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Micropatterned Dynamically Adhesive Substrates for Cell Migration

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We present a novel approach to examine cell migration using dynamically adhesive substrates consisting of three spatially and functionally distinct regions: the first is permanently nonadhesive to cells, the second is permanently adhesive, and the final region is electrochemically switched from nonadhesive to adhesive. We applied a double microcontact printing approach to pattern gold surfaces with carboxylic acid-terminated self-assembled monolayers (SAMs) that permit initial cell adhesion, with methyl-terminated SAMs that permit adsorption of a nonadhesive, and with tri(ethylene glycol)-terminated SAMs that can be electrochemically "switched" to permit cell migration from a prespecified pattern onto a new pattern. Using these substrates, we investigated the migration of epithelial cells from monolayers onto narrow, branching tracks of extracellular matrix in order to characterize how lead cells influence the direction of movement of followers. Time-lapse imaging revealed that, on average, five cells consistently chose one branch before other cells entered the second branch, providing evidence to suggest that intercellular communication plays an important role in guiding the cohesive movement of epithelial sheets. This platform may be of use in furthering our understanding of the mechanisms underlying cellular decision-making during migration in both individual and multicellular contexts.

Introduction

Cell migration is integral to a wide variety of physiologic processes. Epithelial migration plays an important role during normal developmental morphogenesis, as sheets of cells migrate and fold to form specific structures within the developing embryo.[1−5] In cancer progression, the loss of proper migratory control in metastatic cells leads to the formation of new tumors at sites distant from the primary tumor.[6] Following wounding, numerous cell types, including epithelial cells, fibroblasts, immune cells, and endothelial cells, invade the wound bed to fill in and repair the tissue.[7−9] Despite these abundant and critical instances of collective cell movement, the most common assay used to study collective and directed migration in vitro is the scrape-wounding assay. In this assay, a monolayer of adherent cells is scraped and cells are monitored as they invade the denuded area.[10] Recent evidence indicates that epithelial cells several layers away from a scrape-wound edge assist in the movement of the leading edge of cells, suggesting that cells within a multicellular migrating sheet communicate via as yet undetermined means.[11,12] In addition, the migration of cells in certain developmental contexts is restricted to specific areas by a combination of adhesive and repulsive cues that guide cells along appropriate paths.[13] However, because scrape-wounding affords no control over initial organization of cells and where they migrate, addressing the tantalizing prospect of cell-to-cell communication during collective movements has not been studied in detail.

The confluence of micropatterning technologies and novel surface chemistries to generate dynamically adhesive substrates provides a powerful new approach to noninvasively study cell migration. These approaches are based upon spatially arranging cell culture surfaces into regions adhesive to cells and regions that are nonadhesive to cells, and subsequently rendering the nonadhesive regions newly adhesive. In one such approach, microfabricated membranes of poly(dimethyl siloxane) (PDMS) were used to spatially confine cells to openings within the membrane.[14] Cells could then be released from their initial positions by lifting the membrane off of the culture substrate. More recent approaches have focused on utilizing self-assembled monolayers (SAMs) on gold to generate substrates whose surface chemistry can be electrochemically "switched" to expose newly adhesive areas. In one such approach, Mrksich and co-workers designed an elegant system based on electroactive SAMs to capture soluble RGD moieties from solution using a Diels−Alder reaction.[15] Captured RGD rendered previously nonadhesive SAM domains cell-permissive, thus facilitating cell adhesion and migration onto these areas. In a later approach, EG3-terminated SAMs, typically used as a nonadhesive when spatially patterning cells using microcontact printing, were desorbed from the surface following

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an electrochemical pulse, exposing bare gold and permitting the adsorption of ECM proteins and subsequent movement onto the surface.[16] By affording control over the temporal onset of migration from a prespecified geometry, dynamic substrates offer the potential to study how cells transition from a sessile to migratory state as well as the impact of preswitch geometric constraints on subsequent cell movement.[17]

