Using electroactive substrates to pattern the attachment of two different cell populations

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This report describes the development of an electroactive mask that permits the patterning of two different cell populations to a single substrate. This mask is based on a self-assembled monolayer of alkanethiolates on gold that could be switched from a state that prevents the attachment of cells to a state that promotes the integrin-mediated attachment of cells. Monolayers were patterned into regions having this electroactive monolayer and a second set of regions that were adhesive. After Swiss 3T3 fibroblasts had attached to the adhesive regions of this substrate, the second set of regions was activated electrically to permit the attachment of a second population of fibroblast cells. This method provides a general strategy for patterning the attachment of multiple cell types and will be important for studying heterotypic cell-cell interactions.

This paper describes a method to pattern the attachment of two different cell types to a common substrate. This strategy is based on a self-assembled monolayer of alkanethiolates on gold that can be electrically switched from a state that prevents cell attachment to a state that promotes cell attachment (1, 2). Monolayers that are patterned into one set of regions with this electroactive surface chemistry and a second set of regions that promote cell attachment provide a flexible method for patterning two different cell types. The ability to control the locations of different cell types and to vary the distances between cell types in a systematic manner would offer new opportunities for mechanistic studies of heterotypic cell-cell signaling (3, 4). These same methods for patterning cocultures will also be important in cell-based technologies, including sensors for screening libraries of drug candidates and for detecting pathogens in environmental samples (5, 6). In these applications, the active cell often requires heterotypic influences from a second cell to maintain viability and biological activity for the sensing function.

Several methods have been demonstrated for patterning two (or more) cell types to a substrate. One group of strategies uses patterned resists that allow cells to attach only to select regions of a substrate. Removal of the resist then reveals regions of the surface to which a second cell type can attach. In recent work, Toner and coworkers (7, 8) used photolithography to pattern a polymer photoresist on a glass slide. The substrate was treated with a solution of the extracellular matrix protein collagen I to modify the glass surface with an adsorbed layer of the protein and then rinsed with an organic solvent to remove the photoresist and afford a patterned layer of collagen. Hepatocytes attached to this substrate primarily at the protein-coated regions (cells that attached to the other regions could be removed by washing). Fibroblast cells then could attach to these regions to give a patterned coculture. A related method uses physical masks to prevent cell attachment to regions of the substrate (9–11). Whitesides and coworkers (11), for example, applied elastomeric membranes having patterned holes to glass slides and seeded bovine capillary endothelial cells onto the substrate. After cells had attached to both the elastomeric membrane and the exposed regions of the glass slide, the membrane was removed to produce a patterned cell culture. Attachment of a second cell type then could result in a patterned coculture.

A second group of methods patterns multiple cell types by directing the delivery of cells to discrete regions of a substrate. Nanogen (San Diego) has used dielectrophoresis to pattern and separate HeLa cells from red and white blood cells on a microelectrode array (12). This method takes advantage of distinct polarizabilities of different cell types to selectively trap and translate cells with alternating electric fields. Other researchers have used microfluidic channels to direct a suspension of cells to unique regions of a surface (13). An important advantage with these methods is that they avoid the need to pattern the substrate or to remove masks before cell attachment. The constraints of the microfluidic networks, however, limit the generality of geometric patterns and place a lower limit on the distance over which two different cell types can be separated.

In this paper we demonstrate a strategy based on an electroactive mask to direct the attachment of a first cell type, followed by electrochemical modulation of the surface to permit attachment of a second cell type to the previously inert regions (Fig. 1). This method has two important advantages over current methods. First, it does not require extensive or invasive manipulation of the substrate of the type that is currently required in removing masks or microfluidic elements. This feature makes this approach better suited for arranging cells in microsystems, where physical manipulations are not straightforward. Second, this method gives unprecedented control in defining the properties of the substrate, and more importantly, in controlling entirely the receptor-ligand interactions between cell and substrate (14–16). This benefit derives from the use of self-assembled monolayers to tailor the properties of the substrate.

