Degradation of Micropatterned Surfaces by Cell-Dependent and -Independent Processes†

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This paper describes a study to determine the role of active cellular processes in the initial patterning and eventual degradation of different micropatterned substrates. We compared the effects of serum and cell type on the ability of cells to crawl onto the nonadhesive regions of a variety of patterned substrates. Cells initially patterned in the presence of serum onto substrates manufactured using agarose, pluronics, hexa(ethylene glycol), or polyacrylamide as the nonadhesive. While polyacrylamide remained inert and patterned cells for at least 28 days, agarose and pluronics degraded by gradual desorption of the nonadhesive from the surface independently of the presence of cells. Hexa(ethylene glycol) degraded by a time-dependent mechanism that could be accelerated by cell-dependent oxidative processes. In contrast to the other substrates studied, bovine serum albumin (BSA) patterned cells only under serum-free conditions. The serum did not displace BSA from the surface but instead activated cell-secreted proteases that led to degradation of the substrate. These findings illustrate the importance of specific cellular and noncellular processes in the failure of different nonadhesive chemistries commonly used to pattern cells.

I. Introduction

Spatially patterned surface chemistry is a powerful approach to control the placement and geometry of individual cells and to create addressable arrays of uniform or heterogeneous populations. Cellular patterning methods organize the chemical functionalities presented on a surface to promote selective attachment of one or more types of cells to predetermined regions on a substrate. Recently, cellular patterning has been used to dissect the role of cellular geometry on proliferation,[1,2] migration,[3] and differentiation[4,5] of individual cells, as well as that of cell−cell interactions on the behavior of groups of cells.[6,7] Cellular patterns also have been designed for numerous commercial applications, including cell-based biosensors[8] and cellular arrays for high-throughput diagnostics.[9]

Many different techniques have been developed to pattern cells.[10] Although a variety of processes are used, a common theme of these techniques is to pattern adhesive and nonadhesive regions on a surface, such that cells attach to the adhesive regions and are repelled by or unable to attach to the nonadhesive regions. While the adhesive material is generally an adsorbed or printed extracellular matrix (ECM) protein to which cells adhere using specific receptors, the chemical and physical nature of the nonadhesive region varies greatly. These nonadhesive materials typically fall into one of several categories. Chemically, nonadhesives can be grouped as carbohydrates (agarose,[7] mannitol[11]), synthetic polymers (poly(ethylene glycol)[1], polyacrylamide,[12] pluronics[13]), or proteins (albumin[14,15]). Physically, nonadhesive regions are either flat monolayers coplanar to the adhesive regions[11,16] or raised hydrogels.[7,17] They all appear to resist adhesion of cells by preventing nonspecific protein adsorption. The functional lifespan of these nonadhesive materials varies widely in the literature (24 h to 60 days), with pattern failure commonly defined as the invasion of cells onto the nonadhesive region.[10] However, it is unclear whether the differences in performance between patterns are due to differences in the nonadhesive used, or differences in experimental conditions, such as different cell types or feeding protocols. It is also unclear if the presence of cells alters the degradation process. Cells could actively remodel nonadhesive surfaces by secreting locally high concentrations of adhesive proteins and degradative enzymes.[18] Cells could also indirectly degrade nonadhesive surfaces by causing local physical stresses such as pH changes[19] or mechanical strains.[20]

In this study, we determined the contribution of active cellular processes to the failure of patterns of cells cultured

