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Engineering Cell Shape and Function

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An elastomeric stamp, containing defined features on the micrometer scale, was used to imprint gold surfaces with specific patterns of self-assembled monolayers of alkanethiols and, thereby, to create islands of defined shape and size that support extracellular matrix protein adsorption and cell attachment. Through this technique, it was possible to place cells in predetermined locations and arrays, separated by defined distances, and to dictate their shape. Limiting the degree of cell extension provided control over cell growth and protein secretion. This method is experimentally simple and highly adaptable. It should be useful for applications in biotechnology that require analysis of individual cells cultured at high density or repeated access to cells placed in specified locations.

New approaches to drug screening, in vitro toxicology, and genetic engineering focus on the analysis of functional changes within individual cultured cells (*1*). While potentially exciting, these approaches are limited in that cell shape and, hence cell behavior (*2–7*) can vary greatly from cell to cell within the same population in a culture dish. Further, it is difficult to analyze many randomly oriented cells simultaneously or to return to the same cell after measurements have been made in other locations. Thus, a convenient method for physically isolating large numbers of individual cells cultured at high density, controlling their shape, and reproducibly defining their distribution would be extremely valuable.

We now report a simple and flexible method for the construction of tissue culture substrata that contain adhesive islands distributed in defined sizes, shapes, and patterns (Fig. 1) and demonstrate their utility for controlling cell shape, growth, and function. An elastomeric polydimethylsiloxane (PDMS) stamp was used to pattern the adsorption of a hexadecanethiol [$HS(CH_2)_{15}CH_3$] in a self-assembled monolayer (SAM) on gold substrata (Fig. 1A) (*8, 9*). The remaining bare gold regions were derivatized with a polyethylene glycol (PEG)–terminated alkanethiol [$HS(CH_2)_{11}(OCH_2CH_2)_6OH$]; SAMs of this alkanethiolate resist adsorption of proteins (*10*). Through this technique, hexadecanethiolate regions of defined size (lateral dimensions of 2 to 80 μm) that supported protein adsorption were produced on otherwise nonadhesive gold surfaces (Fig. 1B). The exposure of this substratum to the purified extracellular matrix (ECM) protein, laminin, resulted in the formation of protein-coated islands of defined geometry and distribution (Fig. 1C) that corresponded precisely to the patterns formed from SAMs of hexadecanethiolate (Fig. 1B).

The ability of this technique to control cell distribution and shape was explored by the plating of primary rat hepatocytes in hormonally defined medium (*7*) on laminin-coated substrata that were stamped with square and rectangular islands having micrometer-scale dimensions (*9*). Cells attached preferentially to the adhesive, lami-

