Generation of static and dynamic patterned co-cultures using microfabricated parylene-C stencils

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Many biological processes, such as stem cell differentiation, wound healing and development, involve dynamic interactions between cells and their microenvironment. The ability to control these dynamic processes *in vitro* would be potentially useful to fabricate tissue engineering constructs, study biological processes, and direct stem cell differentiation. In this paper, we used a parylene-C microstencil to develop two methods of creating patterned co-cultures using either static or dynamic conditions. In the static case, embryonic stem (ES) cells were co-cultured with fibroblasts or hepatocytes by using the reversible sealing of the stencil on the substrate. In the dynamic case, ES cells were co-cultured with NIH-3T3 fibroblasts and AML12 hepatocytes sequentially by engineering the surface properties of the stencil. In this approach, the top surface of the parylene-C stencil was initially treated with hyaluronic acid (HA) to reduce non-specific cell adhesion. The stencil was then sealed on a substrate and seeded with ES cells which adhered to the underlying substrate through the holes in the membrane. To switch the surface properties of the parylene-C stencils to cell adhesive, collagen was deposited on the parylene-C surfaces. Subsequently, a second cell type was seeded on the parylene-C stencils to form a patterned co-culture. This group of cells was removed by peeling off the parylene-C stencils, which enabled the patterning of a third cell type. Although the static patterned co-culture approach has been demonstrated previously with a variety of methods, layer-by-layer modification of microfabricated parylene-C stencils enables dynamic patterning of multiple cell types in sequence. Thus, this method is a promising approach to engineering the complexity of cell–cell interactions in tissue culture in a spatially and temporally regulated manner.

Introduction

Most cell processes are controlled by the cellular microenvironment comprised of adjacent cells, soluble factors and matrix components. The ability to engineer the complexity of the cellular microenvironment would be useful in the development of tissue engineered constructs and improved cell culture systems.[1,2] For example, stem cells differentiate based on a series of spatially and temporally regulated signals from the extracellular microenvironment.[3,4] To study these environmental cues, it may be beneficial to engineer systems in which the interaction of stem cells with other cells could be controlled temporally and spatially. Microscale technologies are a potentially powerful method of achieving this complexity as they can be used to engineer the cellular microenvironment with high reproducibility and resolution.[1]

Microscale approaches have been used to construct three-dimensional (3D) scaffolds,[5,6] controlled microbioreactors,[7,8] cell and protein arrays,[9] patterned co-cultures[10–12] and for studying cell-matrix[13,14] and cell-soluble factor interactions.[15] In particular, a number of studies have addressed the effect of homotypic and heterotypic cell–cell interactions by using patterned co-cultures.[10,16,17] Previously, stencils of PDMS[18] and parylene-C[19] were used to pattern cells type on a variety of substrates. Furthermore, patterned co-cultures have been generated by changing the surface properties from cell-repulsive to cell-adhesive by electroactive and thermally responsive polymers,[20,21] magnetic forces,[22] oxygen plasma treatment,[12] and layer-by-layer deposition of biomaterials.[11,23] Although these static co-cultures provide the heterotypic interactions with other support cell types, they do not replicate dynamic aspects of the *in vivo* environment. It is known that the dynamics of cell–cell interactions as regulated by embryonic morphogenesis and mechanical factors is a key regulator of cell fate decisions. Thus, the development of patterned co-culture techniques in which cell–cell interactions can be controlled in a dynamic manner is of importance. Recently, Hui and Bhatia have demonstrated the use of a microfabricated interdigitating system to control the dynamic

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interactions of various cell types.[24] Their system uses a microfabricated silicon platform to bring cells in close proximity to each other in a dynamic manner. Here we demonstrate an alternative method of fabricating dynamic co-cultures by using parylene-C microstencils that can be fabricated in a simple and cost effective manner to enable their widespread use in the biological community.