Here, we apply micropatterning technologies to generate dynamically adhesive substrates to investigate spatially controlled, collective cell migration. We have developed methods to pattern substrates with three distinct regions of surface adhesivity—regions that are permanently adhesive, regions that are permanently nonadhesive, and regions that are initially nonadhesive but that can be electrochemically switched to become adhesive. Using microfabricated elastomeric stamps to control the spatial positioning of these different surface domains, we used these substrates to investigate cell migration from a monolayer into a confined space. Specifically, we examined whether Madin Darby canine kidney (MDCK) cells respond to a branch in their migratory path independently or as a multicellular unit, and present data that suggests cooperative migratory behavior on the part of these cells.

Materials and Methods

Materials. The following reagents were used (supplier): octadecanethiol (Sigma), referred to as CH3 thiol; mercaptohexadecanoic acid (Sigma), referred to as COOH thiol; tri(ethylene glycol)-terminated hexadecanethiol (Prochimia), referred to as EG3 thiol; Pluronic F127 (BASF); and fibronectin (BD Biosciences).

Cell Culture. Bovine adrenal microvascular endothelial cells (BAMEC) were obtained from VEC Technologies and were cultured in low-glucose DMEM containing 10% fetal bovine serum (Hyclone), 10 ng/mL EGF, 3 ng/mL bFGF, 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (all unspecified reagents from Invitrogen). Madin Darby canine kidney (MDCK, from ATCC) cells and normal rat kidney (NRK, from ATCC) cells were cultured in high-glucose DMEM containing 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (all from Invitrogen). For time-lapse imaging, cells were cultured in CO_2-independent media (Invitrogen) supplemented with 3.5 g/L D-glucose (Sigma), 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (all from Invitrogen).

Preparation of Stamps. Stamps of poly(dimethylsiloxane) (PDMS; Sylgard 184, Dow Corning) were prepared by casting prepolymer onto photolithographically generated master patterns on silicon, as previously described.[18]

Preparation of Patterned Gold Substrates. Gold-coated coverslips were prepared by electron beam evaporation of 5 nm titanium followed by 20 nm of gold. Patterned substrates containing only CH3 and COOH thiols were prepared by placing a PDMS stamp inked in a solution of 1−2 mM CH3 thiol in ethanol in conformal contact with the gold substrate for 4 min. Substrates were then immersed in a solution of 1−2 mM COOH thiol for 2 min. After rinsing and sterilizing, samples were incubated in 1% Pluronic F127 overnight, followed by 50 μg/mL fibronectin for 1 h. For substrates containing only COOH and EG3 thiols, stamps inked in a solution of 1−2 mM COOH thiol were placed in contact with the gold substrate for 2 min. Substrates were then immersed in a solution of 1−2 mM EG3 thiol for 1 h. After rinsing and sterilizing, samples were incubated in 50 μg/mL fibronectin for 1 h prior to seeding cells.

