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Patterning the Cellular Microenvironment

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Cell biology has been a study of global changes in populations of cells: cell biologists often pool millions of cells that are cultured in a dish into a single measurement. Many, if not most, discoveries of the workings of cells have resulted from such averaging of gross responses of the whole cell population, such as the activation of a signaling pathway by expression of a key protein, or the changes in gene expression that accompany mitogenic stimulation. But what of effects that manifest themselves in small populations of cells, or in local regions of a culture? How does one isolate the effects of local inhomogeneities in the surrounding microenvironment, from one cell to the next? How does one study *subtle* effects?

In order to address these issues, we have adopted an approach that applies techniques common in microfabrication and lithography to the patterning of the cellular microenvironment at cellular length scales. The microenvironment—the collection of mechanical and biochemical signals that are presented to a cell in space and time—consists of many different types of signals. Among these are interactions between neighboring cells, interactions between cells and their underlying extracellular matrix, signals that result from the binding of soluble growth factors, cytokines, and hormones, and mechanical forces that stress cell bodies. Our approach presumes that these signals, and their presentation in space and time, while not completely deterministic, can strongly influence the behavior of a cell. In a culture dish, cells generate local inhomogeneities in these signals, simply by the stochastic nature of their uncontrolled interactions with each other and the culture surface. We use microfabrication technologies to control these interactions and thereby generate a microenvironment that is uniform and spatially well-defined for all cells in a culture, and thus enable the amplification of local effects to global ones that affect all of the culture.

This review is divided into two sections. First, we describe the use of a set of non-photolithographic techniques—soft lithography—to pattern proteins on a substrate and to thereby pattern the position, placement, and shape of cells. Second, we discuss the use of spatially patterned cultures as a tool in cell biology—specifically, as a tool to answer how cell shape and cell-cell interactions affect behavior in endothelial cells.

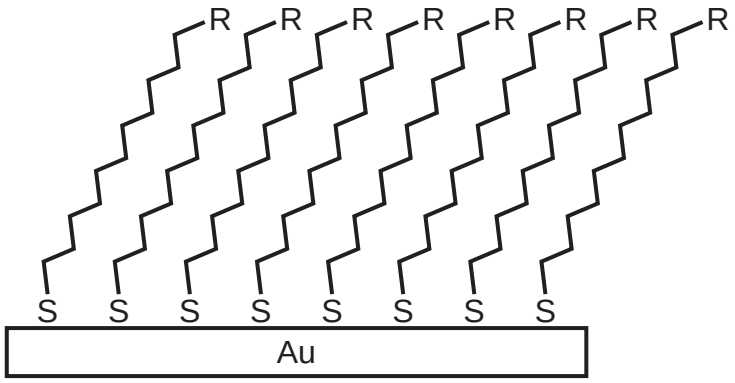
Patterning Cells and Their Microenvironment

One process that we use to pattern the attachment and spreading of cells relies on a technique known as *microcontact printing* (μCP) [1]. Microcontact printing is conceptually similar to macroscale printing: a template with patterned surface relief is inked with a suitable liquid, dried, and brought into contact with a substrate. Ink transfers from template to substrate only where contact occurs. In μCP, the template is a stamp that is cast from the soft elastomer polydimethylsiloxane (PDMS), the ink is a special organic molecule known as an alkanethiol, and the substrate is a thin film of gold (usually evaporated onto glass or silicon).

Alkanethiols are organic molecules that possess the thiol functionality –SH. The sulfur atoms in these molecules exhibit a strong affinity for noble metals such as gold, silver, and copper. Immersion of a thin film of gold into a solution of alkanethiol derivatizes the surface of the gold with a self-assembled monolayer (SAM) of alkanethiolate (Figure 1). In essence, alkanethiols adsorb onto the surface of the gold and organize themselves into a close-packed array in order to maximize the number of thiol headgroups that are in contact with gold atoms and the lateral interactions between neighboring alkyl chains. These SAMs alter the surface properties of the gold, such that the chemical properties of the surface reflect those of the *endgroup* of the thiol.