Design Rationale

The key element in our method to turn on select regions of a substrate is the development of a self-assembled monolayer that presents hydroquinone groups among a background of penta(ethylene glycol) groups. In earlier work, we showed that the hydroquinone group undergoes oxidation when an electrical potential is applied to the underlying gold film to give the corresponding benzoquinone. This benzoquinone (but not the hydroquinone) then undergoes a selective and efficient Diels-Alder reaction with cyclopentadiene to afford a covalent adduct (17–19). When a diene is covalently modified with a ligand, the Diels-Alder reaction results in immobilization of the ligand on the monolayer (19). In this work, we use conjugates of cyclopentadiene and the peptide Gly-Arg-Gly-Asp-Ser-NH_2 (RGD-Cp) (Fig. 2). Because this peptide is a ligand that binds to integrin receptors and mediates cell adhesion (20), the immobilization of this conjugate gives a surface to which cells can attach efficiently (14–16). The glycol groups of the monolayer are critical to this design because they prevent the attachment of cells (they are inert to the nonspecific adsorption of protein)

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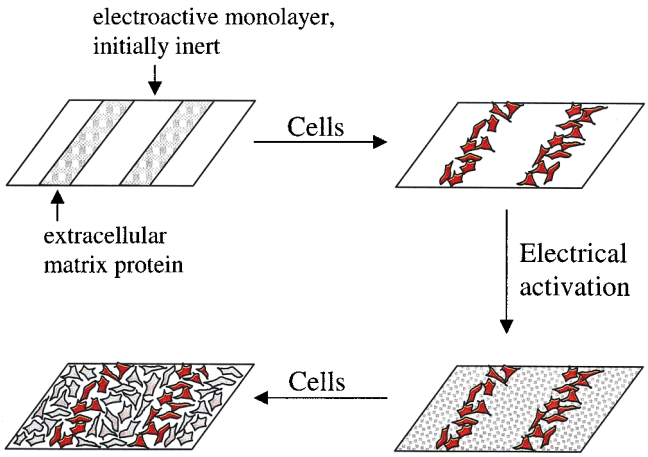

Fig. 1. Strategy for patterning two different cell types to a substrate. The method starts with a surface that is patterned into regions that promote cell attachment (i.e., regions coated with extracellular matrix proteins) and regions that are inert to cell attachment, but that can be converted to promote attachment by the application of an electrical potential. After cells attach to the first set of regions, the inert set of regions can be activated electrically so that a second cell population can attach, generating a patterned coculture.

(21). This scheme, therefore, provides an electrochemical route to turn on a substrate (or select regions of a substrate) for the attachment of cells (22). In the demonstration that follows, a monolayer was patterned into regions to which cells can attach and regions that prevent cell attachment (but that are electrically switchable). In this way, one cell type can be patterned to the substrate and proliferate to completely occupy the patterned region, and then the rest of the substrate can be activated to permit attachment of the second cell type.

Materials and Methods

Preparation of Molecules. (1-Mercapto-undec-11-yl)penta(ethylene glycol) was prepared as described (23). The hydroquinone-terminated alkanethiol was prepared in seven steps from (1-mercapto-undec-11-yl)tetra(ethylene glycol) (23) and 2,5-dimethoxybromobenzene (Aldrich). The conjugate of Arg-Gly-Asp and cyclopentadiene was prepared in three steps from cyclopentadienylacetic acid methyl ester (24) and the peptide Gly-Arg-Gly-Asp-Ser-NH_2. The peptide was synthesized on fluorenylmethoxycarbonyl-Rink amide MHBA resin (Anaspec, San Jose, CA) by using standard protocols (14). All compounds were characterized by mass spectrometry, ^{1}H NMR, and ^{13}C NMR.

Cell Culture. Swiss Albino 3T3 fibroblasts (American Type Culture Collection) were cultured in DMEM (GIBCO) supplemented with 10% FBS, 200 units/ml penicillin, and 200 μg/ml streptomycin (complete medium). Cultures were maintained at 37°C in a humidified atmosphere containing 10% CO_2. Near confluent monolayers of cells were passaged by treatment with a solution of 0.05% trypsin/0.53 mM EDTA (GIBCO). Cells were used between passages 3 and 10. Fluorescence microscopy was performed on a Zeiss Axiovert 135 microscope.

Preparation of Patterned Monolayers. Substrates were prepared by the evaporation of titanium (5 nm) and then gold (15 nm) onto glass coverslips (Corning no. 2, 18 × 18 mm). Microcontact printing was used to pattern hexadecanethiol [$HS(CH_2)_{15}CH_3$] onto gold-coated substrates (25, 26). The substrate was then immersed for 8 h in an ethanolic solution containing the hydroquinone alkanethiol conjugate and (1-mercapto-undec-11-yl)penta(ethylene glycol) (10 μM in quinone, 1 mM in total thiol), to install the electroactive monolayer in the remaining regions of gold. The substrates were rinsed thoroughly with absolute ethanol and dried under a stream of nitrogen. Substrates were immersed in a solution of fibronectin (100 μg/ml in PBS, pH 7.4) for 4 h to adsorb protein to the regions of hexadecanethiolate (27).