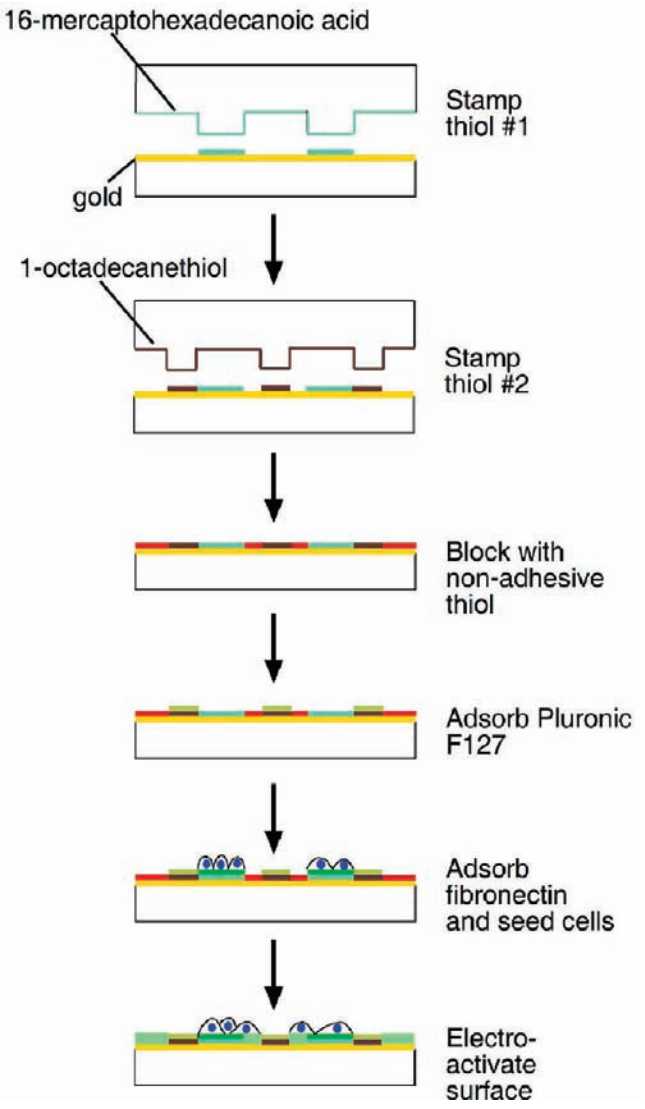

Figure 1. Schematic of method to prepare substrates with three functionally different domains—one adhesive to cells or protein, one nonadhesive to cells or protein, and one initially nonadhesive but that can be electrochemically switched to become adhesive to cells or protein.

For trifunctional substrates, samples were printed using stamps inked with CH3 thiol as above. After removal of the stamp, a fresh stamp containing a second pattern inked with COOH thiol was aligned to the CH3 thiol pattern and printed. The order of CH3 versus COOH thiol stamping was reversed as needed to generate the desired surface chemistry. Samples were then immersed in EG3 thiol for 1 h, rinsed, sterilized, incubated in 1% Pluronic F127 overnight, and coated with 50 μg/mL fibronectin for 1 h before either seeding cells or switching for optimization studies. Substrates ultimately displayed trifunctional, patterned surfaces consisting of CH3, COOH, and EG3 self-assembled monolayers (SAMs).

Electrochemical Desorption of EG3. Prior to application of a voltage pulse to desorb EG3 SAM from the surface, samples were rinsed in Hanks' balanced salt solution (HBSS, Invitrogen) and incubated in an HBSS solution containing 0.2% Pluronic F127 and either 200 μg/mL mouse IgG (Sigma) for substrates without cells or 50 μg/mL fibronectin (BD Biosciences) for substrates containing cells. A potentiostat (Princeton Applied Research; Gamry) was used to apply a voltage potential between the gold substrate (cathode) and a stainless steel counter electrode immersed in the medium. Cells were cultured in the HBSS solution for 1 h, then returned to their normal culture medium.

