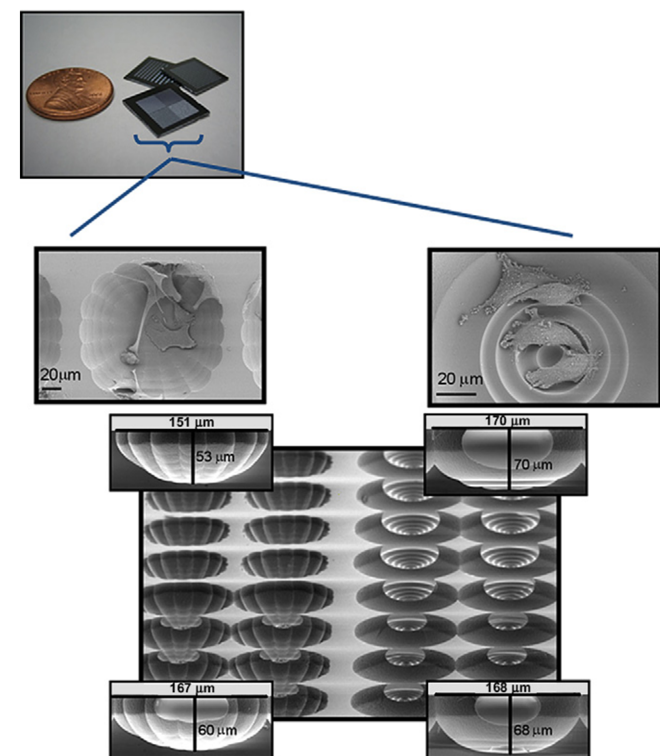

Fig. 1. Photo image of the fabricated devices in silicon and SEM images of the three dimensional (3-D) silicon substrates as cell culture substrates comprising star- and circular-shaped microchambers with the corresponding top view images.

2.2. Cell culture

The well-established cell culture model of invasive human breast cancer, MDA-MB-231 [24], originally isolated from the pleural effusion of a patient with metastatic disease was purchased from the American Type Culture Collection (Manassas, Virginia). Cells were maintained in plastic T-75 cm^2 culture flasks in a humidified incubator at 37 °C. MDA-MB-231 cells were propagated in RPMI culture medium containing 5% fetal bovine serum (FBS, Atlanta Biologicals,Atlanta, Georgia), 4 mM glutamine, 1 mM sodium pyruvate, and penicillin–streptomycin (100 Units/ml). For adhesion analysis the cells were grown on silicon chips for 48 h at 37 °C in humidified 7% CO_2-93% air atmosphere after plating at a density of 1.1×10^4 cells/mm^2.

For experiments involving SAHA, 5×10^4 MDA-MB-231 cells were plated into 12-well multi-well dishes containing 3 mL of culture medium supplemented with 10% FBS and a single 1 cm^2 silicon chip. Cells were incubated for 24 h after plating to allow cells to attach to the substrate and adapt to the fresh medium; then SAHA (1.5 μl) was added directly into the culture medium to a final concentration of 3 μM SAHA in 0.05% DMSO using a 2000× stock solution of SAHA dissolved in 100% DMSO. After exposing cells to SAHA for 20 h, cells were processed for imaging.

2.3. Immunohistochemistry

Cells grown on silicon were washed in Hank's balanced salt solution (HBSS), then fixed with 3% paraformaldehyde (PF) in 250 mM Tris, pH 7.2 for 10 min followed by 6% PF-0.25% Triton X-100 in PBS for 10 min. Cells were washed free of the PF, then vinculin, filamentous (F^-) actin, and cell nuclei were stained at room temperature as follows. Cells were incubated for 30 min with mouse monoclonal anti-vinculin (1:100 in 2% chicken serum albumin-PBS, Abcam, Cambridge, MA), then with rhodamine-tagged goat anti-mouse IgG (1:300 in 2% chicken serum albumin-PBS, Invitrogen, Carlsbad, CA) for 30 min. Next, cells were incubated for 15 min with AlexaFluor488-phalloidin (10 U/ml in 140 mM NaCl-6% bovine serum albumin in 40 mM Tris, pH 7.2, Invitrogen), and Hoeschst33342 (Invitrogen, 30 μg/ml) was added for 5 min. Cells were washed with three exchanges of PBS between each step. The silicon chips were mounted on glass coverslips using ProLong Gold antifade (Invitrogen) and air-dried overnight prior to imaging using confocal microscopy (ZEISS-LSM-510 META) in the reflection mode.

To stain microtubules, the procedures were as described above except a 20 min incubation with mouse monoclonal anti-β-Tubulin I + II antibody (1:1000 in 2% chicken serum albumin in PBS, Sigma) followed by a 20 min incubation with the rhodamine-tagged goat anti-mouse IgG were used. Images of the microtubules were obtained using fluorescence microscopy with a Nikon Eclipse 80i instrument.

2.4. Scanning electron microscopy (SEM)

The cells were fixed in 3.7% formaldehyde in PBS for 10 min 48 h after plating, critical-point-dried and sputter-coated with a thin layer of gold palladium. SEM images were obtained using a Leo ZEISS 1550 instrument.

2.5. Atomic force microscopy

Cells (3×10^4/ml) were grown on type IV Collagen-coated 25 mm round glass coverslips in 35 mm^2 dishes in RPMI culture medium supplemented with 10% FBS for 24–48 h. Immediately prior to the experiments, 20 mM HEPES buffer was added to the culture medium to maintain pH 7.2. Atomic force microscopy instrumentation MFP-3D-Bio (Asylum Research Corporation, Santa Barbara, CA) was used for the force measurement. The AFM was combined with an inverted optical and fluorescence microscope for precise positioning of the AFM cantilever tip over the center of the cell nucleus and cell visualization while force was applied. Soft V-shaped silicon nitride cantilevers, TR400PSA (Olympus), with the nominal length of 200 μm and a spring constant of 0.02 N/m was used for force measurement at the tip velocity of 0.5 μm/s. A ~10 μm glass bead (Duke Scientific) was attached to the cantilever tip to reduce any nonlinearity in deforming stress and to minimize cell damage. All measurements were carried out using a standard fluid cell (Asylum Research) at room temperature.

The Young's modulus of individual cells was computed as detailed previously [6] by using the Hertz's model where, the relationship between the applied force, F, and the indentation depth, δ, can be expressed as:

$$F = \frac{4\sqrt{R}}{3(1-\nu^2)} E \delta^{3/2} \quad (1)$$

R is the radius of the tip, and E and ν are the Young's modulus and Poisson's ratio of the indented cell, respectively. The Poisson's ratio, ν, is assumed to be 0.5 in accordance with the incompressibility assumption usually employed for cells and soft tissues. We used the linear version in δ of the Hertz's model to define the elasticity and initial contact point with the cell. This model leads to Equation (2)

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