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Salivary gland cell differentiation and organization on micropatterned PLGA nanofiber craters

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Abstract

There is a need for an artificial salivary gland as a long-term remedy for patients suffering from salivary hypofunction, a leading cause of chronic xerostomia (dry mouth). Current salivary gland tissue engineering approaches are limited in that they either lack sufficient physical cues and surface area needed to facilitate epithelial cell differentiation, or they fail to provide a mechanism for assembling an interconnected branched network of cells. We have developed highly-ordered arrays of curved hemispherical “craters” in polydimethylsiloxane (PDMS) using wafer-level integrated circuit (IC) fabrication processes, and lined them with electrospun poly-lactic-co-glycolic acid (PLGA) nanofibers, designed to mimic the three-dimensional (3-D) *in vivo* architecture of the basement membrane surrounding spherical acini of salivary gland epithelial cells. These micropatterned scaffolds provide a method for engineering increased surface area and were additionally investigated for their ability to promote cell polarization. Two immortalized salivary gland cell lines (SIMS, ductal and Par-C10, acinar) were cultured on fibrous crater arrays of various radii and compared with those grown on flat PLGA nanofiber substrates, and in 3-D Matrigel. It was found that by increasing crater curvature, the average height of the cell monolayer of SIMS cells and to a lesser extent, Par-C10 cells, increased to a maximum similar to that seen in cells grown in 3-D Matrigel. Increasing curvature resulted in higher expression levels of tight junction protein occludin in both cell lines, but did not induce a change in expression of adherens junction protein Ecadherin. Additionally, increasing curvature promoted polarity of both cell lines, as a greater apical localization of occludin was seen in cells on substrates of higher curvature. Lastly, substrate curvature increased expression of the water channel protein aquaporin-5 (Aqp-5) in Par-C10 cells, suggesting that curved nanofiber substrates are more suitable for promoting differentiation of salivary gland cells.

Keywords

Nanofibers; Substrate curvature; Basement membrane; Cell morphology; Cell differentiation; Salivary gland

1. Introduction

The inability to adequately produce saliva, known as salivary hypofunction, is an important clinical problem affecting millions of Americans and is a primary cause of xerostomia (dry mouth) [1]. The disorder, commonly caused by the autoimmune disease Sjogren's syndrome, radiation therapy, or head trauma, decreases a person's ability to chew, swallow, digest, and speak, and often leads to more serious conditions such as oral infections, tooth decay, and gum disease [2–5]. Treatment options for patients with salivary hypofunction are limited, and usually involve the use of medications which introduce unwanted side effects, or artificial saliva which is temporary and insufficient [6]. A possible remedy for salivary hypofunction involves the development of an engineered salivary gland which can permanently replace lost or damaged acinar tissue [7].

Two main tissue engineering approaches have been investigated in research towards an artificial salivary gland. The first involves allowing salivary gland epithelial cell lines or primary cells to self-assemble within a 3-dimensional medium, namely Matrigel or other natural matrices like hyaluronic acid [8–10]. Although rounded 3-D polarized acinar-like structures have been formed using these methods, these approaches are limited in that they form closed spheroids lacking interconnectivity, and thus lack a mechanism for harvesting saliva. Additionally, Matrigel is nonimplantable in humans. Other approaches have explored the use of flat synthetic and/or natural substrates to organize salivary gland cells into a monolayer [11–13]. Salivary gland cells grown on flat polymeric substrates often fail to assemble tight junctions, which are needed for directional secretion of saliva [14–16], and are unable to form a complex 3-D branching structure, a requirement for producing sufficient surface area to produce adequate amounts of saliva [17]. By adding physical complexity to substrates, it may be possible to achieve acinar cell organization and differentiation which more closely resembles cells grown in 3-D environments, while still achieving an interconnected monolayer of epithelial cells capable of directing saliva.

Electrospun nanofibers have been used in many studies to create substrates having high surface area to mimic the nanoscale structure of the extracellular matrix (ECM) [18–20]. These substrates are often used as scaffolding for mesenchymal cells, allowing for attachment, spreading and infiltration of these cells, which assemble within a 3-D network of fibrous support proteins in the ECM [20,21]. Relatively few groups have used electrospun nanofibers as a scaffold for epithelial cells to grow on top of, that is, using the nanofiber matrix as an artificial basement membrane (BM), which is a specialized extracellular matrix found in close proximity to the epithelium *in vivo* [22,23]. We previously used nanofibers as substrates for salivary gland epithelial cells, and demonstrated the ability of poly-lactic-co-glycolic acid (PLGA) nanofibers to promote a more 3-D epithelial-like cell morphology [24] and to downregulate and localize focal adhesion complexes in adult mouse acinar and ductal cell lines similar to what is seen in primary gland tissue [25], relative to cells grown on non-textured, flat substrates. While the nanofibers themselves promote apicobasal polarization, we recently demonstrated that covalent modification of nanofiber scaffolds with laminin-111 further stimulated apicobasal polarity in two salivary gland epithelial cell lines, while chitosan inhibited polarization [26].

Other groups have manipulated cells by physically constraining them within or on top of micropatterned surfaces, inducing morphological and behavioral changes compared to those grown on flat surfaces [27–34]. Less studied in recent years is the effect of substrate curvature on the organization and differentiation of cells, particularly epithelial cells. Although many epithelial cells are supported by a highly curved concave basement membrane *in vivo*, including those found in branching organs such as the mammary gland, lungs and salivary gland [35], studies involving substrate curvature have been using mostly

non-epithelial cells and/or convexly-curved substrates [36–40]. It remains unclear whether a concave surface might promote epithelial cell organization and differentiation.