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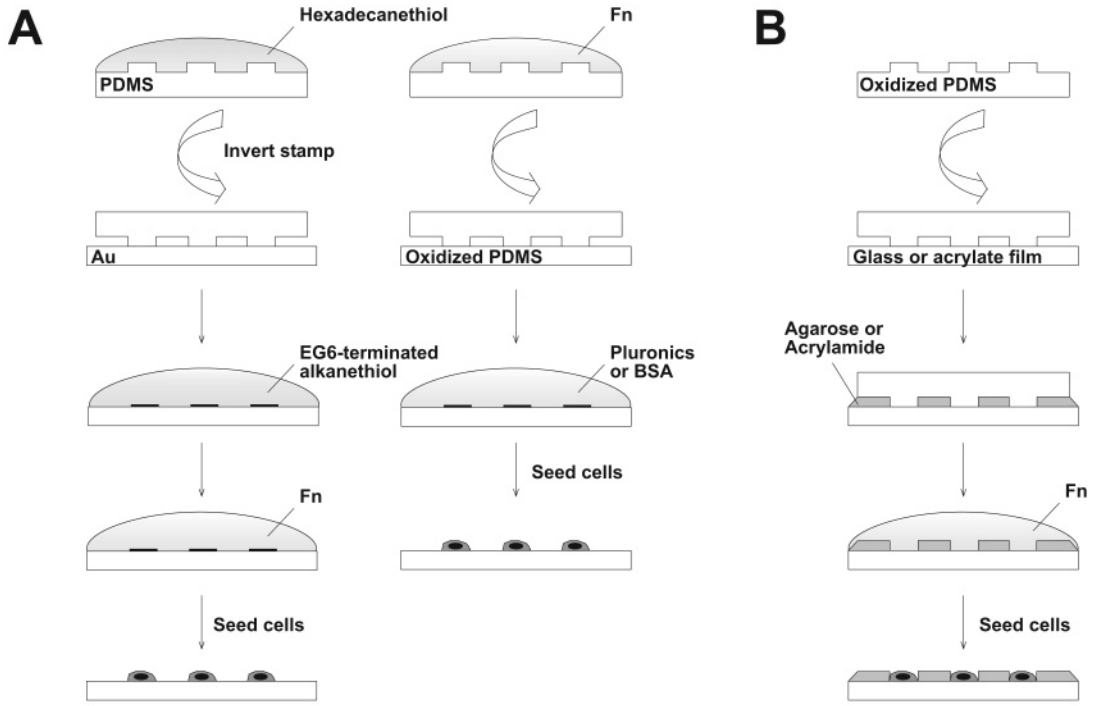

Figure 1. Schematic outline of patterning procedures. (A) Microcontact printing used to pattern EG6 (left), BSA (right), and pluronics (right) substrates. (B) Microfluidics used to pattern hydrogels for agarose and polyacrylamide substrates.

on substrates created with different nonadhesives. We chose to study agarose and polyacrylamide hydrogels, physisorbed pluronics and bovine serum albumin (BSA), and self-assembled monolayers (SAMs) of hexa(ethylene glycol)-terminated alkanethiolate (EG6) as representative examples of nonadhesive materials in each of the different chemical and physical categories described above. Comparing the failure rate of substrates with and without cells, we have identified that the mechanisms of failure of cellular patterns fall into three general categories, depending on the cell type and the nonadhesive: cell-independent degradation, cell-dependent degradation, and combination processes.

II. Materials and Methods

Materials. Hexa(ethylene glycol)-terminated alkanethiol $HS-(CH_2)_{11}(OCH_2CH_2)_6OH$, referred to as EG6, was synthesized as previously described.[21] (Tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane was obtained from United Chemical Technologies (Bristol, PA). The following reagents were purchased from the given suppliers: human fibronectin (Collaborative Biomedical Products); bovine serum albumin (BSA; Intergen Company); Pluronic F108 (BASF); FITC-labeled F108 (AllVivo, Birmingham, AL); agarose (Invitrogen); acrylamide (Invitrogen); *N,N′*-methylenebis(acrylamide) (bis; Invitrogen); 2,2′-azobis(2-methylpropionamidine) dihydrochloride (azobis; Aldrich); minocycline hydrochloride (Sigma); cimetidine (Sigma); 4-methylpyrazole hydrochloride (fomepizole; Sigma).

Cell Culture. Bovine pulmonary artery endothelial cells (ECs; VEC Technologies, Rensselaer, NY) were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 5% calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen). 3T3-L1 pre-adipocytes (L1s; ATCC) were cultured in high glucose DMEM supplemented with 10% calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. After seeding cells onto substrates, the medium was replaced every 2 days. Conditioned medium was removed from cells grown at 50% confluence on tissue culture plastic for 2 days, and centrifuged to remove trace cellular debris prior to use. Cimetidine and fomepizole were added to cells at 30 min after seeding and to culture media at the time of feeding at 22 and 100 μg/mL, respectively. Cells were preincubated with 100 μM minocycline for 2 h before the addition of serum-containing media, which was also supplemented with 100 μM minocycline.

Preparation of Stamps. Stamps of poly(dimethylsiloxane) (PDMS) were made by casting Sylgard 184 (Dow Corning, Midland, MI) on a silicon master with 20 or 50 μm thick, 150 μm square features made by photolithography. To aid in release of the cured PDMS, the master was first silanized overnight with a vapor of (tridecafluoro-1,1,2,2,-tetrahydrooctyl)-1-trichlorosilane under vacuum before casting the PDMS.