Figure 1. (**A**) Schematic of a self-assembled monolayer of alkanethiolate on gold. (**B**) The chemical structures of the two alkanethiols—hexadecanethiol and a hexa(ethylene glycol)-terminated thiol—that we use to pattern protein adsorption and cellular attachment.

A)



B) Hexadecanethiol:

R = -$(CH_2)_4CH_3$

Hexa(ethylene glycol)-terminated alkanethiol:

R = -$(OCH_2CH_2)_6OH$

Figure 1

For instance, hexadecanethiol ($HS(CH_2)_{15}CH_3$) has a nonpolar CH_3 endgroup, and SAMs of this thiol on gold are hydrophobic. Our interest in alkanethiols, and in the SAMs that they form on thin films of gold, stems from the ability of certain types of SAMs to resist protein adsorption. Specifically, SAMs of alkanethiols that exhibit an oligo(ethylene glycol) terminus ($HO(CH_2CH_2O)_n$-, n = 3-7) resist the adsorption of a wide variety of proteins including large "sticky" ones such as fibronectin and small ones such as lysozyme [2]. The strategy behind μCP is to print one type of SAM (usually one that does not resist protein adsorption), and to derivatize the unstamped surface with a second type of SAM (usually one that does resist protein adsorption).

Figure 2 illustrates the process. We first fabricate a textured surface (the "master") from which stamps will be cast. This surface may be made conveniently by photolithography, or may be taken directly from commercially available templates (e.g., corner-cube retroreflectors, diffraction gratings, or grids for electron microscopy). Treatment of the master with a fluorosilane coats the surface of the master with a thin film of fluorocarbon that acts as a mold-release agent; we then pour a prepolymer of PDMS onto the fluorinated master, cure the prepolymer, and peel off the polymerized PDMS from the master. The surface of the PDMS stamp is a replica of the topology of the original master.

We then use a cotton swab to apply a dilute (~1 mM) ethanolic solution of hexadecanethiol to the stamp, and dry the stamp well under a stream of nitrogen to remove ethanol from the stamp. Placing the stamp, inked-side down, onto a thin film of gold allows the alkanethiol to adsorb onto the gold and form a well-ordered SAM; the formation of this SAM requires <5 sec of contact between stamp and gold. Because the stamp is cast from a soft elastomer, the stamp deforms to ensure conformal contact

Figure 2. Schematic outline of microcontact printing of SAMs on gold, and its use in patterning the attachment of cells.