Here, we have built upon the benefits of electrospun PLGA nanofiber substrates in engineering salivary gland tissue by combining nanofibers with lithographically micropatterned curved “craters”, intended to mimic the physical structure of the BM surrounding salivary gland acinar cells at both the nano and microscale levels. Adding craters to growth substrates also significantly increases surface area compared to flat and standard nanofibrous substrates, and thus is a more practical alternative to flat surfaces for creating an engineered salivary gland. We hypothesized that by adding curvature on the same size scale as acini *in vivo* to nanofiber substrates, salivary gland epithelial cells would subsequently undergo apicobasal polarization and differentiate. In this study, we compared the structural organization of two salivary gland cell lines grown on nanofiber-coated micropatterned surfaces of varying curvature with cells grown on flat nanofibers or on Matrigel.

2. Materials and methods

2.1. Materials

Transparency masks were ordered from Infinite Graphics (Minneapolis, MN). 200 mm, <100> single crystal wafers were purchased from Ecotech Recycles (Kalama, WA). P20 adhesion promoter was purchased from ShinEtsuMicroSi Microelectronic Material (Phoenix, AZ). SPR 220 7.0 positive photoresist was purchased from Shipley Inc. (Marlboro, MA). AZ 300 MIF developer was purchased from AZ Electronic Materials (Stockley Park, UK). Polydimethylsiloxane (PDMS) was purchased from Dow Corning (Midland, MI). Sulforhodamine B (SRB) to stain fibers (Cat. No. S-1307) was purchased from Life Technologies (Grand Island, NY). Polylactic-co-glycolic acid (PLGA), with a lactic to glycolic acid ratio of 85:15 and a molecular weight of 95,000 Da, was obtained from Birmingham Polymers (Pelham, AL). Hexafluoroisopropanol (HFIP) was from Sigma—Aldrich, USA (St. Louis, MO). Vectabond Reagent was purchased from Vector Laboratories (Burlingame, California). Glutaraldehyde (Cat No. G5882) and hexamethyldisilazane (Cat. No. H4875) were obtained from Sigma—Aldrich. Matrigel was purchased from BD Biosciences (Bedford, MA). SIMS immortalized adult mouse submandibular ductal cells were generously provided by Dr. Daniel Malamud, and Par-C10 immortalized adult rat parotid acinar cells were generously provided by Drs. Mary Reyland and David Quissell. Antioccludin (Cat. No. 33-1500) antibody was purchased from Life Technologies, and anti-E-cadherin (Cat. No. 610182) was from BD Biosciences. DAPI, (to stain nuclei), Alexafluor-488-phalloidin and rhodamine-phalloidin (to stain actin) were purchased from Life Technologies. Anti-actin (Cat. No. A-3853) was purchased from Sigma Aldrich, USA (St. Louis, MO) and Fitzgerald Labs (Boston, MA), respectively. Anti-aquaporin-5 (Cat. No AQP-005) was obtained from Alomone labs (Jerusalem, Israel). Donkey anti-species cyanine and Dylight dye-conjugated AffiniPure F(ab')₂ secondary antibodies, and donkey serum, were from Jackson ImmunoResearch (West Grove, PA). ECL anti-mouse IgG HRP and ECL anti-rabbit IgG HRP secondary antibodies were obtained from GE Healthcare (Piscataway, NJ). Fluor-Gel mounting media was acquired from EMS (Hatfield, PA), and p-phenylenediamine (PPD) (Cat. No. P6001) was from Sigma—Aldrich.

2.2. Creation of micropatterned crater arrays

A transparency mask was designed using Tanner EDA L-Edit v.15.01 which contained arrays of circles of radius 20, 30, 50, and 80 μm . A 200 mm single crystal silicon wafer was first Piranha cleaned in 3:1 sulfuric acid:30% hydrogen peroxide solution for 2 min, rinsed, dried, and then P20 adhesion promoter was spin-coated at 3000 rpm for 45 s. SPR 220 7.0

positive photoresist was then spin-coated at 500 rpm for 2 min, soft baked at 90 °C for 5 min, and exposed for 360 s in an EV640 contact aligner (Electronic Visions Co., Austria) at a dose of $\sim 4 \text{ mJ/cm}^2$ in soft contact mode. After exposure, the resist was developed for ~ 5 min with gentle agitation in AZ 300 MIF developer. Next, an elevated hard bake at 150 °C for 5 min, which was above the glass transition temperature of the resist, deformed the cylindrical features, yielding rounded “mounds”. Feature shape was then verified using a profilometer and a Leica DM8000 M optical microscope to ensure completion of the process. Next, polydimethylsiloxane (PDMS) was thoroughly mixed using a precursor to crosslinker ratio of 10:1, spin-coated onto the patterned wafer for 90 s at 600 rpm, and cured for ~ 4 h at 70 °C. After curing, the PDMS was carefully peeled off the substrate and immersed in absolute ethanol for 1 h to remove any residual photoresist.