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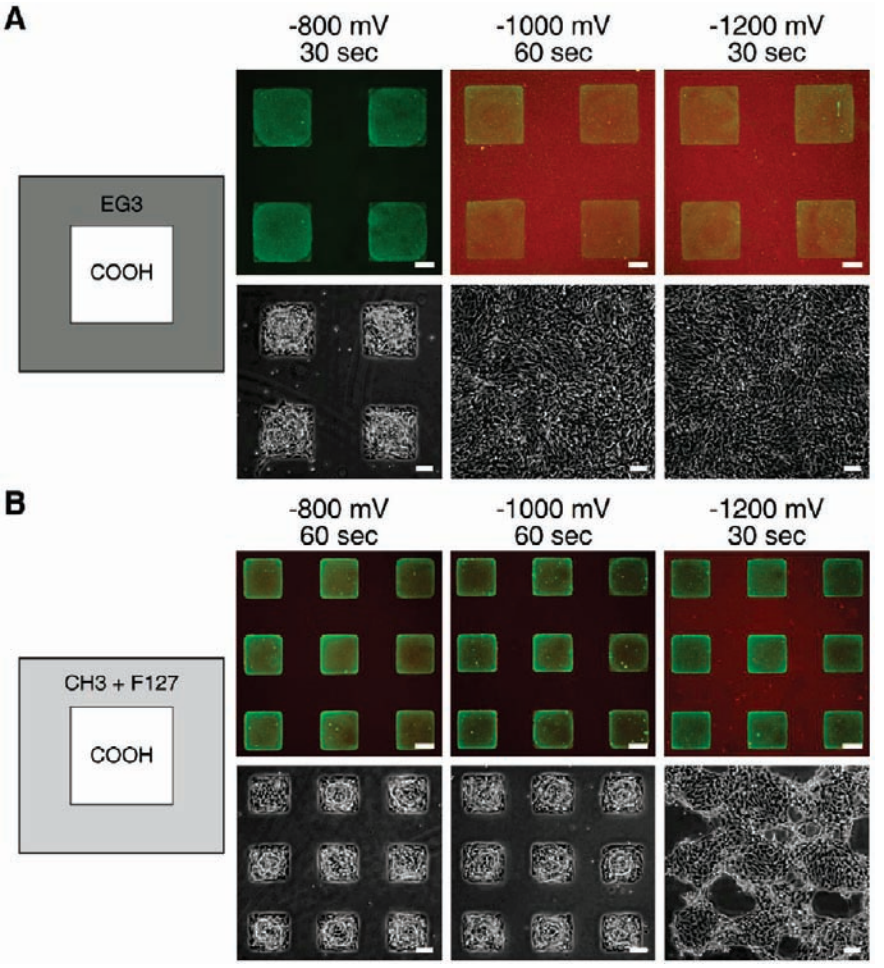

Figure 2. Optimization of switching voltages using bifunctional substrates. Green indicates fibronectin; red indicates mouse IgG adsorbed from solution following electrochemical switch. Mouse IgG was adsorbed for 1 h after switching, followed by fixation and immunostaining. For experiments with cells, fibronectin was adsorbed for 1 h after switching before rinsing and returning to culture medium. Images of cells were taken at least 18 h after switching. (A) COOH/EG3 combination. (B) COOH/CH3+F127 combination. All scale bars indicate 100 μm.

For samples containing only COOH and EG3 SAMs, Pluronic F127 was not added to the desorption solution. Voltages between −800 and −1600 mV and applied potential times between 15 and 90 s were examined.

Immunofluorescence Analysis and Imaging. Immunofluorescence samples were fixed in 4% paraformaldehyde, blocked in 1% bovine serum albumin (Serologicals Corporation), and stained with goat anti-mouse IgG (Invitrogen) and rabbit anti-fibronectin (Sigma), followed by appropriate secondary antibodies labeled with AlexaFluor 546 and AlexaFluor 488 (Invitrogen), respectively. Substrates were imaged on an inverted fluorescence microscope (Eclipse TE200, Nikon) using a 10× 0.30 NA objective and a cooled CCD camera (Diagnostic Instruments).

For time-lapse imaging, samples were placed within a heated and CO_2 controlled stage-top incubator (In Vivo Scientific) mounted to an inverted microscope (Axiovert 200M, Zeiss). Multiple fields were simultaneously recorded using a motorized stage (Marzhauser) controlled by *Axiovision* software (Zeiss). Fields were then analyzed using *Axiovision* software to determine the number and total length of cells that had entered one branch prior to the entrance of a cell into the second branch and the time delay between when cells entered the first branch and when they entered the second branch.