Electrochemistry. Cyclic voltammetry was performed with a Bioanalytical Systems CV-50 potentiostat by using the gold/SAM as the working electrode, platinum wire as the counter electrode, and Ag/AgCl as the reference electrode.

Preparation of Cocultures. Swiss 3T3 fibroblasts were removed from tissue culture substrates by trypsinization and resuspended at a concentration of 60,000 cells/ml in serum-free culture medium. The cells were plated onto a patterned monolayer precoated with fibronectin and allowed to attach for 4 h. The substrate then was transferred to a new culture dish containing complete medium and incubated for 48 h. During this time the cells proliferated to fill the patterned regions completely. The patterned cells were labeled with the dye CellTracker Orange (Molecular Probes) by incubation in media containing the dye (25 μM) for 5 min. After labeling, the cells were rinsed with serum-free DMEM and incubated for 30 min. The inert regions of the substrate were turned on by applying an electrical potential of +500 mV for 10 s to the gold substrate in the presence of serum-free culture medium containing RGD-Cp (2 mM) (22). After 2 h at 37°C, the medium was replaced with complete medium (described above) containing unlabeled fibroblasts at a concentration of 80,000 cells/ml. The substrates were incubated at 37°C for an additional 2 h before the substrate was examined by fluorescence microscopy.

Results and Discussion

We used two populations of Swiss 3T3 fibroblasts, wherein one was labeled with a fluorescent vital dye, to demonstrate the independent patterning of two different cell populations to give a patterned coculture. Microcontact printing was used to pattern a substrate with 300 μm wide lines of hexadecanethiolate followed by immersion of the substrate into a solution of hydroquinone-terminated alkanethiol (HQ) and penta(ethylene glycol)-terminated alkanethiol (EG_5OH) in a ratio of 1:99 to install the electroactive monolayer in the nonprinted regions. The substrate then was immersed in a solution of the extracellular matrix protein fibronectin in PBS (100 μg/ml) for 4 h to coat the hexadecanethiolate regions with an adsorbed layer of protein (Fig. 3). The addition of fibroblasts to this substrate resulted in the attachment of cells only to the fibronectin-coated regions. These patterned cells then were fluorescently labeled with a nonspecific vital dye. A potential of +500 mV was applied to the entire substrate for 10 s to oxidize the hydroquinone to the corresponding quinone, which then could react with RGD-Cp to install the peptide via the Diels-Alder reaction (22). Addition of nonlabeled fibroblasts to the modified substrate resulted in the attachment of these cells to the second set of regions. Fluorescence microscopy showed that invasion of the labeled fibroblasts into regions presenting the peptide was minimal. It is significant that cells attached to fibronectin-coated regions of the substrate were unaffected by the addition of the peptide conjugate.

We found the degree to which cells on the first patterned region of the substrate can migrate onto the second region, and therefore compromise the fidelity of the patterned coculture, depended on the geometric features of the pattern. Fig. 4 compares the extent to which cells confined by either a straight edge or a curved feature migrate out of the patterned regions