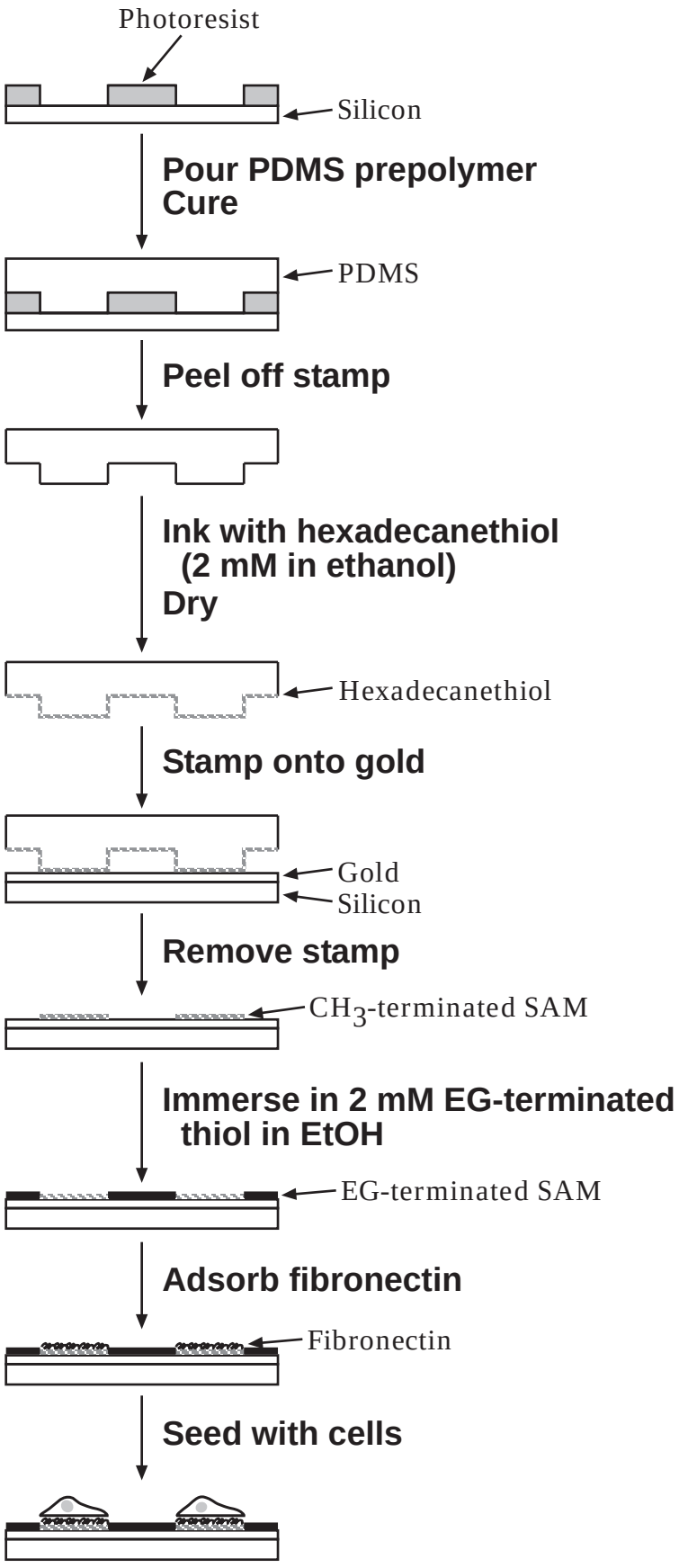

Photoresist
Silicon
Pour PDMS prepolymer
Cure
PDMS
Peel off stamp
Ink with hexadecanethiol
(2 mM in ethanol)
Dry
Hexadecanethiol
Stamp onto gold
Gold
Silicon
Remove stamp
CH3-terminated SAM
Immerse in 2 mM EG-terminated
thiol in EtOH
EG-terminated SAM
Adsorb fibronectin
Fibronectin
Seed with cells

Figure 2

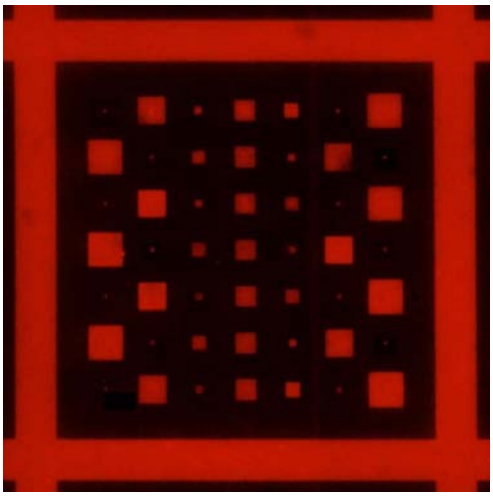
between stamp and gold. Removal of the stamp and subsequent immersion of the stamped surface into a solution of the hexa(ethylene glycol)-terminated alkanethiol $HS(CH_2)_{11}(OCH_2CH_2)_6OH$ derivatizes the unstamped regions with a hexa(ethylene glycol)-terminated SAM, which resists adsorption of protein.