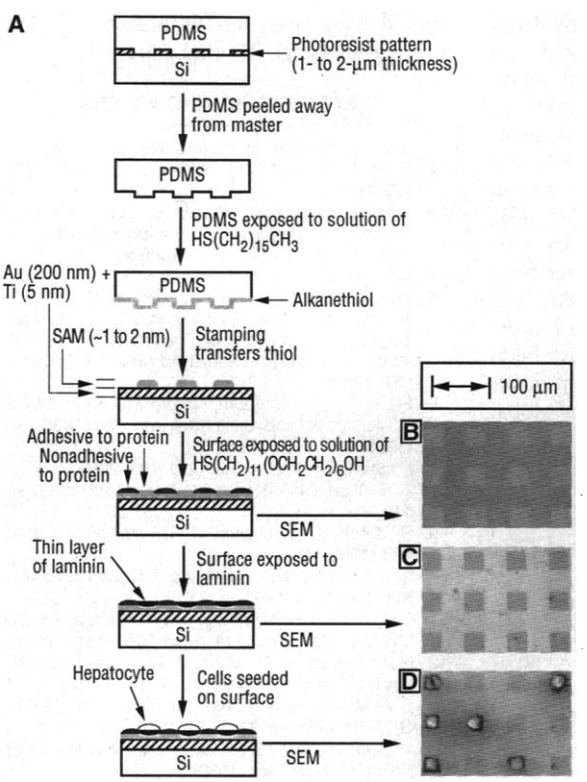

Fig. 1. (**A**) Diagrammatic representation of the method for the fabrication of the rubber stamp and the creation of patterned substrata (*8, 9*). (**B**) Scanning electron micrograph of the substrate after the imprinting of 40 μm by 40 μm squares with hexadecanethiol [$HS(CH_2)_{15}CH_3$] and exposure to PEG-terminated alkanethiol [$HS(CH_2)_{11}(OCH_2CH_2)_6OH$, PEG-thiol]. Adhesive hexadecanethiolate-coated regions appeared light against a darker, nonadhesive background coated with PEG-thiolate, as reported (*18*). (**C**) Scanning electron micrograph of a laminin-coated patterned substratum. Protein coating resulted in a reversal of the light and dark staining patterns (*18*). (**D**) Scanning electron micrograph of a similar laminin-coated substratum containing adherent rat hepatocytes.

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nin-coated islands and were prevented from extending onto surrounding nonadhesive regions (Fig. 1D). The selective attachment of cells to these adhesive islands resulted in the development of regular arrays of individual adherent cells that were aligned single file in both horizontal and vertical columns, extending over large areas (square micrometers in size) of the culture surface (Fig. 2). The adhesive islands limited cell spreading and largely prevented cell-cell contact formation. A few cells (less than 10%) extended ruffling membranes outside of the adhesive areas and bridged adjacent islands. This extension could be reduced by an increase in the width of the nonadhesive boundary. In most cases, cells conformed to the shape of the underlying island with mean projected cell areas consistently within 20% of the designed island area, as determined by computerized image analysis. In many cells, the abrupt prevention of cell spreading at the lateral borders of each island resulted in the formation of cells with corresponding square (Fig. 1D) and rectangular (Fig. 3A) shapes with "corners" that approximated 90°. In contrast, cells plated at the same density on unpatterned, laminin-coated gold substrata spread extensively, formed numerous cell-cell contacts, and exhibited normally pleiomorphic forms (Fig. 3B). Thus, the shape and spatial distribution of cultured cells can be simply controlled with our rubber stamp method to engineer adhesive islands with desired design characteristics and shapes.

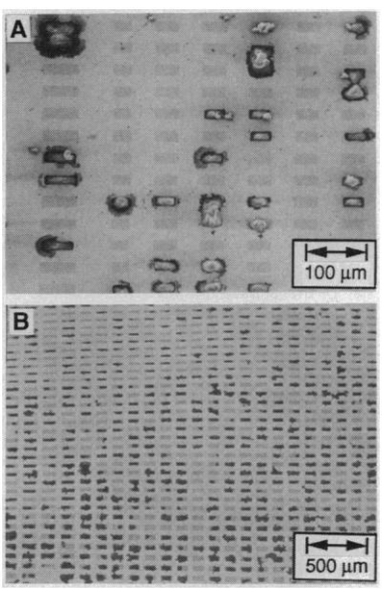

Fig. 2. (**A**) High- and (**B**) low-magnification views of scanning electron micrographs showing a patterned region that contains cells adherent to adhesive islands of varying size. In this study, the patterned region extended over approximately 9 mm^2 without discontinuity. However, on occasion unpatterned areas have been observed within otherwise highly patterned regions.

Because the extent to which a cell spreads influences its growth and function (*2–7*), the maintenance of large populations of cells in specific shapes on patterned substrata could be an effective way to control both the behavior of individual cells and the performance of the entire culture. By fabricating adhesive islands of varying size on a single substratum (Fig. 2), we were able to maintain cells at defined degrees of extension (Figs. 2 and 3A) for extended periods of time, independent of the presence of growth factors (*3*) or the composition (*3*, *6*), mechanics (*4*, *5*), or molecular density (*4*, *7*) of the extracellular matrix. The quantitation of DNA synthesis in hepatocytes cultured on islands of different size in defined medium (*7*) containing saturating amounts of soluble growth factors (epidermal growth factor, insulin, and dexamethasone) revealed significant differences in the number of cells that exhibited nuclear labeling (Fig. 4A). The synthesis of DNA was highest (60% nuclear labeling index) in cells on unpatterned surfaces where cells could spread without restriction. A decrease in the size of the adhesive islands resulted in a progressive reduction in growth: For the smallest islands (less than 1600 μm^2), less than 3% of the adherent cells entered S phase. This result was not due to changes in cell viability; similar percentages of cells incorporated the vital dye, calcein AM (*11*), on all substrata.