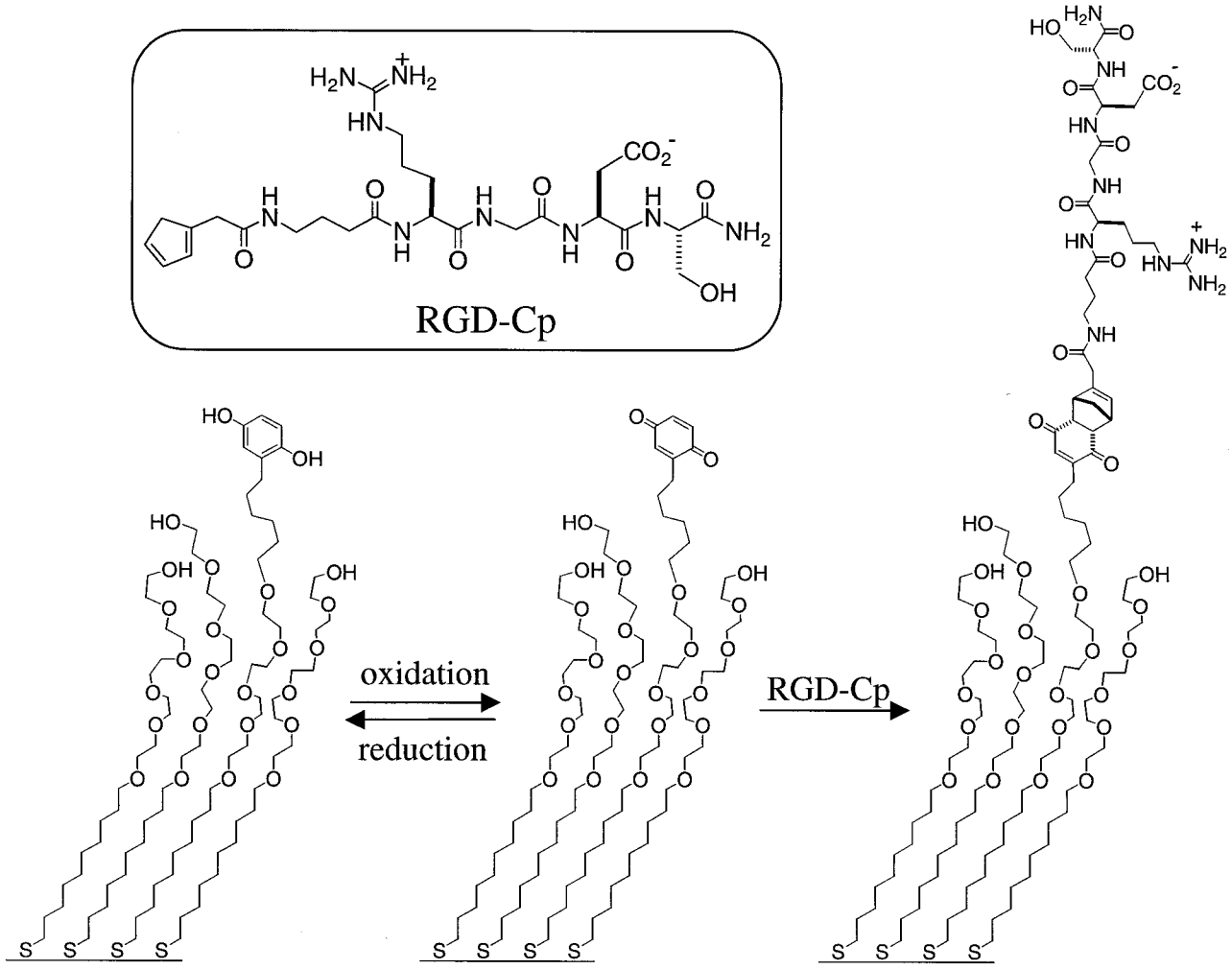

Fig. 2. Molecular strategy for creating substrates that can be electrically switched to permit cell attachment. A monolayer presenting a mixture of hydroquinone groups and penta(ethylene glycol) groups (*Left*) is converted to a monolayer presenting the corresponding quinone groups (*Center*) by application of a potential to the underlying gold (500 mV versus Ag/AgCl). Both monolayers are inert to the attachment of cells. Addition of a conjugate of cyclopentadiene and the peptide Gly-Arg-Gly-Asp-Ser-NH_2 (RGD-Cp) to the monolayer presenting the quinone group results in the Diels-Alder-mediated immobilization of peptide (*Right*). 3T3 fibroblasts attach and spread on the resulting surface. Monolayers presenting the hydroquinone group are unaffected by the treatment with RGD-Cp and remain inert to cell attachment.

after immobilization of RGD-Cp. Cells patterned onto linear features remained completely confined 7 h after immobilization and migrated only to a small extent at longer times (Fig. 4*A*). Cells patterned into circular regions having a diameter of 200 μm, however, migrated within hours after the immobilization of RGD-Cp (Fig. 4*B*). The use of pattern geometry to control the onset cell migration represents a general principle for the design of cocultures and will be useful for a range of methods that rely on masks to prepare cocultures. We believe that the substantial difference in onset of migration is caused by the alignment of the cellular cytoskeletal filaments. On linear patterns (or more generally, on patterns having a very large radius of curvature relative to cell size) the cytoskeleton of cells at the edge is oriented parallel to the edge (27, 28). For migration to occur, it may be necessary to disrupt the parallel organization of the cytoskeleton at the edge of large or linear patterns, delaying the initiation of migration.