2.3. Preparing electrospun PLGA nanofiber-lined craters and flat nanofiber samples

Circular glass coverslips were pre-coated with Vectabond reagent to facilitate polymer adhesion and dried. Immediately prior to electrospinning, coverslips were coated with a thin layer ($\sim 10 \mu\text{l}$) of PDMS precursor/crosslinker to act as an adhesive layer between the PDMS crater array and glass and to adhere fibers near the periphery of the coverslips. Circular arrays of each feature size were cut out of the PDMS sheet using a cork borer, and the cutouts were then placed on top of the PDMS layer. Next, the samples were placed in a 60 °C oven for 180 s to partially cure the PDMS so it could still hold the fibers to the edge of the coverslip but would not flow over the top of the crater array. The coverslips containing the arrays were then placed on foil and electrospun nanofibers were deposited using a 25 gauge needle, 12 kV potential, 15 cm needle-to-spinneret distance, and 3 $\mu\text{l/min}$ feeding rate for 1 h. The electrospinning solutions contained 8% w/w PLGA in HFIP with 1% w/w NaCl and 2.5 μM SRB dye to stain the fibers and delineate the basal side of the cell monolayer for fluorescence confocal imaging. After electrospinning, samples were immediately placed in a 37 °C incubator overnight to fully cure the adhesive PDMS. Flat nanofiber samples were prepared as previously described [25]. Samples were then sterilized using UV irradiation for at least 1 h, and soaked in sterile PBS for three days at 37 °C to promote conformation of nanofibers to the curved craters.

2.4. Matrigel preparation

Using a variation of previously described methods [41], Matrigel basement membrane matrix was added 1:1 to ice-cold complete cell media, pipetted to mix, then spread onto MatTek glass bottom dishes (Ashland, MA) or Lab-Tek II Chamber Slides (Scotts Valley, CA) for samples to be imaged via confocal microscopy. For cells grown in full three-dimensional (3-D) Matrigel, 80 μl of diluted Matrigel was used, and for thin Matrigel 20 μl was spread using a sterile cell scraper. Samples were then incubated for 1 h at 37 °C to polymerize the gel before cell seeding.

2.5. Cell culture

After crater array and flat nanofiber samples incubated in PBS for three days at 37 °C, samples were transferred to complete sterile media of the cell type to be seeded and incubated for 24 h at 37 °C. SIMS media preparation and cell culture is described in Refs. [25,26]. Par-C10, an immortalized adult rat parotid gland acinar cell line was cultured in DMEM-F12 media supplemented with 2.5% FBS, growth factors and 50 mg/ml gentamycin on BD Primaria flasks, as previously described [25,42]. Cells of each type were seeded in a 24-well plate at 70,000 cells/well for crater/nanofiber samples, and 30,000 cells/well for 3-D Matrigel samples to facilitate formation of 3-D acinar-like structures. SIMS were grown on substrates for 96 h, and Par-C10 cells were grown for 120 h to allow cells to achieve confluence.

2.6. SEM characterization

Samples with and without cells were characterized using a Zeiss 1550 field emission scanning electron microscope (Leo Electron Microscopy Ltd., Cambridge, UK; Carl Zeiss, Jena, Germany) as previously described [25]. To determine the crater degree of curvature for each feature size (20,30,50, and 80 μm radius), wafers were cleaved such that mounds of each size were at the edge of the cleaved plane and imaged parallel to the surface of the wafer (cross-sectional). Several images were then annotated using the Zeiss integrated software and ImageJ, and the angle of the tangent line at a distance of 10 μm from the apex of each mound relative to a tangent line at the apex was measured as the degree of curvature.

2.7. Immunocytochemistry and confocal microscopy

Cells were fixed in a 1:1 ratio of freshly prepared 4% paraformaldehyde with 5% sucrose in PBS to complete cell media on ice for 20 min. Fixed samples were then washed twice in PBS–Tween 20 (0.5% Tween), permeabilized for 15 min in 0.1% or 0.4% Triton X-100 for nanofibrous and Matrigel samples, respectively, and blocked for 2 h in PBS-T with 20% donkey serum. Primary antibodies were prepared at a 1:100 dilution in PBS-T + 3% BSA and incubated on a rocker overnight at 4 °C. After washing four times with PBS-T, donkey anti-species secondary antibodies were prepared at a 1:200 dilution in PBS-T + 3% BSA with 5% donkey serum. Nuclei were stained with DAPI (1:5000) and F-actin was detected using rhodamine–phalloidin (1:300) or Alexafluor-488 phalloidin (1:60) included in the secondary antibody solution. Samples were mounted on glass coverslips using Fluor-gel mounting media with 1:100 p-phenylenediamine (PPD) antifade solution, sealed using clear nail polish, and dried before imaging. Laser scanning confocal microscopy was performed using a Leica SP5 laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany) and images were acquired at magnifications of 20 $\times$ using a dry objective or 40 $\times$ and 63 $\times$ using an oil immersion objective. Z slices of thickness 0.8 μm for 40 $\times$ and 0.4 μm for 63 $\times$ were acquired for all samples. All quantitative images within a given experiment were captured at the same depth using the same laser intensity and gain settings so that fluorescent intensities could be compared across samples. Confocal top-down images on nanofibers and craters are displayed as maximum projection images, prepared using the Leica LAS AF software.

2.8. Cell monolayer height measurements

Confocal z-stacks at 63 $\times$ magnification were viewed as cross-sections, and the annotation software in the LasAF program was used to measure the distance from the basal membrane to the apical membrane using the stain for F-actin (and the stained fibers as reference points). At least 8 measurements were acquired and averaged per sample, and at least 2 samples were used for each type. Measurements were graphed, and standard deviations for each set were calculated.