Parylene-C is an inert, non degradable and mechanically robust material that has been widely used as a coating in a variety of applications. For biomedical applications, parylene-C has been used for coating implantable devices and flexible probes. More recently, parylene-C has been used to fabricate microdevices such as neurocages[25] and microfluidic channels.[26] In addition, parylene-C has been used for micro-patterning applications to pattern antibodies,[27] lipid bilayers,[28,29] proteins,[30] and cells.[27,30]

One of the main advantages of parylene-C is that it is mechanically robust (Young's modulus of 3.2 GPa)[31] compared to poly(dimethylsiloxane) (PDMS) ($\sim$0.75 MPa).[32] Therefore, unlike other elastomer stencils it can be easily removed or attached to a surface without tearing. We have observed that parylene-C stencils can form reversible seals on hydrophobic surfaces, such as polystyrene and PDMS. In addition, these stencils were extremely robust and could be used for multiple patterning processes.[19] Moreover, our experiments have demonstrated that more cells adhere to parylene-C in comparison to PDMS.[33]

In this work, we describe two methods of creating patterned co-cultures using parylene-C microstencils such that cell–cell interactions can be controlled in a static or dynamic manner (Fig. 1). In the first approach, static cell co-cultures were created with embryonic stem (ES) cells and NIH-3T3 fibroblasts or AML12 hepatocytes by reversibly sealing the stencil on the substrate. Static patterned co-cultures have been previously demonstrated[12,19] and were generated here to show their difference from dynamic co-cultures. Dynamic co-cultures were created by seeding ES cells with fibroblasts and hepatocytes sequentially. In this approach, the top surface of the parylene-C stencil was initially treated with hyaluronic acid (HA) to reduce non-specific cell adhesion. The stencil was then placed onto a PDMS substrate and seeded with ES cells, which adhered to the underlying substrate through the holes in the membrane. To switch the surface properties of the parylene-C stencils to cell adhesive, collagen was deposited on the parylene-C stencil. Subsequently, a second cell type was seeded on the parylene-C stencils to form a patterned co-culture. This group of cells was removed by peeling off the parylene-C stencil to enable the patterning of a third cell type. This method is a promising approach in engineering the complexity of cell–cell interactions in tissue culture in a spatially and temporally regulated manner.

Materials and methods

Materials

All tissue culture media and serum were purchased from Gibco Invitrogen Corporation and cell lines were purchased from American Type Culture Collection. All chemicals were purchased from Sigma unless otherwise indicated. PDMS
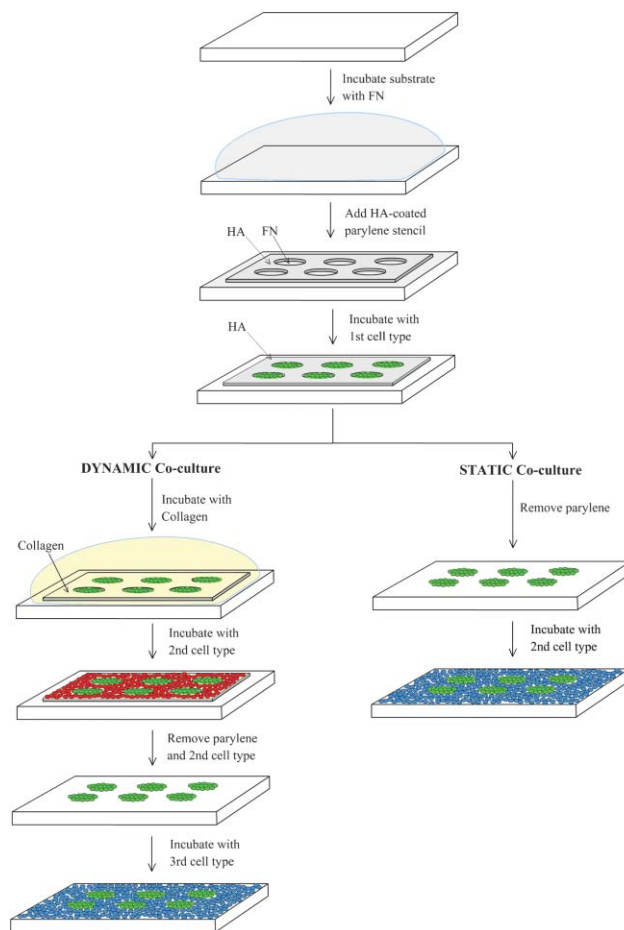