We assessed the differentiated function of hepatocytes cultured on substrata coated with uniformly sized islands by assaying the culture supernatant for the secretion of albumin, a liver-specific product. Hepatocytes cultured on unpatterned substrata rapidly lost the ability to secrete high levels of albumin (Fig. 4B), as previously reported (*12*). In contrast, hepatocytes maintained near normal levels of albumin secretion for at least 3 days when cultured on the smallest adhesive islands (40 by 40 μm) that fully restricted cell extension (Fig. 4B). In general, albumin secretion rates decreased as the size of the adhesive island was increased, and growth was promoted (Fig. 4, A and B).

These data are consistent with studies that show that cells such as hepatocytes can be switched between the states of growth and differentiation in the presence of soluble mitogens by the modulation of cell shape (*4*, *7*). In those past studies, however, cell spreading was prevented by the lowering of the coating density of laminin (from 1000 to 1 ng/cm^2) on otherwise

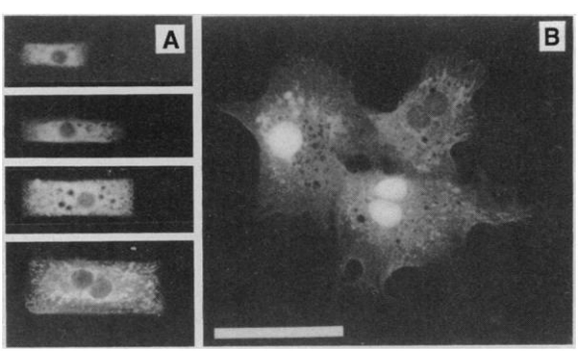

Fig. 3. (**A**) Control of cell shape and size on patterned substrata. Hepatocytes were plated on separate rectangular islands with dimensions of 40 by 15, 60 by 15, 70 by 20, and 80 by 30 μm, respectively, from top to bottom. Only cells that fully conformed to the shape of the island are shown. (**B**) Cells adherent to an unpatterned substratum. Cell bodies were visualized by the overexposure of fluorescent micrographs of cells that were stained for incorporated bromodeoxyuridine (BrdU) with an indirect immunofluorescence DNA synthesis assay. Nuclear uptake of BrdU (DNA synthesis) was only observed in the largest cells on the unpatterned substratum in this figure. All photos were printed at the same magnification (scale bar, 80 μm).

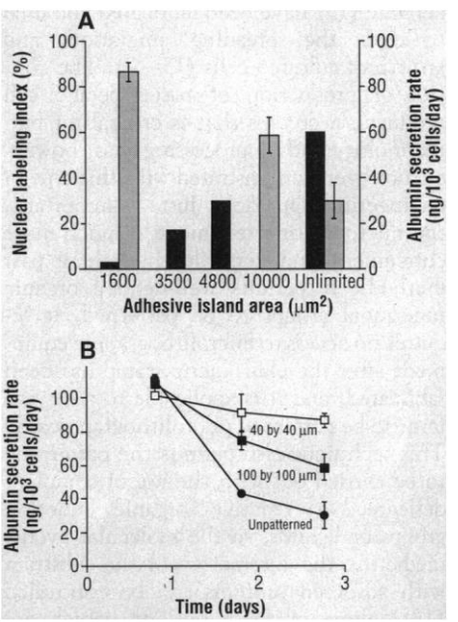

Fig. 4. (**A**) Relation among adhesive island area, DNA synthesis (black bars), and albumin secretion (gray bars). DNA synthesis was measured by quantitating the nuclear incorporation of BrdU (from 24 to 36 hours of culture). Albumin secreted into the medium from 56 to 80 hours after plating was measured with a quantitative enzyme-linked immunosorbent assay (ELISA) technique. (**B**) Maintenance of albumin secretion within hepatocytes cultured on adhesive islands of varying size.