2.9. Cell–cell junction protein localization measurements

In order to qualitatively assess polarity in cells grow on each substrate, images were acquired from cross-sectional views of z-stacks, and the images were analyzed using ImageJ. Using the freehand tool, lines were drawn across the monolayer of cells within craters and on flat nanofibers, and intensity histograms of the channel containing the protein of interest were generated along the lines using the RGB Profile Plot plugin. For each sample, a line near the apical side and a line near the basal side of the cell monolayer were drawn, and a representative histogram for each sample type is shown. Each cross-sectional fluorescence confocal micrograph and corresponding intensity plot are representative of six measurements taken per substrate type.

2.10. Western blot analysis

Cells grown on crater/nanofiber samples for 96 h (SIMS) or 120 h (Par-C10) were lysed to obtain total protein using ice-cold RIPA buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl₂, 1.5 mM EGTA, 1% Triton-100, 1% Na-deoxycholate, 0.1% SDS, protease and phosphatase cocktail inhibitors, Roche). Cell lysates were pooled from 6 wells of each sample type in a 24-well plate and each well was lysed for 10 min. All lysates were centrifuged at high speed (17,000× g) for 20 min, and the supernatant was removed for total protein quantification using a Micro-BCA assay kit (Pierce, Rockford, IL). Lysates were prepared using protein loading buffer, normalized for equal protein loading, and 10–20 µg of protein was run per lane as previously described [25,43]. At least three experimental runs for each protein were conducted. A representative blot for each protein is shown, along with the corresponding plot of band intensities quantified using ImageJ.

2.11. Statistical analysis

Sample sets containing three or more samples were analyzed using a one-way analysis of variance (ANOVA) and Bonferroni's post-test in GraphPad Prism 5 software. If two sample sets were compared, a Student's *t*-test was used. A value of *p* < 0.05 for both methods was considered to be statistically significant.

3. Results

3.1. Development and characterization of PDMS craters

To create PDMS crater arrays, an appropriate photolithography mask was designed to yield features on the same size scale as acini *in vivo* and with controllable levels of curvature. After determining the average radii of mouse adult submandibular gland acini to be 16.0 ± 4.3 µm (female) and 13.7 ± 2.4 µm (male) by measuring in fixed tissue sections, and 25.94 ± 7.64 µm average radius for Par-C10 acinar cell line spheroids in 3-D Matrigel, appropriate sized features were engineered such that the smallest features would be close to these ranges. Features having radii of 20, 30, 50, and 80 µm were prepared. A test mask was designed having large spacing between features to determine the increase in radius of the circles after lithographical patterning. After experimentally determining the developed resist feature radii to be 6 µm larger than the circle radii on the mask, 15 µm was selected as an appropriate spacing between the edges of each circle, and a final mask was designed with features arranged in a hexagonal close-packed conformation to minimize space between the features. Arrays of each feature size were laid out so to fit on a 200 mm wafer. After processing, the mound arrays were highly -ordered and contained negligible non-uniformities observed between mounds as determined using SEM (Fig. 1A, B). Next, the degree of curvature for each feature size was analyzed using cross-sectional SEM and average degrees of curvature were plotted in Fig. 1C. 20 µm radius mounds contained the highest degree of curvature, and 80 µm radius mounds contained the lowest. After transferring the mounds to PDMS, craters retained the shape of the mounds, yielding identical negative copies, or craters across each array (Fig. 1D).

3.2. Lining PDMS craters with PLGA nanofibers

For comparison, three nanofibrous substrates were used for subsequent cell growth experiments. Flat nanofibers were used as a substrate containing no curvature, 80 µm radius craters as a substrate exhibiting low curvature, and 30 µm radius craters as a substrate having high curvature. 20 µm radius craters were not selected to be used as a high curvature substrate because these failed to achieve full fiber conformation to the surface after PBS incubation described in the experimental methods. In order to accurately assess the effect that curved nanofibrous surfaces have on salivary gland epithelial cells, it was important to ensure that all the craters contained nanofibers which conformed to the curved surfaces. Fig.

1E shows a 30 μm radius crater post-incubation lined with nanofibers. Due to the deformation of the fiber matrix in order to achieve conformity, the spacing between fibers outside of the crater (Fig. 1Ei) was less than those inside the crater (Fig. 1Eii). However, the spacing between fibers within the craters of every size was all less than 5 μm , and most was less than 1 μm . This spacing size minimized the interaction between the cells and the PDMS substrate below the fibers, given that the diameter of SIMS and Par-C10 cells is $\sim 10\text{--}15\ \mu\text{m}$.

3.3. SIMS cell growth on crater arrays

To test the hypothesis that salivary gland epithelial cells can be affected by the curvature of the substrates, cells were seeded on samples containing craters of increasing curvature that were precoated with nanofibers. It was important to ensure that the cells conformed to the shape of the craters and grew in a monolayer. A preliminary experiment was performed to optimize cell seeding density using the adult mouse ductal cell line, SIMS. Immediately after seeding, cells settled within the craters (Supplementary Fig. 1), however after 96 h of growth, it was found through scanning electron and confocal microscopy that SIMS became 100% confluent on crater arrays of all sizes and conformed to the shape of the curved craters (Fig. 2A–C). The arrangement of cells was determined by staining for nuclei (DAPI) and F-actin (phalloidin), and it was found that nuclei tended to preferentially localize within craters of higher curvature (Fig. 2D–F). It was also observed that SIMS grown in 30 μm radius craters and flat nanofibers (Fig. 2D, F) grew in nearly perfect monolayers, whereas those grown in 80 μm radius craters (Fig. 2E) tended to overlap somewhat within the arrays.