Patterning Substrates with EG6, BSA, and Pluronics by Microcontact Printing (μCP). EG6 substrates were patterned by μCP of SAMs as previously described[16] (Figure 1A). Briefly, glass coverslips were coated by electron beam evaporation with 2.0 nm of Ti, followed by 15 nm of Au. Stamps were inked in a solution of 2 mM hexadecanethiol (Sigma) in ethanol, dried under nitrogen, and placed in conformal contact for 2 s with the Au-coated coverslips. The unstamped regions of the coverslips were coated with a nonadhesive SAM by immersing in a 2-mM solution of EG6 in ethanol for 1 h. Substrates were rinsed and sterilized in ethanol and incubated in 25 μg/mL fibronectin in PBS for 2 h.

BSA and pluronics substrates were prepared by μCP of fibronectin as previously described[22] (Figure 1A). Flat PDMS was oxidized under UV/ozone (UVO cleaner, Jelight Co., Inc., Irvine, CA). Stamps were coated with a 25 μg/mL solution of fibronectin in PBS for 2 h, rinsed in PBS, dried, and placed in conformal contact for 2 s with the flat oxidized PDMS. The unstamped regions of the oxidized PDMS were coated for 2 h with either 1% BSA or 1% F108 pluronics in PBS.

Patterning Substrates with Agarose or Polyacrylamide Hydrogels by Microfluidics (μFluidics). Agarose substrates were prepared as previously described[7] (Figure 1B). A solution of 0.6% agarose/40% ethanol in water was perfused through the channels created by sealing a stamp against a SuperFrost slide (Fisher Scientific) and dried under vacuum. Peeling off the stamp unmasked regions of bare glass on the substrate. Substrates

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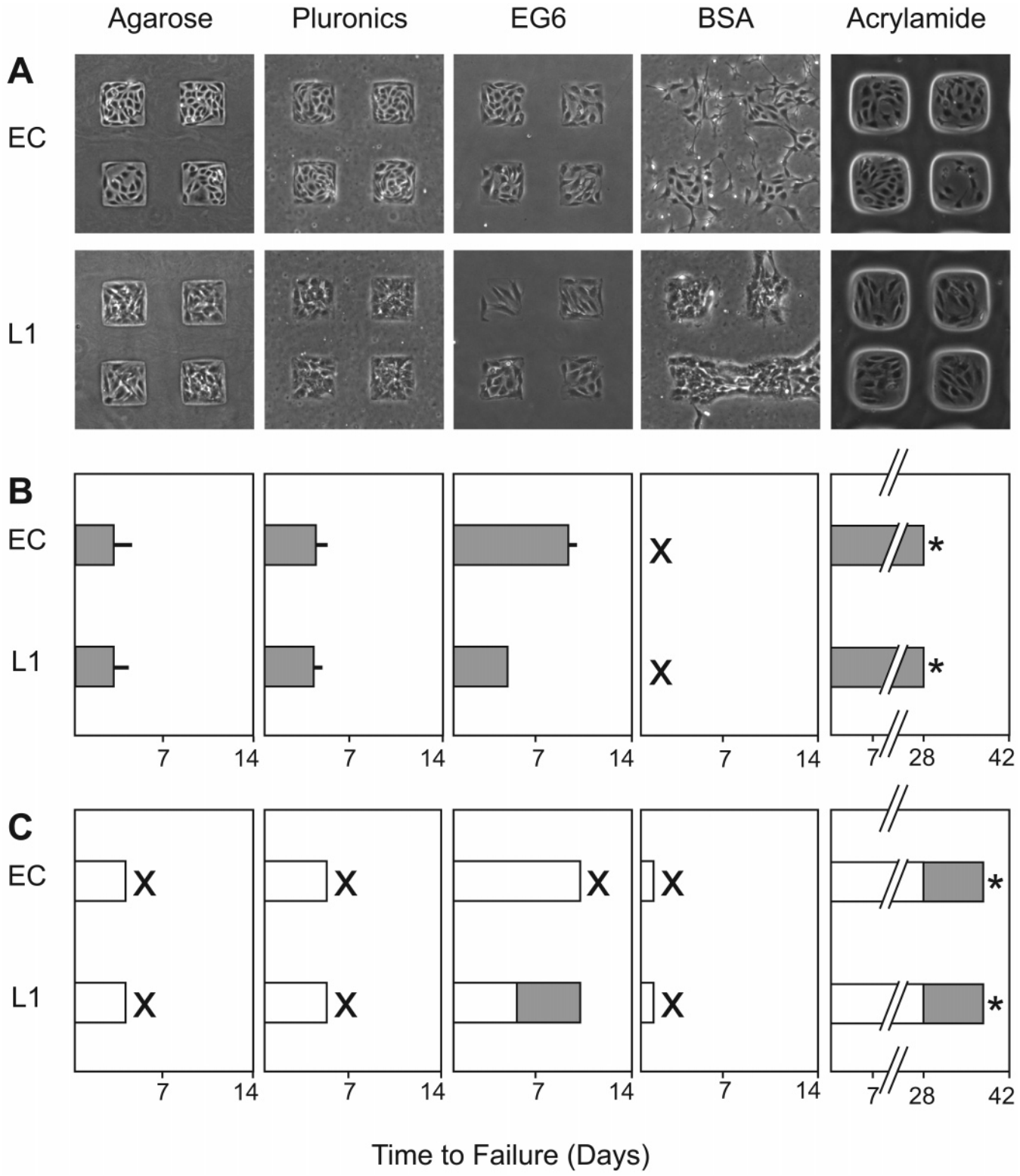