nonadhesive plastic dishes. For this reason, it was not possible to separate the effects caused by modulating cell shape from those induced by altering cell-ECM contacts. Most, if not all, past models used to study shape-dependent growth control have had similar limitations. In contrast, the method based on microprinting allowed us to restrict hepatocyte spreading while maintaining a high laminin density locally. If cell-ECM contact formation is the controlling variable, then raising the local ECM density by a thousandfold [relative to past studies (*7*)] should have had dramatic effects on DNA synthesis. Instead, we found that growth remained almost completely inhibited within poorly spread cells in the smallest laminin-coated adhesive islands. Thus, it appears that it is the degree to which the cell extends and not the density at which the ECM ligand is presented that dictates whether the cell will grow or differentiate. This type of approach, which uses molecularly defined, patterned surfaces and serum-free culture conditions, should facilitate further analysis of the basic mechanism of coupling between cell shape and function as well as other fundamental biological processes that involve changes in the forms of cells or in the contacts between them.

Adhesive islands of palladium on a nonadhesive underlayer of cellulose acetate (*13*), agar (*14*), or poly-hydroxyethylmethacrylate (*15*) have been fabricated and used to study the spreading, migration, and growth of cultured cells (*13–17*). The control of production of tissue-specific cell products, a process that is critical for biotechnology and bioprocessing, has, however, not been demonstrated with this type of engineering approach. Just as important, the microprinting technique is much more convenient and versatile than these past methods: It permits well-defined organic functional groups to be patterned, it requires no access to microlithographic equipment after the elastomeric stamp has been fabricated, and it is applicable to areas too large to be patterned photolithographically. This technique also permits the patterning to be carried out with the use of complex, delicate, or reactive organic function groups or ligands, so the molecular events mediating the interaction of the substrata with adsorbed proteins can be controlled (*18*). None of these options, which are critical for sophisticated biological applications, have been available in the past.

The microprinting technique therefore provides a simple and convenient method to engineer the shape, distribution, and function of individual cells and to control the overall performance of a cell culture. Once the stamp is fabricated with photolithography, it can be used repeatedly and routinely. Because the stamp is pliable, it could potentially be used to pattern curved surfaces. The flexibility in surface chemistry permits the preparation of patterned surfaces with varying degrees of surface charge, wettability, interfacial energy, and resistance to protein adsorption. The need for serum in the culture medium can also be circumvented by coating of the patterned substrata with purified ECM proteins and by the use of a chemically defined medium, methods that we used in the present study. This ability to avoid the use of serum may be a particular advantage for uses that involve screening for growth factors and other cell-specific functional modulators. In the future, it may be possible to engineer the entire culture system by the incorporation of synthetic peptides (such as Arg-Gly-Asp peptides and Fab fragments) or genetically engineered proteins (such as antibodies) as specific cell-adhesive ligands into the patterned monolayers.

This method may also provide a new way to control cell shape and function in both small- and large-scale cell cultures. For example, the prevention of cell extension with the use of patterned surfaces could be used to synchronize large populations of cells in interphase without pharmacological intervention, to increase rates of production of specific proteins (*7*), or to stimulate relevant metabolic or secretory pathways (*16*). Patterned surfaces provide a unique capability to culture cells at very high density while, at the same time, isolating each cell from its neighbors and holding it in a predetermined location. In principle, an index number could be assigned to each patterned cell on the basis of its location. This indexing would facilitate the choice of cells with desired characteristics and the ability to return to them multiple times. This latter property may be very useful for the automation of drug screening and procedures in toxicology that use single-cell functional microassays; for applications in genetic engineering that require micromanipulation or microinjection, such as the generation of transgenic animals; and for the fabrication of sheets of cells in defined patterns and shapes for use in tissue engineering.

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