3.4. SIMS cell monolayer heights with varying substrate curvature

To test determine whether substrate curvature has an effect on the subcellular organization of salivary gland cell monolayers, SIMS grown on substrates of varying curvature were stained for F-actin and nuclei, and confocal z-stacks were acquired to measure the height of cell monolayers. Nanofibers were stained using a red dye (SRB) that was incorporated into the polymer solution preelectrospinning to delineate the basal surface and verify that the cells were in direct contact with the substrate. The SRB-stained nanofibers were determined to be suitable indicators of basal membrane location since the cells were found in all cases to be growing directly on top of the nanofibers, and were used in lieu of a basolateral protein marker for all subsequent experiments. A series of Z-stack images was acquired for cells in craters of varying curvature, as well as on flat nanofiber controls. As illustrated in Fig. 3A and B, SIMS grown on 30 μm radius craters had substantially greater cell monolayer heights than those grown on flat nanofiber controls. The monolayer heights, including those grown on 80 μm radius craters, are quantified in Fig. 3D. A progressive increase in cell height was observed as substrate curvature increased.

Additionally, SIMS cells were grown on Matrigel samples as a positive control since epithelial cell lines are known to organize in Matrigel in a more *in vivo* like manner than on flat surfaces [9,41,44,45]. A 3-D Matrigel sample was used to determine the average cell monolayer height of cells growing as rounded aggregates containing a central lumen, which most closely resembles how the cells assemble *in vivo*. After staining cells for F-actin and nuclei, cell monolayer height measurements were acquired (Fig. 3C, quantified in Fig. 3D). Average SIMS cell monolayer heights in Matrigel were $8.3 \pm 0.91\ \mu\text{m}$, as compared to those on 30 μm radius craters, which were $9.5 \pm 0.52\ \mu\text{m}$. Finally, thin layers of Matrigel were prepared on the bottom of glass bottom dishes such that cells could not penetrate into the gel and would be forced to assemble as a flat monolayer, to determine if the increased cell layer height detected for SIMS cells grown in 3-D Matrigel was due to the 3D physical nature of the gel or simply to its chemical composition. The average thin Matrigel cell monolayer height was found to be significantly lower than any other platform tested (Supplementary Fig. 2A, quantified in Fig. 3D), indicating that the SIMS cell height in 3-D Matrigel is due to

the reorganization of the cells in 3-D and is not simply due to chemical signaling. This result further suggests that cell monolayer height is a response to substrate curvature and the ability of the cells to organize in a manner analogous to that in the 3-D gel.

3.5. Cell–cell junction protein expression and localization in SIMS with varying substrate curvature

Given the observed increase in cell monolayer height in SIMS grown on craters of increasing curvature, it was hypothesized that this phenomenon may be related to achievement of apicobasal polarity, or a cell's ability to separate proteins apically or basally based on their location *in vivo*, and possibly also due to increased expression of cell–cell junction proteins. To test this hypothesis, SIMS cells were immunostained and immunoblotted to detect the transmembrane adherens junction protein, E-cadherin, as well as the transmembrane tight junction protein, occludin. Both tight and adherens junctions are located apically along the lateral membrane of salivary gland epithelial cells *in vivo*, though the tight junction is more apically-localized [14,15,46,47].

First, expression and localization of the adherens junction protein, E-cadherin, was evaluated. As before, three substrates were used; 30 μm radius nanofibrous craters, 80 μm radius nanofibrous craters, and flat nanofibers. It was found after immunostaining that SIMS grown on all substrates expressed E-cadherin, and the protein was relatively well localized to the plasma membranes (Fig. 4A). Western blot analysis was conducted to determine total E-cadherin expression in SIMS cells on substrates of varying curvature. There was found to be no significant difference in the total expression of the protein across the three substrate types (Fig. 4B).

Next, SIMS were evaluated for localization and total expression of the tight junction protein occludin. The protein was found to be localized to the plasma membranes of cells grown on all substrate types (Fig. 5A). Unlike E-cadherin, occludin protein expression increased significantly with increasing substrate curvature, as revealed by Western blot (Fig. 5B). Additionally, localization of occludin in SIMS grown on 30 μm radius craters was compared to those grown on flat nanofibers using cross-sectional views of confocal z-stacks of immunostained cells (Fig. 5C). There was a very stark contrast in apical localization of occludin in cells grown on 30 μm radius craters, which exhibited exclusively apical localization, to those grown on flat nanofibers, which displayed fluorescence over the entire length of the lateral membranes. Additionally, fluorescence intensity line profile plots were generated, showing the intensity of occludin fluorescence along lines drawn through the apical and basal side of each cell monolayer (Fig. 5D). For SIMS on the 30 μm radius crater, a line drawn through the apical side of the monolayer has peaks of markedly higher intensity than those of the basal profile, which was mostly noise. In contrast, SIMS grown on the flat nanofiber sample exhibited apical and basal peaks which had comparable intensities. It can be concluded, therefore, that increasing the degree of nanofibrous substrate curvature increases expression and apical localization of the tight junction protein occludin in SIMS cells.