Results

We hypothesized that SAMs consisting of multiple terminal groups of differing hydrophobicity could be used to generate patterned, trifunctional surfaces with regions that were permanently adhesive, regions that were permanently nonadhesive, and regions that were initially nonadhesive but could be electrochemically switched to become adhesive (Figure 1). To determine the optimal conditions for preparing such a surface, we first examined SAM surfaces prepared with paired combinations of thiols. COOH-terminated SAMs adsorb extracellular matrix proteins such as fibronectin and therefore permit cell adhesion. In contrast, regions of EG3-terminated SAMs resist protein adsorption and consequently cell adhesion. Previous reports have demonstrated that EG3-terminated thiol can be desorbed from the gold surface via a voltage potential to generate newly adhesive domains.[16] To confirm the dynamic control over substrate adhesivity on bifunctional surfaces consisting of COOH and EG3 terminated SAMs, we seeded BAMECs onto 300 μm × 300 μm islands and observed that they migrated out of the patterns after pulses below −1 V, but not at larger voltages (Figure 2A). Immunofluorescence analysis of switched surfaces demonstrated that EG3 was desorbed from the surface and protein in solution adsorbed onto newly exposed regions (Figure 2A). We then examined whether permanently nonadhesive regions could be generated by the adsorption of Pluronic F127 onto SAMs generated from CH3 thiols. We have demonstrated previously that Pluronic F127 binds strongly to hydrophobic but not hydrophilic domains and thereby renders the surface nonadhesive.[18] On bifunctional substrates of COOH

