Regiospecific Control of Protein Expression in Cells Cultured on Two-Component Counter Gradients of Extracellular Matrix Proteins

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This work describes the use of microfluidic tools to generate covalently immobilized counter gradients of extracellular matrix (ECM) proteins laminin and collagen I. Using these platforms, we demonstrate control of the expression levels of two proteins linked to cell cycle progression by virtue of the spatial location of cells on the gradients, and hence by the local ECM environments in these devices. In contrast to physisorbed gradients, covalently immobilized protein patterns preserved the gradient fidelity, making long term cell studies feasible. This method of precisely controlling local cell environments is simple and broadly portable to other cell types and to other ECM proteins or soluble factors. Our approach promises to enable new investigations in cell biology that will contribute to the establishment of biological design rules for controlling cell growth, differentiation, and function.

Introduction

The identification and study of the constellation of factors that control cell behavior is key to many cell biology studies. Both the quantification of components in the tissue microenvironment and their quantitative control are formidable obstacles to determining the biological design rules that control cell behavior in vivo. There is an urgent need for in vitro models that enable investigations of cell behavior in complex but precisely controlled microenvironments. Although the ever-increasing ability to fabricate and pattern structures at the microscale has long promised the crypt–villus axis.5,6 Importantly, the regiospecific expression profiles of laminin isoforms along the crypt–villus axis (Figure 1A) correspond to the transition of epithelial cells from the proliferative crypt zone to the villus, where they undergo terminal differentiation.7 Specific laminin–integrin interactions influence cell cycle progression through various intracellular signaling pathways.8 In addition, integrins expressed on intestinal epithelial cells exhibit regiospecific distribution patterns along the crypt–villus axis, thus adding an additional level of complexity to investigations of ECM–epithelial cell interactions in vivo.9 Although laminin isoforms induce differentiation in some epithelial cell lines,9 a major question remains: to what extent does the ECM microenvironment control stem cell differentiation? The lack of in vitro models that adequately simulate the in vivo ECM environment has been a chief obstacle to addressing this issue.


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By harnessing the distinct properties of fluid flow at the microscale, most notably laminar flow, microfluidic platforms enable the study of cells in precisely defined microenvironments, including both immobilized and diffusional gradients. The lack of turbulent mixing at the microscale generates laminar flow of parallel fluid streams of different composition that mix only by slow interdiffusion. This in turn generates stepwise solution gradients that can be used to pattern surfaces with different proteins and different cells, while enabling the spatial control of the medium surrounding individual cells, to selectively expose cells or parts of cells to soluble triggers. Recently, Jeon et al. demonstrated the use of smooth gradients of interleukin-8 in solution, to study the impact of differences in the expression of protein markers for cell cycle progression that were linked directly to the local laminin/collagen I concentration ratio defined by the spatial position of the cells on the gradient. These results illustrate the broad potential utility of these novel platforms for further investigations of cell behavior, including differentiation, migration, and proliferation that are governed by the cell microenvironment.

**Materials and Methods**

**Substrate Preparation.** The substrates consisted of self-assembled, carboxy-terminated alkanethiol monolayers on gold films. Glass slides (Fisher Scientific, Pittsburgh, PA) were cleaned by boiling in a piranha solution (HCl:H2O2:H2O = 1:1:1) for 20 min. They were subsequently rinsed with water and ethanol and dried with nitrogen. Gold (A-1-Coin Buyers, Champaign, IL) was thermally deposited onto the glass slides in a thermal evaporator (Cooke Vacuum Products, Inc., Norwalk, CT) at a base pressure of 10⁻⁶ Torr. An adhesion promoter, chromium (R.D. Mathis Co., Long Beach, CA), with a thickness of 10 Å was first deposited onto the glass slides at a rate of 0.1 Å/s. The gold films were then deposited at 1 Å/s with a final thickness of 500 Å. After deposition, the gold substrates were immersed overnight in a solution of 1 mM 16-mercaptohexadecanoic acid and 11-mercaptoundecan-1-ol (Sigma, St. Louis, MO) at a 3:1 molar ratio (1 mM total alkanethiol). The substrates were then rinsed with ethanol and dried with filtered nitrogen.