3.6. Par-C10 cell arrangement and monolayer heights with varying substrate curvature

To determine if an acinar cell line would respond to a 3-D curved substrate similarly to a ductal cell line, Par-C10 acinar cells were grown on nanofibrous craters of various levels of curvature, as well as flat nanofibers containing no curvature, 3-D Matrigel, and thin Matrigel. A comparison of top-down images stained for nuclei reveals that Par-C10 cells spread out more than SIMS on fibrous substrates of all levels of curvature, and the nuclei do not preferentially localize within the craters (Fig. 6A–C). In general, Par-C10 cells displayed lower cell monolayer heights on all of the nanofiber-coated samples (Fig. 6D, E, quantified

in Fig. 6G). Although the differences in average heights between nanofibrous samples were not as pronounced as with SIMS, there still was a statistically significant difference between cell heights of Par-C10 cells grown on 30 μm radius craters and all other substrates tested. As with SIMS, Par-C10 cells grown on thin Matrigel had a significantly lower average cell monolayer height than those on 30 μm radius craters (Supplementary Fig. 2B, quantified in Fig. 6G). Cells grown in 3-D Matrigel, however, showed a markedly higher average height than any of the other substrates (Fig. 6F, quantified in Fig. 6G). These results suggest that Par-C10 cells grown on 30 μm radius craters provide a more permissive environment for assembling a 3-D acinar phenotype as compared to those grown on flat nanofibers or 80 μm radius craters.

3.7. Cell–cell junction protein expression and localization in Par-C10 cells with varying substrate curvature

As with SIMS, immunostaining and immunoblotting was performed on Par-C10 cells, examining localization and total expression of cell–cell junction proteins E-cadherin and occludin. The adherens junction protein E-cadherin was first evaluated. Immunostained cells showed that the protein was expressed in cells grown on 30 μm and 80 μm radius craters, as well as on flat nanofibers (Fig. 7A). Although the protein was somewhat membrane-localized, there was a significant amount of cytoplasmic localization of E-cadherin in this cell line; however the levels of cytoplasmic E-cadherin did not appear to be substrate-dependent. Western blot analysis revealed that there was no difference in the total protein expression levels of E-cadherin as the level of substrate curvature increased (Fig. 7B).

The tight junction protein, occludin, was also evaluated in Par-C10 cells. Immunostaining revealed that occludin was expressed in cells on all substrate types, and staining was confined to the membranes of the cells (Fig. 8A). Total expression of occludin was found to increase significantly as substrate curvature increased, especially on 30 μm radius craters (Fig. 8B). Even though Par-C10 cell monolayer heights were generally lower than those of SIMS cells, it was apparent from cross-sectional fluorescence confocal images that occludin was more apically-localized in cells on 30 μm radius craters than in cells on flat nanofibers (Fig. 8C). The increased apical localization in Par-C10 cells on 30 μm radius craters was measured by capturing fluorescence intensity profiles (Fig. 8D). The line profile for the apical region of the cell monolayer on the 30 μm radius crater demonstrated significantly larger peaks than the profile of the basal region, whereas both profiles for cells on flat nanofibers are similar. This data indicates that tight junction formation in Par-C10 cells is enhanced by increased nanofibrous substrate curvature.

3.8. Aquaporin-5 expression in Par-C10 cells grown on substrates of varying curvature

The increased apical polarization of the salivary gland cell lines suggests that functional differentiation may follow. As an early indicator of acinar differentiation in Par-C10 cells, total expression of a major water channel protein in salivary gland acinar cells, aquaporin-5 (Aqp-5), was determined by Western blot (Fig. 9). For these experiments, an additional crater of radius 50 μm was included in the analysis and shown in the representative blot and corresponding band intensity plot. It was determined that nanofibrous substrate curvature increases expression of Aqp-5, as cells grown on 30 μm radius craters showed significantly higher levels of Aqp-5 protein, as compared to cells grown on nanofibrous substrates containing less curvature. A slight but negligible increase in Aqp-5 expression was seen when increasing curvature from flat to 80 μm radius craters.

4. Discussion

Currently, there is no concrete platform for engineering an artificial salivary gland that has been shown to fully recreate the environment in which epithelial cells interact with *in vivo*. As a result of the multifaceted 3-D nature of the salivary gland and basement membrane *in vivo*, current approaches for engineering an implantable salivary gland have been restricted by both material selection and engineering limitations. Currently, 3-D gel-based substrates allowing self-assembly of acinar cells, and flat polymeric surfaces which force cells to assemble as a monolayer are used, each presenting practical and theoretical benefits and restrictions. Here, the approach of organizing cells on polymeric substrates was chosen since it allows control over the arrangement of cells, and innovative engineering approaches were implemented to add structural complexity that was to date only exhibited in 3-D gel platforms or *in vivo*. We built upon previous studies demonstrating benefits of growing salivary gland cell lines on nanofibrous substrates as compared to flat surfaces [24–26] by adding curvature under the nanofibers, intended to mimic the nano and microscale structure of the basement membrane surrounding salivary gland epithelial cells.

Photolithography proved to be an effective method for uniformly and reproducibly generating arrays of mounds to be used as a mold for patterning craters into PDMS. The degree of curvature of the mounds was increased by decreasing the feature radius. Since the temperature used for photoresist reflow was above the glass transition temperature, but lower than the melting point of the resist, the contact angle for each size mound varied, resulting in differences in curvature. By exploring surface treatment of the wafer or varying the reflow temperature, it may be possible to further control the degree of curvature for various feature sizes.