Figure 2. Time course of failure depends on cell type and nonadhesive. (A) Phase contrast images of EC or L1 cells on different patterned nonadhesive substrates prepared as described in Materials and Methods at 24 h following initial seeding. (B) Bar graph showing time to failure of EC and L1 on different substrates. (C) Bar graph showing time to failure of EC and L1 on different substrates after preincubation with media. Substrates were preincubated with media for the period of time indicated by the white bars, and cells were subsequently seeded on the substrates. Gray bars indicate period of clean patterning of cells on these preconditioned substrates. X indicates seeded cells never conformed to the pattern. An asterisk indicates that pattern did not fail for the duration of the experiment. Error bars indicate standard deviation of two independent experiments.

were sterilized in ethanol, washed in PBS, and incubated in a 25 μg/mL solution of fibronectin in PBS for 2 h.

Polyacrylamide substrates were made as follows: A stamp was oxidized in air plasma (~200 mTorr, 10 min) and placed in conformal contact with an acrylate film (SERVA Electrophoresis). A solution of 9.5% acrylamide/0.5% bis/20 mg/mL azobis in water was perfused through the channels between the stamp and the film and cured and covalently attached to the acrylate by exposure to UV light (6 min at 365 nm; Electro-Lite Corp.). After the stamp was removed, the substrate was oxidized under UV/ozone, sterilized in ethanol, rinsed in PBS, and incubated in a 25 μg/mL solution of fibronectin in PBS.

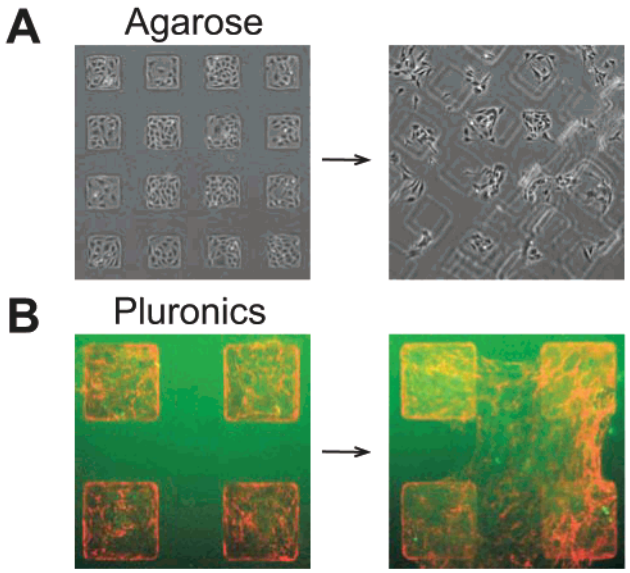

Figure 3. Agarose and pluronics substrates fail by cell-independent mechanisms. (A) Phase contrast images of ECs on agarose substrates 1 day before (left) and 1 day after (right) agarose lifts off. The agarose film can be seen out of focus floating above the substrate (right). (B) Fluorescence images of fluorescent pluronics (green) and fibronectin (red) 1 day before and 1 day after L1s invade.