**Uniformly-Coated Protein Substrates.** Patterns of proteins with uniform mass coverage were generated inside a microfluidic network that was molded in poly(dimethylsiloxane) (PDMS) (Dow Corning, Midland, MI). A PDMS microfluidic network (Figure 1B) was placed onto a gold substrate coated with a self-assembled monolayer (SAM). The gold substrate and the PDMS slab were assembled, carboxy-terminated alkanethiol monolayers on gold films. Glass slides (Fisher Scientific, Pittsburgh, PA) were cleaned by boiling in a piranha solution (HCl:H2O2:H2O = 1:1:1) for 20 min. They were subsequently rinsed with water and ethanol and dried with nitrogen. Gold (A-1-Coin Buyers, Champaign, IL) was thermally deposited onto the glass slides in a thermal evaporator (Cooke Vacuum Products, Inc., Norwalk, CT) at a base pressure of 10⁻⁶ Torr. An adhesion promoter, chromium (R.D. Mathis Co., Long Beach, CA), with a thickness of 10 Å was first deposited onto the glass slides at a rate of 0.1 Å/s. The gold films were then deposited at 1 Å/s with a final thickness of 500 Å. After deposition, the gold substrates were immersed overnight in a solution of 1 mM 16-mercaptohexadecanoic acid and 11-mercaptoundecan-1-ol (Sigma, St. Louis, MO) at a 3:1 molar ratio (1 mM total alkanethiol). The substrates were then rinsed with ethanol and dried with filtered nitrogen.

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I/BSA were mixed at 25%, 50%, 75%, and 100% collagen. After rinsing the channels with buffer, the Plexiglass and PDMS slabs were peeled off, and the substrates were blocked with 0.3 mL of a 10% (w/w) solution of BSA in PBS for 15 min at room temperature. The substrates were rinsed with PBS and sterile water and then dried in air.

**Surface ECM Gradient Preparation.** The microfluidic gradient tool (Figure 1B), which was used to generate patterns of protein gradients, comprises a network of microchannels in PDMS with the modified gold substrate as the base. The microchannels were fabricated using rapid prototyping and soft lithography. Inlet and outlets were created using a steel punch. Polyethylene tubing inserted into these ports served to deliver fluid to the microchannels. The gold substrate and the PDMS microchannels were brought into contact and “sandwiched” between two PDMS slabs. To prevent leakage during injection, the assembly was clamped together with binder clips. Bubbles were purged from the microchannels by first injecting ethanol and then rinsing the channels with distilled water followed by acetate buffer (0.5 M sodium acetate, pH 5.5). A 0.5 mL volume of EDC/NHS solution was then injected through the microchannel. After 15 min at room temperature, the channels were rinsed with 0.5 M of acetate buffer to remove unretracted EDC/NHS. Approximately 0.2 mL of laminin and collagen I (BD Biosciences, Bedford, MA) in 0.5 M acetate buffer at pH 5.5 were then pumped through the microchannels at a flow rate between 0.3 and 0.9 μL/min at 4 °C using a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA). The unreacted groups on the substrate were then blocked to prevent nonspecific adsorption with 0.2% of 10% bovine serum albumin (Sigma) in PBS for 10 min at room temperature. The PDMS mold was then removed from the substrate, and the exposed gold surface was blocked with 10% BSA in PBS for 15 min at 4 °C. Physically adsorbed gradients were prepared similarly without using the EDC/NHS coupling agents.

**Surface ECM Gradient Characterization.** Immobilized gradients of laminin and collagen I were stained with, respectively, anti-laminin (rabbit, 1:50 in 10% BSA/PBS, Sigma) and anti-collagen I (mouse, 1:100 in 10% BSA/PBS, Sigma) for 10 min at room temperature. After rinsing with PBS, secondary antibodies, anti-rabbit IgG labeled with FITC (1:200 in 10% BSA/PBS, Sigma), and anti-mouse IgG labeled with Alexa Fluor 568 (1:100 in 10% BSA/PBS, Molecular Probes, Portland, OR) were applied for 10 min. The substrate was then rinsed with PBS, treated with an Antifade kit (Molecular Probes) to maintain the fluorescence intensity, and then covered with a coverslip. Fluorescent images were visualized on a Nikon Optiphot-2 microscope (Fryer, Carpentersville, IL) equipped for epifluorescence. Digital images were captured with Image-Pro Plus software, version 3.3 (Media Cybernetics, Silver Spring, MD).