Both salivary gland epithelial cell lines examined in this study were able to form a confluent monolayer and conform to the nanofibrous craters. Although the arrays were optimized to maximize the curved substrate surface area, a small amount of surface area between craters in each sample was flat. This may have had a minor effect on the Western blot analysis, which was done by lysing all the cells on the arrays. In the future, it may be possible to restrict cell attachment to the craters by coating the flat regions with an anti-cell adhering polymer such as polyethylene glycol (PEG). In both ductal SIMS and acinar Par-C10 cell lines, there was a significant increase in cell monolayer height as nanofibrous substrate curvature was increased from none (flat nanofibers) to the highest level (30 μm radius craters). It is of interest that the acinar cell line was not as responsive to substrate curvature as was the ductal cell line; the reasons for this are unknown. Although the molecular mechanism driving epithelial cell response to substrate curvature remains unclear, our results indicate that curvature stimulates polarity in epithelial cells, which promotes assembly of the tight junction protein, occludin, at the apical cell surface. Tight junctions are important in maintaining a semi-permeable seal between epithelial and other cells at the apical region of the lateral membrane to regulate paracellular transport, an important feature in functioning acinar epithelial cells [14–16,48]. Of the two junction proteins assayed in this study, occludin, a protein exclusive to tight junctions, showed the greatest response to increasing substrate curvature, as both expression and apical localization significantly increased in cells on 30 μm radius craters for both SIMS and Par-C10 cells. This suggests that by forcing a salivary gland epithelial cell monolayer into a curved conformation, there may be a facilitation of tight junction assembly within the cells. In future studies, major aims are to understand the mechanisms behind the behavior exhibited by epithelial cells forced to grow on curved substrates. Additionally, we will build upon the results of this study and those in our recent study demonstrating that apicobasal polarity increases in cells grown on laminin-functionalized nanofibers [26]. It may be that Par-C10 acinar cells require both

physical and chemical cues to assemble into fully polarized cell monolayers with cell heights comparable to those in cells grown in 3-D Matrigel.

One of the most promising results in this study was that expression of the water channel protein aquaporin-5 (Aqp-5) increased significantly on 30 μm radius craters as compared to flat nanofibers. Aqp-5 has been used as a marker of acinar differentiation in several studies, and is a good indicator of acinar functionality [49–51]. It has been demonstrated previously that there is an interaction between Aqp-5 expression and tight junction assembly, as Aqp-5-deleted mice exhibited decreased expression of tight junction proteins, including occludin [52]. Thus, future studies may examine whether there is an effect on Aqp-5 expression when tight junction protein expression and/or localization is enhanced on nanofibrous craters.

To our knowledge, this is the first development of a substrate surface which combines the engineering of curved surfaces with a coating of electrospun nanofibers with the intention of mimicking the physical structure of the basement membrane surrounding many epithelial cell types in branching organs. From a physiological standpoint, it has clear benefits over flat surfaces or flat nanofibers given its physical resemblance to the basement membrane. It is also a more practical alternative to current platforms, adding necessary surface area needed to secrete adequate amounts of saliva as compared to flat surfaces, and controlling the arrangement of cells so that eventually an incorporated device which can direct saliva can be achieved.

5. Conclusions

In this study, wafer-level photolithography, photoresist reflow, replica molding, and electrospinning were combined to create a growth surface containing nano and microscale features intended to mimic the physical structure of the basement membrane surrounding salivary gland acinar epithelial cells while increasing surface area of the scaffold. The PDMS substrates containing PLGA nanofiber-lined, micropatterned “craters” induced a subcellular organizational change in both ductal SIMS and acinar Par-C10 salivary gland epithelial cell lines, significantly increasing the average height of cell monolayers grown within craters containing the highest level of curvature, as compared to cells grown on flat nanofibers. Nanofibrous substrate curvature did not have an effect on expression or localization of the adherens junction protein E-cadherin; however, curvature significantly increased both expression and apical polarity of the tight junction protein occludin in both SIMS and Par-C10 cells. Additionally, increasing curvature under Par-C10 cells was found to increase expression of an acinar differentiation marker, aquaporin-5, a major water channel protein in salivary gland acinar cells. In conclusion, 3-D nanofibrous craters have promise in advancing the development of an artificial salivary gland, and could help resolve shortcomings of currently-used platforms in the field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Creation and characterization of nanofibrous crater arrays. (A, B) After thermal reflow, photoresist mound arrays are highly ordered and uniform. (C) Plot shows a decrease in degree of curvature as feature radius increases. “Crater radius” refers to the radius of the circle used in the photolithography mask and is independent from the height of the mounds or the depth of the craters. Due to the nearly identical mound size within each array, standard deviations are negligible. (D) After transferring the arrays to PDMS, craters are formed which exhibit the negative shape and same order as the mounds. (E) Once PLGA nanofibers have been deposited onto craters and incubated in PBS for 3 days and cell media for 1 day, fibers conform to the crater curvature and samples are ready for cell seeding. As a result of fiber stretching during conformation, pore size of fibers deposited on the flat areas (Ei) are smaller than those within the crater (Eii).

Fig. 2.

SIMS mouse submandibular cell line conforms to the shape of 30 μm radius nanofibrous craters after 96 h of growth. SEM images show cell conformity from (A) top-down and (B) angled views. (C) A tilted view of a fluorescence confocal Z-stack 3-D projection with SIMS stained for F-actin (green, phalloidin), and nuclei (blue, DAPI), conforming to a crater lined with nanofibers (red). The Z-plane is denoted by the arrow. The arrangement of nuclei on (D) 30 μm, (E) 80 μm radius craters, and (F) flat nanofibers, reveals that nuclei preferentially localize within the craters, more obviously on 30 μm radius craters. Dotted circles outline a portion of the craters in each sample. On 80 μm radius craters, some regions exhibited cell overlapping (red arrows), whereas on 30 μm radius craters and flat nanofibers, cells grew largely as one monolayer over the entirety of the samples.