To establish that the attachment of the second cell population to the surface was mediated by the peptide ligand alone, we added soluble GRGDS peptide (2 mM, 2 h) to a patterned coculture (described above). Because the attachment of the second cell population is mediated solely by binding of cell surface integrin receptors to the immobilized peptides, the soluble peptide is expected to inhibit this interaction and lead to detachment of cells (14–16). Fig. 5 shows that the second cell population did indeed detach from regions that presented peptide among an inert background, but that the first cell population remained attached to regions to which fibronectin was adsorbed. This result demonstrates that adhesion of fibroblasts to the protein-coated regions of the substrate occurs in an RGD-independent manner. These data are consistent with those found in a study of the long-term attachment of bovine capillary endothelial cells to fibronectin (15). This result also illustrates three features of this methodology: first, it shows that the electrochemical modulation of the substrate does not compromise the integrity of the monolayer; second, it shows that the second cell population is attached biospecifically via cell surface integrin receptors to the newly installed RGD peptide ligand; and third, this methodology can selectively release cells, which allows for both spatial and temporal control in studies of either heterotypic or homotypic cell–cell interactions.

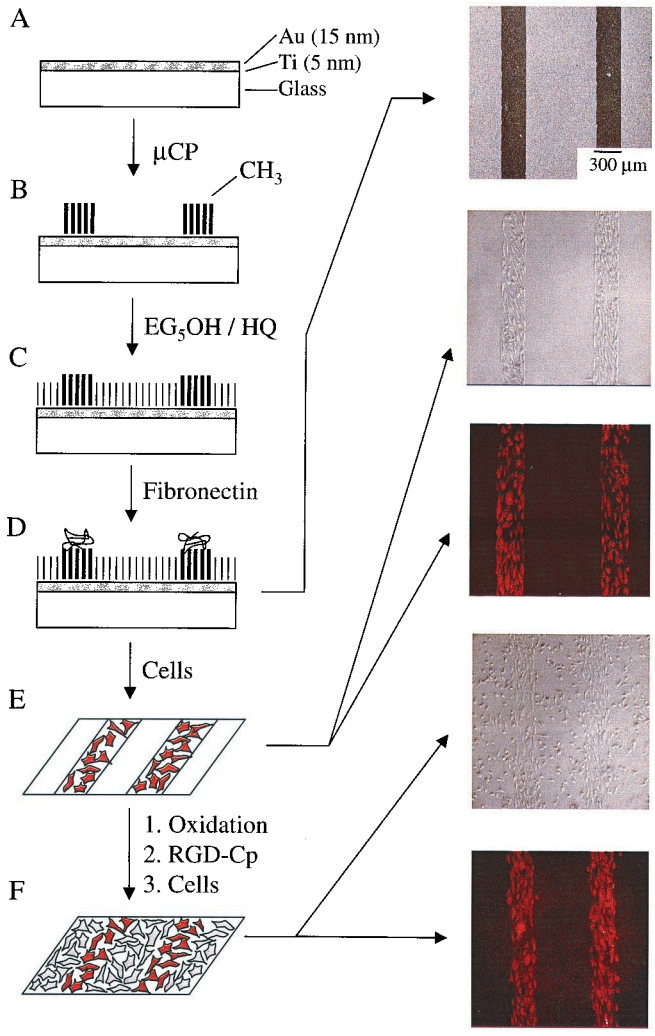

Fig. 3. Use of an electroactive substrate to pattern two cell populations into a coculture. (*A*) Substrates were prepared by evaporating titanium (5 nm) and then gold (15 nm) onto glass coverslips. (*B*) Microcontact printing was used to pattern hexadecanethiolate into lines that are 300 μm wide and separated by 800 μm. (*C*) A second monolayer was assembed on the remaining regions of gold by immersing the substrate into a solution of hydroquinone-terminated alkanethiol (HQ) and penta(ethylene glycol)-terminated alkanethiol (EG_5OH). (*D*) The substrate then was immersed in a solution of fibronectin in PBS for 4 h. A scanning electron micrograph shows that protein adsorbed only to the methyl-terminated regions of the monolayer. (*E*) 3T3 fibroblasts attached only to the regions presenting fibronectin, and when cultured in serum-containing media, divided to fill these regions entirely. The surrounding inert monolayer strictly confined the cell to the rectangular regions. (*F*) Electrochemical oxidation of the monolayer in the presence of media containing RGD-Cp (2 nM) led to the immobilization of the peptide. Fluorescence microscopy shows that the two cell populations are patterned on the substrate. All micrographs were taken by ×5 magnification.