Adsorption of cell-adhesion extracellular matrix (ECM) proteins—fibronectin, laminin, or collagen—onto these surfaces coats the hydrophobic, CH_3-terminated SAMs with a monolayer of protein; the other regions remain uncoated. When seeded on such a surface on which proteins are adsorbed, cells attach and spread only on the islands of ECM proteins (Figure 3). Cells are unable to extend onto surfaces that resist protein adsorption; without adhesive chemical functionalities on those regions, the surface receptors of a cell cannot latch on. In this way, we can control the shapes and degrees of spreading of cells: we can place well-spread cells next to rounded cells, and square cells next to circular cells [3]. We have used this approach successfully to pattern the deposition of many types of cells, such as Madin-Darby canine kidney cells, 3T3 fibroblasts, smooth muscle cells, capillary endothelial cells, macrophages, and PC12 neural cells; we suspect that this approach may be applied to pattern most, if not all, cell types.

The principal benefits of μCP are three-fold. First, in contrast to patterning methods that are based on photolithography, μCP does not subject any of the patterning materials to harsh chemical or physical treatments [4]. This feature is critical for the incorporation of biological molecules, which are very labile. All printing occurs at room temperature in the ambient atmosphere, and all post-printing steps occur in aqueous solutions that do not denature proteins. The gentle nature of μCP allows the patterned surfaces with adsorbed proteins to retain the functional properties of the SAMs and

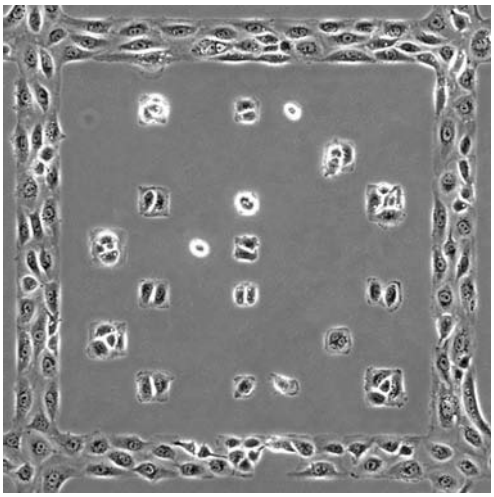
Figure 3. (**A**) A fluorescence micrograph of fibronectin that adsorbed onto a patterned SAM of hexadecanethiol. (**B**) An optical micrograph of normal rat kidney cells that were allowed to attach and spread on patterned fibronectin.

A)



100 µm

B)



100 µm

Figure 3

proteins. Second, the use of a soft deformable stamp as the patterning element allows patterning over large areas. We have been able to stamp three-inch-wide silicon wafers in one impression, and expect that stamping larger substrates is straightforward, provided that masters of that size exist; reel-to-reel printing may also be possible. Third, the process is cheap and relatively convenient to use. Multiple (>100) stamps may be cast from a single master, and each stamp may be used for >100 printings without degradation of the pattern. If needed, stamps may even be replicated from other stamps. The only materials needed in μCP that are not commercially available are the hexa(ethylene glycol)-terminated alkanethiol and thin evaporated films of gold on glass. These materials are available through a three-step chemical synthesis and access to an electron-beam evaporator, respectively.

Recently, we have explored the use of other materials with various aspects of μCP to eliminate these last two obstacles to widespread use of the technique. We and others have found that one other type of surface besides an oligo(ethylene glycol)-terminated SAM resists protein adsorption and cell attachment in the presence of serum: an adsorbed monolayer of a (ethylene oxide-propylene oxide-ethylene oxide) copolymer on hydrophobic surfaces. It appears that these molecules—commercially available under the name of Pluronics—can be printed onto polystyrene in a process analogous to μCP, while retaining their ability to resist protein adsorption. Many other types of surfaces have been synthesized and are claimed to resist the adsorption of protein, but whether these surfaces can resist degradation for periods of time *in the presence of serum* remains to be seen. Because even the adsorption of small amounts of ligand ($<10^{-5}$ ligand/surface atom) suffices for cell attachment onto a surface [5], each

surface must be tested in actual cell culture in order to determine the applicability of that surface in patterning the attachment and spreading of cells.