Fig. 3.

Confocal top-down and cross-sectional views illustrating differences in height of SIMS cell monolayer on a (A) 30 μm radius crater, (B) flat nanofibers, and in (C) 3-D Matrigel. Cells on fibers are stained for F-actin (phalloidin, green) and nuclei (DAPI, blue). Nanofibers are stained red. Cells in Matrigel were stained for F-actin (phalloidin, green), and nuclei (DAPI, blue). (D) Measured heights of SIMS cell monolayers on craters of various sizes, flat nanofibers, and Matrigel. Measurements in craters were acquired at the center (bottom) of the craters. Thin Matrigel is a layer of Matrigel deposited such that it is too thin for cells to penetrate into and grow in full 3-D, whereas 3-D Matrigel is deep enough for cells to grow within the gel. *** indicates data point is significantly different from others (ANOVA, $p < 0.001$).

Fig. 4. (A) Confocal top-down images of nuclei (DAPI, blue) and expression of adherens junction protein E-cadherin (green) in SIMS cells grown on nanofibrous substrates of varying curvature. The protein is expressed and localized mostly to cell membranes on all substrate types. (B) A representative Western blot shows no definitive trend in E-cadherin expression as substrate curvature increases. Normalized band intensities are plotted beside blot image. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

(A) Confocal top-down images of nuclei (DAPI, blue) and expression of tight junction protein occludin (green) in SIMS cells grown on nanofibrous substrates of varying curvature. The protein is expressed and localized to cell membranes on all substrate types. (B) A representative Western blot shows an increase in occludin expression as substrate curvature increases. Normalized band intensities are plotted beside blot image. (C) Cross-sectional views of confocal z-stacks stained for nuclei (DAPI, blue), occludin (green), and fibers (red) show that occludin is more apically-localized (top of the cells) in SIMS on 30 μm radius craters as compared to those grown on flat nanofibers, where the protein fluorescence extends from the apical to the basal side of the cells. (D) Profiles of occludin fluorescence intensity for lines drawn through the apical (blue) and basal (red) side of SIMS cell monolayers on 30 μm radius craters and flat nanofibers, respectively. Cells on the crater show markedly larger peaks for a line drawn through the apical region of the cell monolayer as compared to the basal profile, whereas cells on flat nanofibers have similarly-sized peaks for both apical and basal regions. Areas of locally-intense staining in (C) and their corresponding graphical peaks in (D) are numbered.

Fig. 6.

Par-C10 cell organization on nanofibrous substrates of varying curvature. The arrangement of nuclei in (A) 30, (B) 80 μm radius craters, and (C) flat nanofibers reveals that Par-C10 cells are more spread than SIMS, and nuclei do not preferentially localize within craters. Dotted circles outline a portion of the craters in each sample. Confocal top-down and cross-sectional views showing height of Par-C10 cell monolayer on (D) 30 μm radius crater (E) flat nanofibers, and (F) 3-D Matrigel respectively. The cross-sectional views reveal that cell monolayer layer heights for both nanofibrous substrates are distinctly lower than cells grown in 3-D Matrigel. Cells are stained for F-actin (phalloidin, green) and nuclei (DAPI, blue). Nanofibers are stained red. When Par-C10 heights were quantified (G), cells grown on 30 mm radius craters had significantly higher monolayer heights than cells on substrates of lesser curvature, and those growing in 3-D Matrigel exhibited strikingly higher cell layer heights than any of the other samples (ANOVA ** $p < 0.01$, *** $p < 0.001$).

Fig. 7. (A) Confocal top-down images of nuclei (DAPI, blue) and expression of adherens junction protein E-cadherin (green) in Par-C10 cells grown on nanofibrous substrates of varying curvature. The protein is expressed and marginally localized to cell membranes on all substrate types. (B) A representative Western blot shows no definitive trend in E-cadherin expression as substrate curvature increases. Normalized band intensities are plotted beside blot image. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 8. (A) Confocal top-down images of nuclei (DAPI, blue) and expression of tight junction protein occludin (green) in Par-C10 cells grown on nanofibrous substrates of varying curvature. The protein is expressed and localized to cell membranes on all substrate types. (B) A representative Western blot shows that occludin expression increases as substrate curvature increases, especially on 30 μm radius craters. Normalized band intensities are plotted beside blot image. (C) Cross-sectional views of confocal z-stacks stained for nuclei (DAPI, blue), occludin (green), and fibers (red) show that occludin is more apically-localized (top of the cells) in Par-C10s on 30 μm radius craters as compared to those grown on flat nanofibers, where the protein fluorescence extends from the apical to the basal side of the cells. (D) Profiles of occludin fluorescence intensity for lines drawn through the apical (blue) and basal (red) side of Par-C10 cell monolayers on 30 μm radius craters and flat nanofibers, respectively. Cells on the crater show markedly larger peaks for a line drawn through the apical region of the cell monolayer as compared to the basal profile, whereas cells on flat nanofibers have similarly-sized peaks for both apical and basal regions. Areas of locally-intense staining in (C) and their corresponding graphical peaks in (D) are numbered.

Fig. 9.

Water channel aquaporin-5 (Aqp-5) expression in Par-C10 cells on engineered substrates. A representative Western blot and corresponding normalized band intensity plot showing that expression of Aqp-5 increases when cells are grown on 30 μm craters as compared to substrates of lesser curvature. An additional intermediate crater size of 50 μm radius was analyzed in these experiments.