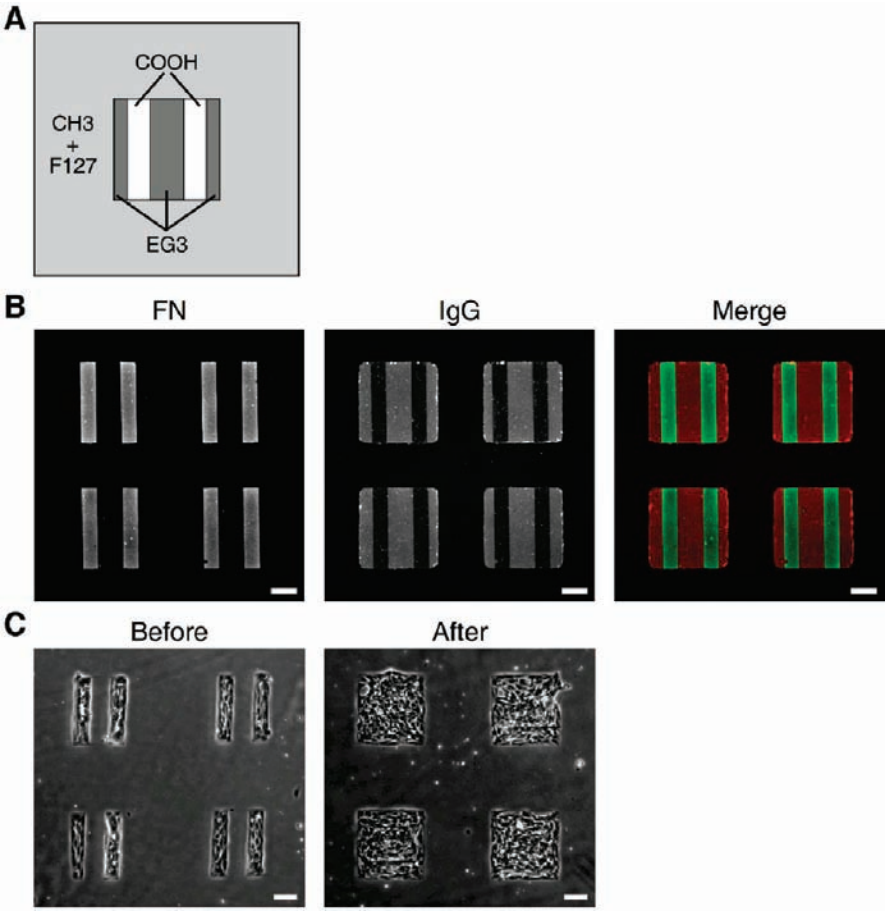

Figure 3. Generation of trifunctional substrates. (A) Schematic of the trifunctional SAM pattern following microcontact printing. (B) Three-color micropatterned substrates following electrochemical switch at −1 V for 60 s. Mouse IgG (red) was adsorbed for 1 h after switching, followed by fixation and immunostaining. Green indicates fibronectin that was adsorbed prior to switching. (C) Cell migration onto EG3-desorbed areas. Cells were cultured for at least 24 h before applying the electrochemical switch at −1 V for 60 s. Fibronectin was adsorbed for 1 h after switching before rinsing and returning cells to culture medium. The "after" image was taken at least 24 h after switching. All scale bars indicate 100 μm.

and CH3 SAMs coated with Pluronic F127 and fibronectin, cells adhered to the COOH SAM domains but not the regions containing CH3 SAMs. Upon application of electrochemical pulses of −0.8 V or −1 V for 60 s, patterns were maintained, but at more negative voltages such as −1.2 V, proteins from solution adsorbed onto the surface and enabled cells to migrate out of their patterns (Figure 2B). Interestingly, the presence of 0.2% Pluronic F127 in the switching solution was essential to maintain the nonadhesive barrier even at lower voltages, suggesting that the electrochemical pulse might compromise the nonspecific interaction between Pluronic and the CH3-terminated thiol.

These observations suggested that we could generate trifunctional surfaces by using a −1 V pulse for 60 s wherein dynamically adhesive regions consisting of EG3 SAMs are "switched" while CH3/Pluronic domains remain nonadhesive. To test this possibility, we sequentially stamped CH3 and COOH thiols onto a gold surface, immersed the gold into a solution containing EG3 thiol, and finally immersed the surface in a solution containing Pluronic F127 (Figure 1). The COOH thiol only reacted with the surface in regions where the CH3 thiol stamp had not made contact. This stamping configuration resulted in a trifunctional surface pattern in which "lines" of COOH thiol alternated with "lines" of EG3 thiol within islands surrounded by CH3/Pluronic (Figure 3A). After incubation in fibronectin, immunofluorescence samples were switched at −1 V for 60 s in the presence of IgG to examine the extent of protein adsorption onto the trifunctional surface. As per the bifunctional studies, new protein (IgG) only adsorbed onto the regions originally consisting of EG3-terminated SAM (Figure 3B). Cells were seeded onto parallel substrates and attached in lines corresponding to COOH thiol. After voltage application, cells migrated out of the lines to fill in the island (Figure 3C), but did not extend beyond the island onto regions of CH3/Pluronic for several days (data not shown). These findings demonstrate that, by tailoring the adhesivity of different regions of a SAM surface, a trifunctional surface could be developed for investigating dynamic cell migration events.

We next applied these micropatterned, trifunctional surfaces to examine the migratory behavior of Madin Darby canine kidney (MDCK) epithelial cells, as they encountered a symmetric branch in their migratory path. We hypothesized that because epithelial cells have strong cell−cell adhesions and exhibit sheet-like movement in which cells at the leading edge seem to influence movement of following cells, such cells may branch asymmetrically as opposed to symmetrically.[11,12,19−21] To test this hypothesis directly using micropatterned, trifunctional substrates, we stamped 1500 μm × 1500 μm squares of COOH thiol followed by