We have also examined patterning techniques that use a PDMS membrane, instead of a stamp, as the patterning element (Figure 4). In this process, a thin film of PDMS is spun and cured on a patterned master; removal of the PDMS from the master results in a thin PDMS membrane with holes [6]. We then place the membrane on top of a culture dish, adsorb ECM proteins on the dish through the holes in the membrane, remove the membrane, and block non-specific adsorption of proteins onto the uncoated regions with Pluronics. Alternatively, we can seed cells directly through the holes in the membrane, allow the cells to spread to the perimeter of the holes, and remove the membrane [7]. These techniques have the advantage over SAM-based approaches in that they may be used with commercially available reagents to pattern many materials, including polystyrene. A limitation of the use of membranes, however, is that only certain topologies are amenable to this sort of patterning; for instance, donut-shaped islands of ECM protein are difficult to fabricate by using membranes.

Other soft lithographic techniques that have been applied towards the patterning of proteins and cells on various substrates are multi-color patterning by flowing through multiple microfluidic channels [8], sub-cellular localization of proteins by laminar flow patterning [9], and direct stamping of proteins [10].

Applications in Cell Biology

We have used spatially confined cell cultures to examine the effects of cell shape on behavior in the case of capillary endothelial cells [11, 12]. During morphogenesis, dramatic changes in cell shape appear to correlate with changes in the functional state

Figure 4. Schematic of the use of PDMS membranes to pattern proteins and cells.