To quantify intensity changes across the gradients, 15 intensity profiles across the laminin and collagen I gradients were measured and averaged for each gradient to give the mean fluorescence intensity (MFI) profile. The fluorescence intensity was then calibrated in terms of the protein surface density, using surface plasmon resonance.

**Calibration of Protein Surface Densities by Surface Plasmon Resonance (SPR).** The covalent attachment of laminin to monolayers was quantified using a home-built SPR. The SPR cell (0.25 mL volume), containing a uniform self-assembled alkanethiol monolayer, was rinsed consecutively with water and acetate buffer (0.5 M sodium acetate/acetate acid, pH 5.5) for 15 min. This was followed by activation with EDC/NHS at 5 °C. Physically adsorbed gradients were prepared similarly with EDC/NHS coupling agents. Between 0.3 and 0.9 μL/min of EDC/NHS solution was then injected through the channel for 5 min. Laminin or collagen I was injected through the cell at a rate of 0.5 μL/min. Finally, the SPR cell was rinsed with acetate buffer, followed by water for 10 min at a flow rate of 4 μL/min. The optical thickness Δd of the bound protein was calculated by fitting Fresnel dispersion equations to the change in the plasmon resonance angle, δ. A calibration curve was determined using the refractive index n of the sample material in the dry state.

With this slope, the changes in the resonance angle δ can be directly related to the optical thickness, Δd of the adsorbed material. The changes in the effective optical thickness, Δd, are readily converted into protein surface coverage by the following equation:

\[
\Gamma = \rho d
\]

In this study, we used a refractive index of 1.46 for both the laminin and collagen I films prepared under identical conditions. We first quantified the surface densities of covalently bound laminin and collagen I films prepared with protein solution concentrations of 0.025, 0.01, and 0.005 mg/mL, and then quantified the corresponding fluorescence intensities of stained laminin and collagen I films prepared under identical conditions. The calibration curve, obtained by relating the absolute protein density to the MFI, was then used to quantify the densities of laminin and collagen I on both the uniform protein stripes and the ECM gradients.

**Cell Culture.** The IEC-6 rat small intestine epithelial cells were cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with a final glucose concentration of 25 mM, 10% fetal bovine serum (PBS, Gemini Bio-Products, Woodland, CA), penicillin (10 000 U/mL), streptomycin (10 000 μg/mL, BioWhittaker, Walkersville, MD), fungizone (250 μg/mL, BioWhittaker), and 0.1 μg/mL bovine insulin (Invitrogen, Carlsbad, CA). Caco-2 human colon adenocarcinoma cells were cultured in DMEM supplemented with 10% PBS, 10 mg/mL transferrin, 2 mM glutamine, 1 mM pyruvate, 10 mM Hepes, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. CHO cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 0.1% fungizone. Cells were maintained at 37 °C in a humidified incubator with 5% CO2. Cell monolayers (~90% confluence) were dissociated by incubation with trypsin-verseine (BioWhittaker) for 5 min at 37 °C. Cells were resuspended in 10 mL of supplemented DMEM and centrifuged for 7 min at 1800 rpm. Cell pellets were resuspended in serum-free medium consisting of supplemented DMEM without FBS.