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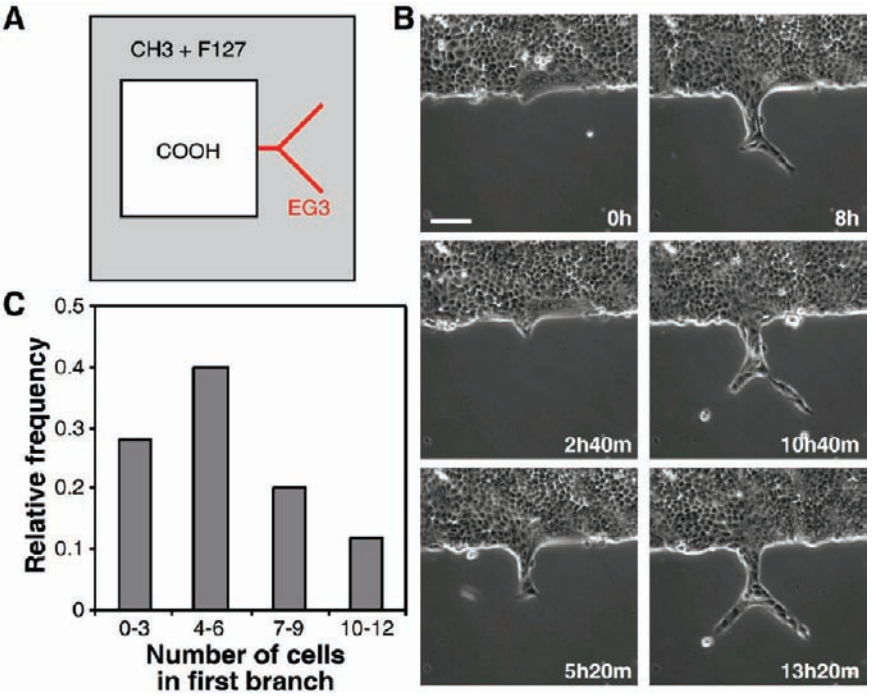

Figure 4. Geometric control of cell migration. (A) Patterning schematic for restricted MDCK migration on trifunctional patterned surfaces. (B) Snapshots of cells migrating into branched pattern. Cells were cultured for 2 days until they reached confluence within the 1500 μm $\times$ 1500 μm COOH-adsorbed squares. Samples were then electrochemically switched at -1 V for 60 s, and fibronectin from solution was adsorbed for 1 h before returning cells to normal media and initiating time-lapse imaging. Snapshot labeled 0 h was taken an arbitrary time after the surface was electrochemically switched but prior to commencement of cell migration. Scale bar indicates 100 μm. (C) Histogram of the number of cells in the first branch at the start of migration into the second branch, based on 25 time-lapse videos.

an inverse pattern of 25-μm-thick branches with CH3 thiol, such that EG3 thiol would adsorb to the gold in the branched pattern (Figure 4A). The pattern was designed to permit cells to migrate approximately 100 μm from the edge of the square before encountering the branch point. Cells initially adhered to the large squares consisting of COOH-terminated SAM and fibronectin. After culturing for 2 days to allow cells to reach confluence within the 1500 μm $\times$ 1500 μm squares, samples were switched, and fibronectin from solution was adsorbed for 1 h before replacing with normal media. Samples were imaged using phase contrast time-lapse microscopy over a period of 24$-$48 h to track cellular migration onto the branched patterns (Figure 4B, Supporting Information Video 1). Cells migrated smoothly until the bifurcation point, at which point the lead cell extended lamellipodial protrusions into both branches before selecting one direction and turning. When comparing between experiments, the lead cell did not select one branch preferentially, but rather distributed between the two branches randomly. Interestingly, several cells in close proximity to the lead cell also turned and followed in the same direction for an extended period of time before another cell initiated migration into the second branch. We observed asymmetric branching behavior, wherein one branch contained multiple cells before the second branch contained any cells, in approximately 80% of the cases (n = 25 patterns total) (Figure 4C). To quantify and assess the strength of this asymmetry, we recorded the number of and total distance traveled by cells that entered one branch before any cells entered the second branch. We found that on average five cells entered the first branch (Figure 4C) and migrated a distance of 80 μm over a period of 2.4 h before any cells extended lamellipodia into the second branch. The likelihood of observing 5 cells choose the same branch if the cells behave independently (branched in either direction with a probability of 0.5) is (0.5),[5] or $\sim$3%. Interestingly, it appeared as though the protrusion of cells into the first branch retarded the movement of cells into the second branch, as often cells poised to enter the second branch did not do so until the first branch had extended some distance. These experimental results suggest that cells migrating in a group do not move independently; their migratory behavior is linked to that of their neighbors. The mechanisms underlying such cell-to-cell communication during movement remain to be explored. These experiments demonstrate the utility of using micropatterned, trifunctional dynamic substrates to study collective cell movements.