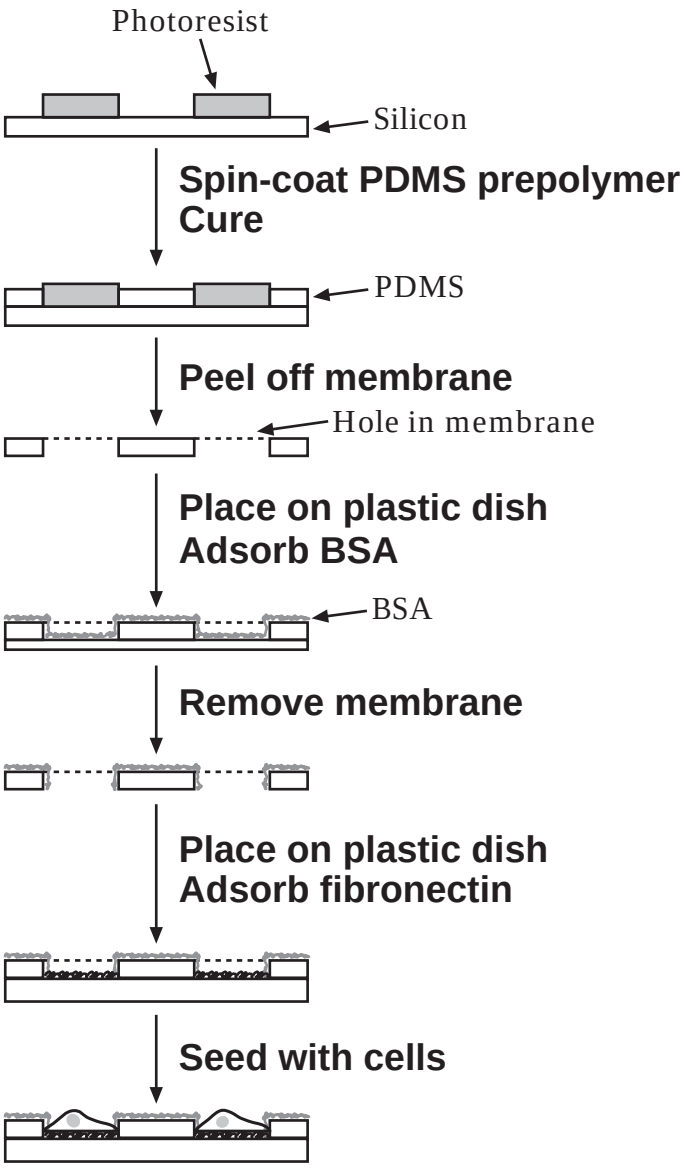
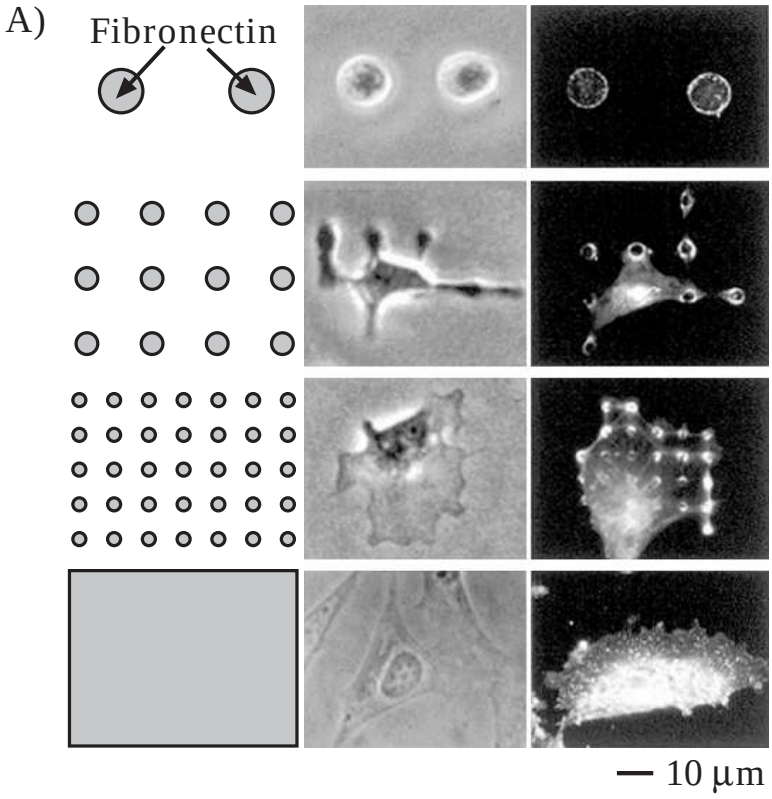

Photoresist
Silicon
Spin-coat PDMS prepolymer
Cure
PDMS
Peel off membrane
Hole in membrane
Place on plastic dish
Adsorb BSA
BSA
Remove membrane
Place on plastic dish
Adsorb fibronectin
Seed with cells

Figure 4

of the cells. Whether these changes in cell shapes are merely indicative of a functional change in the cell, or instead are partly responsible for the trigger of differentiation, remains hotly debated. Early studies had implicated that growth (proliferation of cells) and apoptosis (programmed death of cells) were regulated by the degree to which cells spread on a surface [13, 14]. The larger the area that a cell occupies on a surface, the more likely the cell appeared to progress through the cell cycle, and the less likely the cell entered the apoptotic program. Though suggestive, these studies could not establish a definitive link between cell area and changes in behavior, because cell-ECM contacts correlated proportionally to cell area in these experiments. That is, was the area of cell spreading or the area of cell-ECM contact responsible for causing the growth or death of a cell?