**Analysis of Protein Expression.** Cell cycle progression and exit were monitored by measuring the expression of proliferating cell nuclear antigen (PCNA) and a cyclin-dependent kinase inhibitor (p27), respectively, via immunofluorescence. The expression of the cell markers PCNA and p27 was determined by immunofluorescence 1 day and 3 days after the cells were cultured on uniform protein stripes and on the gradients. During the cell cycle, PCNA is expressed during the S-phase, which indicates that cells progress through the cell cycle. In contrast, p27 is expressed during the G1-phase, if cells exit the cell cycle and undergo a G0 growth arrest. These two expression markers thus indicate differences in cell cycle progression in response to the local environment. Cells were fixed with 3.7% formaldehyde (Fisher, Fair Lawn, NJ) for 30 min and then blocked with 4% PBS in PBS for 15 min at 4 °C. Cells were then incubated with a primary anti-PCNA antibody (PC-10, [1:40], Santa Cruz Biotechnology, Santa Cruz, CA), anti-p27 primary antibody (C-19, [1:50], Santa Cruz Biotechnology), and DAPI (1:200 of 150 μg/mL stock solution, Sigma) for 30 min. Cells were then rinsed with PBS and stained with a goat anti-mouse IgG conjugated with Alexa Fluor 568 (1:200, Molecular Probes) and a goat, anti-rabbit IgG labeled with FITC (1:200, Molecular Probes) to maintain the fluorescence intensity, and then covered with a coverslip. Fluorescent images were captured with a Nikon Optiphot-2 microscope and analyzed with Image-Pro Plus software.

**Materials and Methods.**

fluorescence intensities were measured from 10–20 cells. For the gradient substrates, the fluorescence intensities were then measured from 80–100 cells. The fluorescence intensity was analyzed with ImageJ software version 1.30 (NIH). The intensity of PCNA and p27 secondary antibody staining was quantified from the average intensity of 200 μm² spots within individual cells. Five spots within each cell were measured and averaged. The population averaged fluorescence intensity was then determined by averaging the intensities from a population of cells at the same distance from the wall, and hence in defined extracellular environments. The error bars reflect general experimental variability due to, for example, sample to sample variability or slight photobleaching. A population of cells was defined as cells that attached within a 190 μm region at a defined distance from the wall. The location of the cells within each population was determined with ImageJ software.

The results of PCNA and p27 staining are represented graphically as the mean ± standard deviation from at least three similar experiments. The student’s t-test for two samples, assuming unequal variances, was used to compare the fluorescence intensity of p27 and PCNA along the gradients. We used the data analysis package from Microsoft Excel (Microsoft Corp., Seattle, WA). A P value of 0.05 was considered statistically significant.

Results

Protein Surface Densities. In this work we covalently immobilized both laminin and collagen I on carboxy-terminated SAMs via EDC/NHS linking chemistry. Surface plasmon resonance measurements confirmed the irreversible protein attachment to these EDC/NHS activated monolayers (Figure 2). The difference between the initial and final water baselines gave the optical film thickness Δd of the protein adlayer. The protein surface densities calculated with eq 1 are given in Table 1, as are the corresponding mean fluorescence intensities measured from identical protein films. As shown in Table 1, lowering the solution concentration of laminin from 0.025 to 0.01 mg/mL decreased the MFI due to immobilized laminin by ~40%.

The surface densities of laminin on substrates modified with mixed solutions of laminin and collagen I were determined using the calibration data in Table 1. Figure 2B gives the protein densities on uniform micropatterned strips of laminin/collagen I films prepared with laminin molar percentages of 0%, 20%, 40%, 60%, 80%, and 100%.

Covalently Immobilized Counter Gradients of Laminin and Collagen I. The initial protein concentrations and flow rate affected the profiles of the immobilized gradients in the main channel (Figure 3A–E). We controlled the gradient profile by decreasing the flow rate from 0.9 to 0.3 μL/min. The initial protein solution concentrations were also varied from 7.5 to 2.5 mg/mL. Increasing the concentration of both proteins smoothed the gradient profile and increased the gradient steepness (Figure 3A–C). In contrast, increasing the flow rate changed the profile from a continuous gradient to a stepwise gradient (Figure 3C–E).

Initially, we initiated the surface patterning by injecting one protein solution at one inlet and a buffer solution at the other inlet. This approach, however, did not generate a surface gradient (data not shown). Injecting a single protein solution simply results in a saturated substrate since the immobilization reaction is irreversible and fast. Furthermore, the amount of covalently attached protein is only limited by the surface concentration of carboxyl groups, so that even dilute protein solutions will saturate the surface over time. SPR experiments (Figure 2A) indeed showed that the SAM surfaces were saturated with laminin within 1 h of injecting an 0.1 mg/mL solution. These studies therefore established conditions required to generate smooth, immobilized protein gradients with the microfluidic gradient maker in Figure 1B.