Discussion

We developed a technique to generate trifunctional dynamically adhesive substrates using microcontact printing to pattern gold surfaces with carboxylic acid-terminated self-assembled monolayers (SAMs) that permit initial cell adhesion, with methyl-terminated SAMs that allow adsorption of a nonadhesive, and with tri(ethylene glycol)-terminated SAMs that can be electrochemically switched from nonadhesive to adhesive. While CH3-terminated SAMs are typically used to adsorb protein and enable cell adhesion, we demonstrate here that in conjuction with Pluronic F127 these domains can be rendered inert. We identified the optimal voltage, time, and solution conditions that permit desorption of EG3-terminated regions while retaining the nonadhesive character of Pluronic-coated regions. At voltages beyond this threshold of -1 V, both the EG3-terminated and Pluronic-coated regions failed. It is unclear whether these higher voltages lead to desorption of CH3-terminated SAM from the gold surface or if they weaken the van der Waals interactions between Pluronic and the CH3 terminus. However, the presence of Pluronic F127 in solution is necessary even at -1 V to preserve the nonadhesiveness of CH3/F127 domains, suggesting that the Pluronic to CH3 SAM interface is the weak link.

These surfaces provide a powerful tool to spatially modulate adhesivity and generate complex cell culture substrates for a variety of applications. The ease with which COOH thiols and CH3 thiols can be microcontact printed permits precise control over where cells can and cannot form adhesions. While bifunctional dynamically adhesive SAM surfaces can be used to control the initial geometry of cell adhesion, cells migrate randomly on the surface following switching.[16,17] Trifunctional surfaces enable control over both initial pattern geometry as well as the geometry of switched areas, permitting studies into how cells adapt from one set of ECM constraints to another. While we have focused on applying these substrates to investigate cell migration, they may be applicable to a variety of other scenarios, for example, to capture a second cell type in a specified geometry adjacent to prepatterned cells as a means to investigate heterotypic cellular interactions.

The application of these substrates to investigate the coordinated movement of epithelial cells following a change in surface adhesivity revealed novel cooperative behavior between cells within the sheet. While the mechanisms underlying this behavior are unclear, mechanical forces transmitted through the sheet as well as biochemical signals are likely to contribute. Junctions between epithelial cells, including cadherin-mediated linkages as well as tight junctions and desmosomes, may transmit force across many cell lengths as a means of guiding movement.[22−25] Indeed, the apparent inhibition of migration into the second branch could in part be caused by mechanical forces exerted by the forward movement of cells along the first branch. Alternatively, adherens junctions between adjacent cells have recently been shown to trigger biochemical signaling that biases the migration direction of adjacent cells.[26,27] Regardless of the exact mechanism, the distance and numbers of cells that migrate into the first branch before initiation of second branch migration suggest the presence of an attenuating signal sent by leading cells. Interestingly, these data suggest that the position of cells within a migrating sheet may dictate their position at a later time and act as an important cue during developmental patterning. Multifunctional dynamically adhesive substrates provide a general tool to explore these questions in a more spatially controlled fashion in vitro, and have begun to enable novel insights into the mechanisms underlying epithelial migration.

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Supporting Information Available: We have included a time-lapse video of MDCK cells migrating into a branched pattern as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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