Several methods have been demonstrated for patterning heterotypic cell cultures. Because no one method is best suited for all applications, the development of several methods will provide a general capability for patterning multiple cell types. The method described in this paper uses an electrical mask (1, 2, 22) to control the attachment of a cell population and then permits attachment of a second cell population. In addition to providing a less invasive means to activate a second set of regions to cell attachment, this method is well suited for controlling the attachment of cells to microelectrode arrays and to other substrates that have electrically conductive elements. We believe

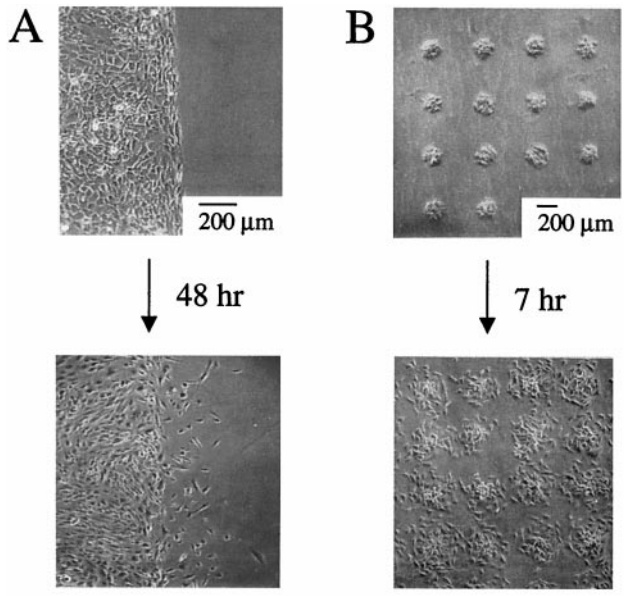

Fig. 4. The onset of migration into the second set of regions after electrochemical activation and peptide immobilization depends on the geometric features of the pattern. Cells patterned along a straight edge (*A*) showed little migration after 48 h, whereas cells patterned to 200-μm circles showed extensive migration after only 7 h (*B*). All micrographs were taken at ×5 magnification.

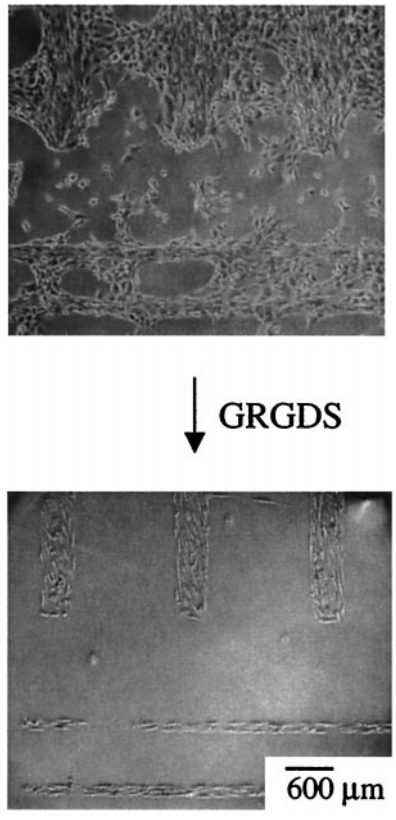

Fig. 5. Selective detachment of cells adhered to regions of the substrate presenting the RGD peptide among penta(ethyene glycol) groups. A patterned coculture was prepared as described in Fig. 3 *Left*. Addition of soluble peptide (GRGDS, 2 mM for 1 h) resulted in the detachment of cells only from the regions of the monolayer presenting peptide conjugates, indicating the specificity of the cell-substrate interaction. Micrographs were taken at ×5 magnification.

this method will be most important for fundamental studies of heterotypic cell–cell interactions.