ECM Control of Protein Expression. The effects of relative laminin and collagen I ratios on PCNA and p27 expression were studied for cells cultured on uniform laminin and collagen I films. Prior studies demonstrated that both rat intestinal crypt-like IEC-6 cells and Caco-2BE cells differentiate or proliferate when grown on laminin or collagen I, respectively. On these uniform, mixed protein films, the cell cycle progression of IEC-6 cells, as demonstrated by PCNA expression, increased with an increasing ratio of collagen I to laminin (Figure 4A; P < 0.05). The p27 expression level, which correlates

<p>| Table 1. Mean Fluorescence Intensities (MFIs) and Surface Density of Uniform Laminin and Collagen I Films |
|-------------------------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th><strong>solution concn</strong> (mg/mL)</th>
<th><strong>laminin</strong></th>
<th><strong>collagen I</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025</td>
<td>220 ± 5</td>
<td>30300 ± 909</td>
</tr>
<tr>
<td>0.01</td>
<td>160 ± 5</td>
<td>17090 ± 513</td>
</tr>
<tr>
<td>0.005</td>
<td>100 ± 4</td>
<td>9130 ± 270</td>
</tr>
</tbody>
</table>

with cell cycle exit, increased with increasing ratios of laminin to collagen I ($P < 0.05$). In comparison, the expression of PCNA in Caco-2 BBE cells (Figure 4B, $P < 0.05$) and CHO cells (Figure 4C, $P < 0.05$) increased with increasing collagen I/laminin ratios.

The cell behavior on gradients was then quantified on the basis of the measured protein expression levels of cells at different positions on the 750-micron-wide gradient pattern. In this work, IEC-6, Caco-2BBE, and CHO cells cultured on the laminin/collagen I gradients exhibited distinct levels of gene expression related to cell cycle progression or exit. These were tracked with the local concentrations of ECM proteins at different positions of the gradient (Figures 6–8). Immunofluorescent staining of IEC-6 cells demonstrated that cells adhering to the highest laminin concentration of 27500 pg/dm² exhibited the highest p27 expression levels. The p27 expression dropped in proportion to the local laminin concentration (Figure 6, $P < 0.05$). Conversely, PCNA expression was greatest in cells located at the lowest laminin concentration of 7500 pg/dm² (highest collagen I concentration). Decreasing expression levels correlated directly with the decrease in collagen I levels in the gradient (Figure 6). These results demonstrate the direct control of the cell cycle by the precise composition of an engineered ECM microenvironment.

Similar measurements were performed with Caco-2BBE and CHO cells (Figures 7 and 8; $P < 0.05$ for each) on identical counter gradients. With both cell types, the level of p27 expression paralleled increasing laminin concentrations, and PCNA expression increased with increasing collagen I concentrations. These observations similarly demonstrate the control of cell cycle kinetics with the microfabricated ECM profiles.

The individual effects of laminin and collagen I on the cell cycle progression were studied with IEC-6 cells cultured on uniformly coated substrates of mixed films of laminin/BSA or collagen I/BSA (Figure 5). BSA was used as the second “inert” component and is presumed to have no effect on the protein expression levels. The inclusion of this background protein is necessary to block the nonspecific adsorption of other proteins in the medium. In this case, the p27 expression increased with increasing laminin (Figure 5A, $P < 0.05$), but it was relatively insensitive to the collagen density (Figure 5B, $P < 0.05$). On the other hand, the PCNA expression increased with increasing collagen I (Figure 5B) and also with increasing...
laminin (Figure 5A). These results differ from that seen on the mixed laminin/collagen I substrates and suggest that the p27 and PCNA expression levels reflect the combined effect of both proteins.

Prior investigations using the gradient generator in Figure 1B showed that soluble gradients of single proteins can be immobilized on solid surfaces by physisorption. Similar “control” studies, which are illustrated in Figure 9, show that the physisorbed gradients (Figure 9A) are not stable over time. Physisorbed counter gradients of laminin and collagen become diffuse within 24 h, when stored in culture medium in the absence of cells (Figure 9B). In contrast, the chemisorbed gradients were stable, even in the presence of cells, after 3 days (Figure 9C).