Immunofluorescence Analysis and Imaging. For detection of adhesive and nonadhesive regions, substrates were fixed in 4% paraformaldehyde, blocked in 1% BSA in PBS, incubated in a solution of rabbit anti-fibronectin antibody (Sigma), and detected with Alexa 594-conjugated goat anti-rabbit antibody (Molecular Probes). Images were obtained with an inverted fluorescence microscope (Eclipse TE200, Nikon) fitted with an Orca CCD camera system (Hamamatsu). All images were obtained with identical exposures.

III. Results and Discussion

We compared the longevity of five different nonadhesive coatings frequently used to pattern cells: agarose,[7] pluronics,[13] EG6,[1,2] BSA,[15] and polyacrylamide.[12] Substrates were prepared such that the geometry (150 μm squares) and chemical presentation (fibronectin) of the adhesive regions were identical. We defined pattern fidelity as the specific localization of cells to the patterned adhesive regions (fibronectin) and not to the nonadhesive regions; pattern failure was thus defined as a breakdown in pattern fidelity, with cells attaching or migrating onto the nonadhesive regions. Cells were seeded on substrates on day 0, and pattern fidelity followed until the patterns failed or until a maximum period of 1 month had passed (Figure 2A). We compared endothelial cells (ECs) and preadipocytes (L1s); both cell types were fed every 2 days. The duration of pattern fidelity differed dramatically between different substrates and between different cell types on the same substrate (Figure 2, parts A and B). For example, neither type of cell formed patterns on BSA substrates, but patterns of both lasted at least 28 days on polyacrylamide substrates. On EG6 substrates, patterns of ECs lasted several days longer than patterns of L1s. The eventual failure of a nonadhesive may arise through multiple mechanisms, including physical degradation of the nonadhesive, detachment of the nonadhesive material from the surface, and active remodeling of the nonadhesive by cells. Nonadhesives likely differ in their propensity for each of these mechanisms of failure, which may depend on the type of cell to which the nonadhesive is exposed.

To understand the mechanism of failure for the different substrates, we first tested whether the degradation of the pattern was due to passive properties of the surface or to active cellular processes. We compared the longevity of patterning of the different substrates in the presence or absence of cells (Figure 2C). Substrates were either cultured with cells or preincubated with media alone until the substrates with cells failed. One day after the patterns with cells failed, we plated cells on the substrates pretreated with media. For all substrates, media was replaced every 2 days for the length of the experiment. Neither type of cell patterned on pretreated agarose or pluronics substrates, indicating that the surfaces degraded prior to the introduction of cells. The degradation of these surfaces thus appears to be a time-dependent, cell-independent process. One common property of agarose and pluronics substrates is that the nonadhesive material is physisorbed onto the surface.[7,13] Thus, these patterns could fail if the nonadhesive readily desorbs. Indeed, failure of the agarose substrates was associated with the agarose physically lifting off the glass surface (Figure 3A), a process visible by phase contrast microscopy. The agarose sheet lifted off at the same time without cells as with either cell type (Figure 2; data not shown).

Similarly, patterns on pluronics substrates degraded simultaneously with cells or with media alone (Figure 2, parts B and C). To visualize the presence of the pluronics on the surface, we patterned substrates using a FITC-tagged pluronics, which has been shown to inhibit protein adsorption and cell adhesion as well as the untagged pluronics.[13] By fluorescence microscopy, the pluronics localized to the nonadhesive regions (Figure 3B). Substrates were fixed and stained for fibronectin prior to and after pattern failure. At 1 day prior to the invasion of cells onto the pluronics-coated regions, fibronectin and FITC-tagged pluronics could be clearly distinguished by fluorescence microscopy. However, at 1 day after the invasion of the cells, the pluronics immunofluorescence had decreased and was no longer uniform, and fibronectin localized to regions where the cells had invaded.

These results suggest that patterns of cells generated by physisorbing nonadhesive materials on the substrate can fail through cell-independent desorption. The hydration of agarose causes it to swell, which may mechanically peel it from the surface. For pluronics, the presence of serum proteins can facilitate desorption of the polymer from the surface,[23] a process that may be sufficient to support the migration of some types of cells onto the pluronics-coated regions. Past studies have demonstrated that the fidelity of pluronics substrates may be cell type-specific,[13] although it remains unclear whether this effect was due specifically to the cells or to differences in manufacturing of the substrates.