With μCP, it was possible to vary cell area and cell-ECM contact area independently. We plated human and bovine capillary endothelial cells onto ECM islands that were arranged as a series of dots (Figure 5A). Some of these patterns consisted of large dots that were separated by large distances; cells seeded on these patterns attached and spread on one island only, and could not bridge across to another, neighboring island. In this case, the island size determined the total cell area, which was equivalent to the total area of cell-ECM contact. Other patterns consisted of small dots that were separated by small distances; cells seeded on these patterns attached and spread on multiple islands simultaneously, so that the total cell area was several times larger than the total area of cell-ECM contact. By varying the diameters and separations of dots, we could indirectly change the cell area or area of cell-ECM contact separately. We used standard assays for apoptosis and proliferation to assess the behavior of cells seeded on these islands, and found that changes in apoptotic or

Figure 5. (A) Capillary endothelial cells that are plated on circular islands of fibronectin bridge across multiple islands when the islands are closely spaced. **(B)** In the three patterns shown (10-μm-diameter circles separated by 20 μm, 5-μm-diameter circles separated by 10 μm, and 3-μm-diameter circles separated by 7 μm), cells spread to greater extents on the smaller islands, although the total cell-ECM contact area remains constant. The proliferative and apoptotic rates correlate with area of cell spreading, rather than with area of cell-ECM contact. (Adapted from [11].)


A)
Fibronectin
10 µm

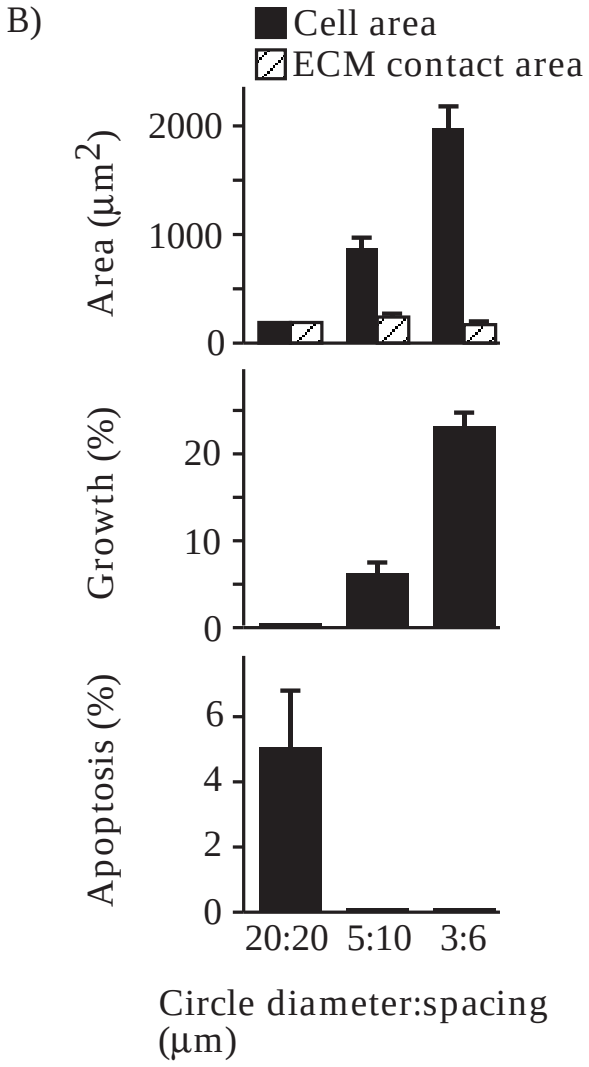

B)
Cell area
ECM contact area
Area (µm2)
2000
1000
0
Growth (%)
20
10
0
Apoptosis (%)
6
4
2
0
20:20
5:10
3:6
Circle diameter:spacing (µm)

Figure 5

proliferative rate changed solely with cell area, rather than with area of cell-ECM contact (Figure 5B).