**Discussion**

Using microfluidic tools, we successfully generated immobilized counter gradients of two physiologically relevant ECM proteins, laminin and collagen I, that individually promote either cell cycle exit or progression. Covalent immobilization ensured ECM stability, and hence the preservation of the local ECM composition over the course of the study. The generation of gradient profiles allowed us to locally control the ECM concentration, which permitted the direct control of the cell cycle kinetics.

The quality of the gradients formed clearly depends on parameters that affect mass transport within the channels: namely, the solute concentrations and the solution flow rates. In this work, the characteristics of the gradient profiles were achieved empirically, resulting in relatively uniform increases in protein concentrations across the width of the channels.

With all three of the cell lines, p27 expression correlated directly with the local laminin concentration in the gradient. Increasing the local concentration of laminin in
the gradient increased the expression of p27 markers. Similarly, with all three of the cell lines, PCNA expression correlated directly with the local collagen I concentration in the gradient. Thus, by culturing IEC-6, Caco-2BBE, and CHO cells on the covalently immobilized gradients, we achieved the control of cell cycle progression through the local ECM composition generated with these devices.

The individual effects of laminin and collagen I in a BSA background differed from these seen with cells cultured on the gradients. These findings confirm that the expression profiles of IEC-6 cells cultured on the laminin/collagen I gradients are determined by the relative, local amounts and identities of the two proteins on the surface.

It is important to point out that the cell behavior reported in this investigation reflects the local concentration on the gradient rather than the protein concentration difference across the cell diameter. In particular, with regard to the expression markers used in this work, the protein gradient does not appear to influence the global cell behavior. Nevertheless, the platform presented in this work enabled the rapid assessment of cell responses to multiple laminin/collagen I ratios within a single channel. Obtaining the same information with multiple, parallel protein stripes of uniform concentrations (see Figure 3B) required more fabrication steps, reagents, and time. For example, at least 20 separate protein “stripes” were used to generate the data in Figure 2B. Thus, the efficiencies inherent in this system provide additional advantages in the use of such tools for cell studies.

Alternately, the gradients themselves, rather than the average local ECM composition, may direct other cell behavior such as migration or axon extension. While this was not the focus of the studies presented here, cell behavior distinctly driven by the protein concentration profile is the subject of future work.16 This investigation defined the design rules, which determine the characteristics of covalently immobilized protein gradients, and further demonstrated the control of gene expression through the precise control of the local ECM composition.

We attribute the regiospecific cell behavior over the course of the study to the influence of the ECM profile generated with the gradient tool, because of the stability of the covalently bound protein gradients. Immunofluorescence staining of the protein gradients before and after cell culture demonstrated the stability of ECM patterns for up to 3 days in the presence or absence of cells. This indicates that the immobilized protein gradients did not degrade and that the cells did not significantly remodel the ECM during the assays. By contrast, in control experiments physisorbed protein gradients maintained in media became diffuse when kept in media for 1 day (Figure 9B). We also carried out all of our cell culture experiments in serum-free medium, to prevent changes in the ECM composition through the nonspecific adsorption of serum proteins. We were thus able to manipulate cell behavior directly through precise control of the local protein concentration as defined by their spatial position on the protein gradient.

Conclusions

This work demonstrates the fabrication of stable, covalently immobilized gradients of different ECM pro-
teins, and the spatial variation of cellular responses directed by these engineered ECM patterns in vitro. IEC-6, Caco-2, and CHO cells exhibited regiospecific expression of p27 and PCNA, markers that are linked to the cell cycle progression, when cultured on immobilized counter gradients of the ECM proteins laminin and collagen I. This prototype system thus represents a powerful and versatile methodology for creating in vitro models of ECM environments for uniquely and quantitatively testing such hypotheses. It is simple and broadly portable to various cell types and other ECM proteins and overcomes the major problem of gradual gradient remodeling that has limited the use of physisorbed gradients to short-term biological studies.

These platforms with covalently immobilized gradients promise to engender a wide range of previously difficult, quantitative investigations of cell growth and differentiation in response to different protein combinations in a variety of concentration profiles. These microfluidic tools will thus enable new investigations of fundamental cell biology, as well as contribute to the establishment of quantitative biological design rules for controlling cell growth, migration, differentiation, and function.

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