In contrast to agarose and pluronics substrates, whose degradation exhibited no cell type-specificity, the failure of EG6 was dependent on the type of cell patterned. Patterns of ECs and L1s failed at different times after plating (Figure 2): L1s failed after 4 days and ECs after 9 days. Preincubation of these substrates for 5 days in media did not appear to significantly affect the longevity of patterning of L1s, while substrates that had been preincubated in media for 10 days no longer patterned ECs (Figure 2C). These findings suggest that the EG6 nonadhesive degrades gradually by a cell-independent mechanism, and L1s possess active cellular processes that can increase this natural rate of degradation of EG6. Short-chain poly(ethylene glycol)s are oxidized and degraded in vivo by enzymes including alcohol dehydrogenase (ADH)

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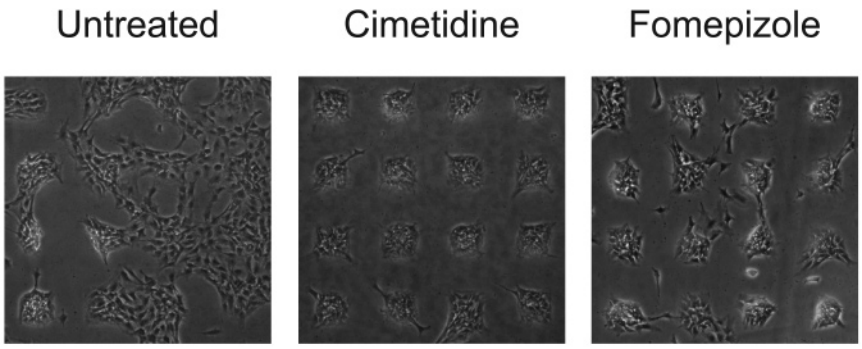

Figure 4. EG6 substrates fail by combination mechanisms. Phase contrast images of L1s on EG6 substrates cultured with (cimetidine, fomepizole) or without (untreated) ADH inhibitors 1 day after failure of untreated patterns.