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1. Mrksich, M. (2000) *Chem. Soc. Rev.* **29,** 267–273.
2. Yousaf, M. N. & Mrksich, M. (2000) in *New Technologies for Life Sciences: A Trends Guide*, ed. Wilson, E. (Elsevier, London), pp. 28–35.
3. Folch, A. & Toner, M. (2000) *Annu. Rev. Biomed. Eng.* **2,** 227–256.
4. Pins, G. D., Toner, M. & Morgan, J. R. (2000) *FASEB J.* **14,** 593–602.
5. Kapur, R., Giuliano, K. A., Campana, M., Adams, T., Olson, K., Jung, D., Mrksich, M., Vasudevan, C. & Taylor, D. L. (1999) *Biomed. Microdev.* **2,** 99–109.
6. Voldman, J., Gray, M. L. & Schmidt, M. A. (2000) *Annu. Rev. Biomed. Eng.* 2401–425.
7. Bhatia, S. N., Balis, U. J., Yarmush, M. L. & Toner, M. (1998) *Biotechnol. Prog.* **14,** 378–387.
8. Bhatia, S. N., Balis, U. J., Yarmush, M. L. & Toner, M. (1999) *FASEB J.* **13,** 1883–1900.
9. Folch, A., Jo, B. H., Hurtado, O., Beebe, D. J. & Toner, M. (2000) *J. Biomed. Mater. Res.* **52,** 346–353.
10. Folch, A. & Toner, M. (1998) *Biotechnol. Prog.* **14,** 388–392.
11. Ostuni, E., Kane, R., Chen, C. S., Ingber, D. E. & Whitesides, G. M. (2000) *Langmuir* **16,** 7811–7819.
12. Cheng, J., Sheldon, E. L., Wu, L., Heller, M. J. & O'Connell, J. P. (1998) *Anal. Chem.* **70,** 2321–2326.
13. Chiu, D. T., Jeon, N. L., Huang, S., Kane R.S., Wargo, C. J., Choi, I. S., Ingber, D. E. & Whitesides, G. M. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2408–2413. (First Published February 18, 2000, 10.1073/pnas.040562297)
14. Houseman, B. T. & Mrksich, M. (1998) *J. Org. Chem.* **61,** 7552–7555.
15. Roberts, C., Chen, C. S., Mrksich, M., Martichonok, V., Ingber, D. E. & Whitesides, G. M. (1998) *J. Am. Chem. Soc.* **120,** 6548–6555.
16. Houseman, B. T. & Mrksich, M. (2001) *Biomaterials* **22,** 943–955.
17. Yousaf, M. N., Chan, E. W. L. & Mrksich, M. (2000) *Angew. Chem. Int. Ed.* **39,** 1943–1946.
18. Chan, E. W. L., Yousaf, M. N. & Mrksich, M. (2000) *J. Phys. Chem. A.* **104,** 9315–9320.
19. Yousaf, M. N. & Mrksich, M. (1999) *J. Am. Chem. Soc.* **121,** 4286–4287.
20. Ruoslahti, E. (1996) *Annu. Rev. Cell Dev. Biol.* **12,** 697–715.
21. Mrksich, M. & Whitesides, G. M. (1997) *Am. Chem. Soc. Symp. Ser.* **680,** 361–373.
22. Yousaf, M. N., Houseman, B. T. & Mrksich, M. (2001) *Angew. Chem. Int. Ed.* **40,** 1093–1096.
23. Pale-Grosdemange, C., Simon, E. S., Prime, K. L. & Whitesides, G. M. (1991) *J. Am. Chem. Soc.* **113,** 12–20.
24. Pei-Hung, Y., Zhen, P. & Johnston R. F. (1996) *J. Organomet. Chem.* **509,** 123–129.
25. Singvi, R., Kumar, A., Lopez, G. P., Stephanopoulous, G. N., Wange, D. I. C., Whitesides, G. M. & Ingber, D. E. (1994) *Science* **264,** 696–698.
26. Chen, C. S., Mrksich, M., Huang, S., Whitesides, G. M. & Ingber, D. E. (1997) *Science* **276,** 1425–1428.
27. Mrksich, M., Dike, L. E., Tien, J., Ingber, D. E. & Whitesides, G. M. (1997) *Exp. Cell Res.* **235,** 305–313.
28. Dike, L. E., Chen, C. S., Mrksich, M., Tien, J., Whitesides, G. M. & Ingber, D. E. (1999) *In Vitro Cell. Dev. Biol. Animal* **35,** 441–448.