Our studies suggest that cellular regulation through interactions with the ECM do not rely only on the chemical ligation of receptors on the cell membrane. Given the same chemical inputs (the same substrate, proteins, and growth factors in the media), a cell may alter its behavior in response to its shape. Cellular attachment to the ECM may therefore combine signaling through chemical factors with mechanical and structural effects that result from changes in cell shape. The nature of this sensor of shape may consist of coupling between the cytoskeleton and nucleus: in all of our experiments, an increase in nuclear area accompanies an increase in cell area, and previous work has indicated that the degree to which a nucleus is spread may determine the pattern of gene expression in that nucleus, perhaps by changing the intranuclear structure. We are currently fabricating substrates that will allow us to decouple the effects of cytoplasmic area and nuclear area on cellular behavior.

At intermediate cell areas that do not promote cell growth or death, we have found hints of cellular differentiation. As in studies of the effects of cell spreading, previous work has demonstrated that cultured cells often exhibit differentiated behaviors only when spread to a moderate extent [15]. We have examined this possibility with bovine capillary endothelial cells by plating these cells onto thin (~10 μm wide) lines of ECM protein. On these patterns, cells spread to a moderate degree, and are also allowed to bind to neighboring cells. Over the course of a week, a fraction of cells appear to detach from the underlying substrate, and rearrange to form “tubes” that possess an inner lumen (Figure 6) [12]. The morphology of these structures appears similar to that of a capillary; currently we are testing the functionality of these

Figure 6. (A) Capillary endothelial cells seeded on 10-μm-wide lines of fibronectin are moderately spread (area ~ 1000 μm^2), and form tube-like structures. **(B)** Cell seeded on 30-μm-wide lines of fibronectin are well-spread (area ~ 2200 μm^2), and do not form tubes. Cells are labeled with a cytoplasmic dye. (Adapted from [12].)

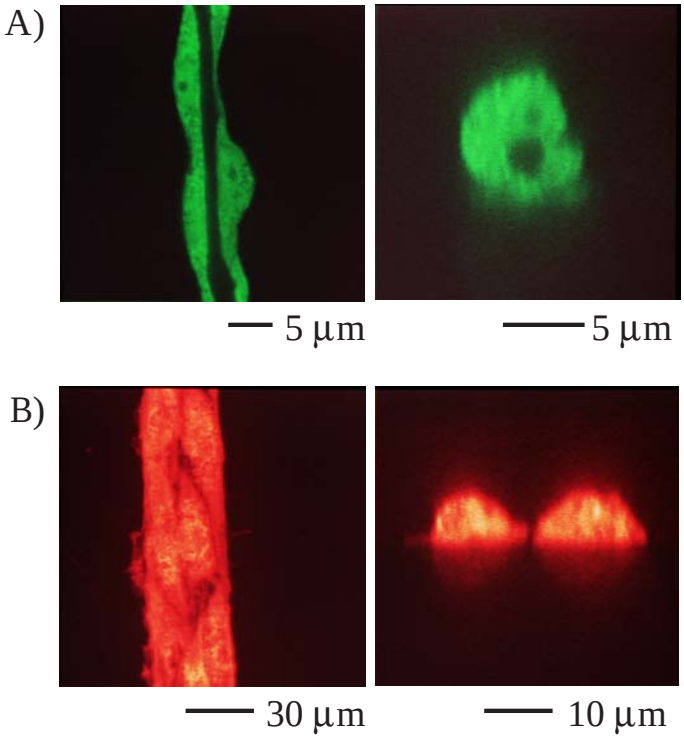


Figure 6

tubes to see if they can support fluid flow. It appears that the unique positioning orientation, and contact of cells with their neighbors and the substrate provides cues that we still have not unraveled.

Conclusions

Our adaptation of microfabrication techniques to cell culture enabled the formation of spatially well-defined cultures, with which we could address specific questions regarding the role of spatial presentation of adhesion in cell behavior. These techniques have sub-micrometer resolution, and may be applied to localize signals with sub-cellular spatial resolution. We are also developing techniques to vary the microenvironment of a cell with time. This set of techniques will enable further fundamental studies in cell biology, and expand the range of tools with which we can manipulate and control cell behavior.

Acknowledgments

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