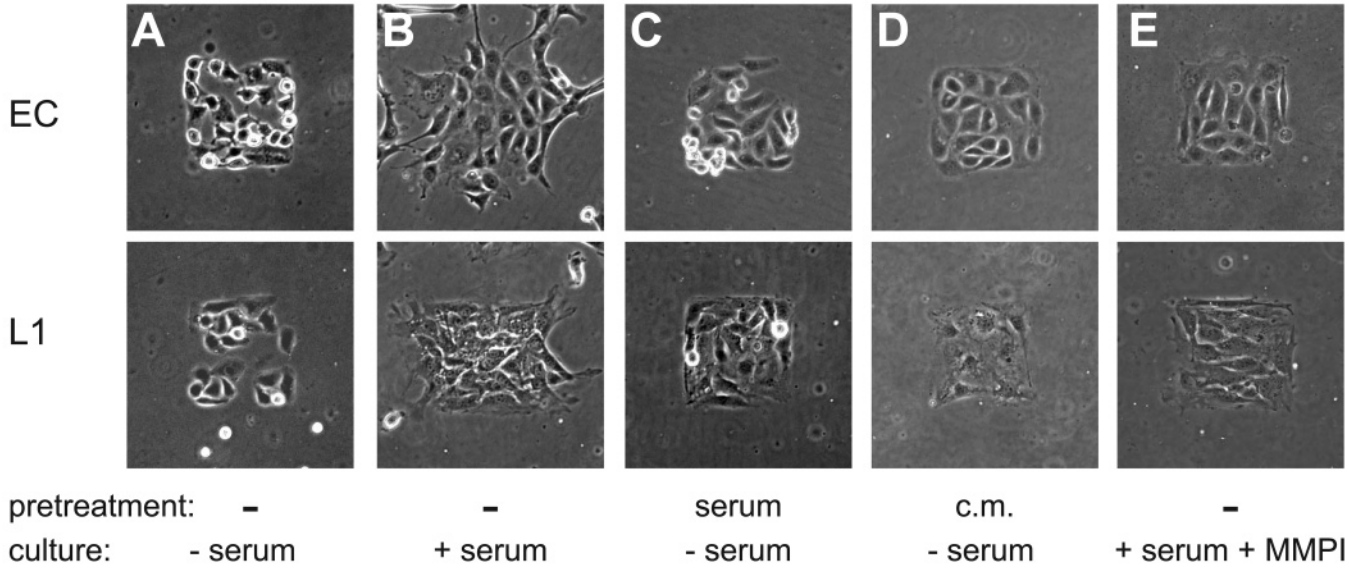

Figure 5. BSA substrates fail by cell-dependent mechanisms. (A) Phase contrast images of ECs and L1s at 18 h after patterning on BSA substrates in serum-free media. (B) Phase contrast images of ECs and L1s first plated on BSA substrates in serum-free media, 18 h after the addition of serum-containing media. (C) Phase contrast images of ECs and L1s at 18 h after plating in serum-free media on BSA substrates that had been preincubated with serum-containing media for 24 h. (D) Phase contrast images of ECs and L1s at 18 h after plating in serum-free media on BSA substrates that had been preincubated with conditioned media (c.m.) for 24 h. (E) Phase contrast images of ECs and L1s first plated on BSA substrates in serum-free media, 18 h after the addition of serum-containing media including MMP inhibitor (MMPI).

and aldehyde dehydrogenase.[24] The L1 pre-adipocytes likely produce these enzymes that are typically associated with fatty acid metabolism and thereby cause a faster breakdown of the patterns. Indeed, culturing L1s on EG6 substrates in the presence of fomepizole or cimetidine, two known inhibitors of ADH activity,[25] reduced the rate of degradation of these substrates (Figure 4). ADH inhibitors also appear to slow the failure of patterns of ECs (data not shown). Even while inhibiting ADH activity, patterns of both cell types eventually broke down, supporting the existence of a cell-independent degradative process.

Other studies have demonstrated the failure of patterns of cells using oligo(ethylene glycol)-terminated SAMs and conjectured that this failure results from the degradation of the nonadhesive regions through autoxidation of the EG moiety.[11,26] Here, we find that the degradation of the nonadhesive EG6 occurs through a combination of cellular and noncellular processes, where the cellular process depends on whether the type of cell patterned on the substrate can produce specific oxidative enzymes. The ability of cells to actively alter EG6 implies that nonadhesive surfaces are not inert under all conditions or with all types of cells.

Finally, we investigated the failure of BSA to pattern cells. Although neither cell type patterned on substrates with BSA as the nonadhesive in our experiments (Figure 2), reports indicate that BSA can be used to pattern cells in serum-free conditions.[15] To confirm these reports, we seeded cells on BSA-patterned PDMS substrates in serum-free media and found that, indeed, cells patterned cleanly under serum-free conditions (Figure 5A) and remained so for up to 2 days. These findings suggest that proteins in serum may adsorb onto BSA-coated regions and thereby facilitate cell adhesion. To test this possibility, we allowed cells to attach and spread on the BSA substrates under serum-free conditions for 5 h, and then replaced the media with serum-containing media. The subsequent addition of serum-containing media allowed the cells to invade onto the BSA regions within 18 h, although ECs and L1s migrated with different kinetics (Figure 5B).

To determine whether serum was directly altering the surface, we preincubated substrates in serum-containing media for 24 h. Cells subsequently plated in the absence

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of serum patterned cleanly on these pretreated substrates (Figure 5C), implying that BSA was not replaced or altered by the preincubated serum. It therefore appeared that the degradation of these patterns required the simultaneous presence of cells and serum, suggesting that invasion results from serum-induced activation of cellular processes that in turn remodel the nonadhesive regions. Cells can remodel their microenvironment by secreting enzymes including serine proteases (plasmin[27]), cysteine proteases (cathepsin B[28]), aspartic proteases (cathepsin D[29]), and matrix metalloproteases (MMPs, including collagenases[30]). To determine whether serum caused cells to secrete a protease into the media that would lead to degradation of BSA, we cultured cells in the presence of serum and then preincubated this conditioned media on cell-free substrates for 24 h. Cells plated without serum on these substrates again patterned cleanly (Figure 5D), suggesting that if cells secrete a factor in response to serum which then degrades BSA, that factor might be chemically labile or otherwise effective only at high concentrations near the surface of cells. To determine if a protease was locally activated by serum-induced cells to degrade BSA, we cultured cells in the presence of specific protease inhibitors. Inhibitors of serine proteases, cysteine proteases, or aspartic proteases did not halt the invasion of ECs and L1s (data not shown). However, the serum-dependent invasion was inhibited when cells were exposed to the MMP inhibitor minocycline[31,32] (Figure 5E). Washing out the MMP inhibitor allowed cells to invade at rates similar to untreated cells, suggesting that the inhibition of migration is reversible and not due to cytotoxicity. The MMP inhibitor does not directly compromise general cell migration, as it did not inhibit the migration of cells onto fibronectin. Given that MMPs have been shown to be activated by growth factors present in serum,[33] our data suggest that activation of MMPs is necessary for the degradation of and invasion of cells onto the BSA-coated regions of the patterns.

Taken together, these results suggest that the degradation of nonadhesives can result from active cellular processes, such as the secretion of proteolytic enzymes. Indeed, the mechanism of failure of EG6 and BSA highlights the importance of enzymes and other secreted factors, such as ADH and MMPs, in pattern fidelity. It is possible that cells secrete additional enzymes not elicited in this study that could degrade other inert surfaces. Furthermore, the susceptibility of a nonadhesive to degradation by cell-secreted enzymes may be influenced by the underlying substrate. Since the degradative cellular processes that remodel the local microenvironment depend on the types of cells being patterned and their culture conditions, the design of patterned substrates should take into account both factors. For some applications, pattern breakdown might be desirable: Knowledge of the specific enzymes that degrade each of these nonadhesive materials could be used to design cell-based biosensors to detect the activation or inhibition of certain enzymes. The ability of cells to affect micropatterned substrates can be as important to pattern fidelity as the effect of the micropatterned substrates on the cells.

The fidelity of cellular patterns is also dependent on cell-independent modes of failure. The lifespan of patterns on agarose and pluronics substrates was clearly limited by desorption of the nonadhesive from the surface. Desorption of the nonadhesive may be specific to the materials and patterning methods used in this study. However, these data cannot rule out the possibility that such desorption is a general phenomenon of physisorbed nonadhesives and dependent on the binding strengths between the nonadhesive and the surface. If covalently attached to the surface, patterns of cells on agarose and pluronics substrates might have a longer time course to failure. For long-term patterning or shelf life, covalent attachment of the nonadhesive to the substrate will likely be necessary for most types of cells.

In addition to the cell type, serum, and type of nonadhesive, other factors regarding the design of patterned substrates not addressed here may affect their stability, such as the geometry of the adhesive regions.[34] The adhesive geometry, cellular organization, and soluble factors in the media can all affect the behavioral state of the cell, including its invasiveness and enzymatic activity. Thus, even apparently unimportant factors such as the frequency at which media is changed could significantly alter pattern longevity. Although reducing the concentration of serum in the media or placing cells on a low feeding schedule can induce quiescence and increase pattern stability in some cases,[35] limiting the use of serum would severely constrain the applicability of the substrate given that the behavior of cells in low serum is dramatically different from their normal physiology. In the end, each cell type might need to be tested for compatibility with each patterning technique. Given this possibility, it remains unclear whether a superior nonadhesive exists for all applications. In these studies, we found that our cells did not degrade substrates prepared with polyacrylamide covalently attached to the surface. The superior nature of these polyacrylamide substrates is likely due to a combination of properties, including the physical nature of the hydrogel, its final topology, and covalent attachment to the surface. Other investigators have demonstrated high pattern fidelity using mannitol-terminated SAMs on Au (25 days for 3T3 fibroblasts[11]) or interpenetrating polymer networks (60 days for primary bone cells[12]) as the nonadhesive. It remains to be determined whether these apparently more stable nonadhesive materials can, under all operating conditions, remain inert—by resisting protein adsorption, physical degradation, desorption, and all forms of cellular attack.

IV. Conclusion

In this study, we compared the lifespan of different nonadhesive patterning techniques using the same cell types and feeding protocols. We have identified multiple mechanisms of failure, including both cell-independent and cell-dependent processes, for substrates patterned with agarose, pluronics, EG6, and BSA as the nonadhesive material. Our results demonstrate that substrates patterned using chemisorbed polyacrylamide exhibit a high lifespan of pattern fidelity. These substrates may be useful for applications requiring long-term patterning of cells,

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such as studies of differentiation or quiescence or cell-based bioreactors.

In sum, the active, degradative mechanisms orchestrated by cells play an important part in the breakdown of surfaces and failure of patterns. Understanding these mechanisms will be helpful for the future design of the nonadhesives used in cellular patterning as well as medical applications. Intelligent engineering of designer surfaces will require a thorough understanding of the interactions between cells and surface components to achieve optimal pattern fidelity, shelf life, and function.

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