Fig. 1 Schematic diagram of the process used to generate static and dynamic co-cultures.

was purchased from Sylgard, Dow Corning. Collagen Type-1 Rat Tail (BD Biosciences) 500 µg mL^{-1}, Fibronectin (FN) 5 µg mL^{-1} and HA from rooster comb 5 mg mL^{-1} were prepared by diluting in sterile water.

PDMS mold fabrication

PDMS molds were fabricated by mixing silicone elastomer and a curing agent in a 10 : 1 ratio. The mixture was then degassed under vacuum until all the air bubbles were removed. The mixture was cured at 70 °C for 2 h. The PDMS was then cooled to room temperature, cut into pieces and then washed with ethanol prior to use.

Cell culture

All cells were manipulated under sterile tissue culture hoods and maintained in a 95% air/5% CO$_2$ humidified incubator at 37 °C. NIH-3T3 fibroblasts were maintained in 10% fetal bovine serum (FBS) in Dulbecco's modified eagle medium (DMEM). AML12 murine hepatocytes were maintained in a medium comprised of 90% of 1 : 1 [v/v] mixture of DMEM and Ham's F-12 medium with 5 µg mL^{-1} transferrin, 5 ng mL^{-1} selenium, 40 ng mL^{-1} dexamethasone and 10% FBS. Confluent flasks of NIH-3T3 and AML12 were fed every 3 to 4 days and passaged when 90% confluent. Mouse embryonic

stem cells (mES) (R1 strain) were maintained on gelatin treated dishes on a medium comprised of 15% ES qualified FBS in DMEM knockout medium. The mES cells were fed daily and passaged every 3 days at a subculture ratio of 1 : 4.

Preparation of parylene-C stencils

To fabricate parylene-C stencils, a thin film of parylene-C was deposited on silicon wafer using a PDS 2010 Labcoater 2 Parylene Deposition System (Specialty Coating Systems, Indianapolis, IN, USA). In this process, a 3 step deposition process was used, comprised of parylene vaporization, pyrolysis, and deposition. The conditions for vaporization were 150 °C and 1 Torr, during which the parylene-C dimer sublimed into a gaseous dimer form (di-*para*-xylylene). The dimer was next fed into a furnace (690 °C and 0.5 Torr) to generate the monomer (*para*-xylylene). The monomer in the deposition chamber (kept at 25 °C and 0.1 Torr) condensed on exposed surfaces and polymerized to form poly-*para*-xylylene. The thickness of the final parylene membrane is determined by the amount of dimer fed into the furnace; 1 gram of dimer adds 0.5 μm to the thickness of the parylene membrane. For our experiments, 10 μm-thick parylene stencils were used. The height was measured with a surface profilometer (Dektak 3ST, Sloan Technology) and varied only between 9.8 μm and 10 μm (data not shown). We found that thicker membranes (specifically, 20 μm) were less flexible and did not adhere to or detach from substrates with the same consistency.

To form patterns in the parylene membranes a 200 nm thick aluminium layer was initially deposited on the parylene-C layer to create a hard mask during the reactive ion etching. A thin photoresist (Shipley, S1813) layer was spun and exposed to define the patterns on the wafer (Quintel aligner). The aluminium mask was next etched in an aluminium etchant (PAN Etchant) at 50 °C for 30 s. The exposed parylene-C film was etched using dry etching in an inductively coupled plasma (ICP) reactive ion etching system (Plasmatherm 790) with O_2. Following this step, the aluminium mask was removed in the aluminium etchant at 50 °C for 2 min. Parylene-C was removed from the silicon wafer by cutting with a scalpel and lifting with fine edged tweezers. This simple removal is made possible by coating the silicon wafer with hexamethyldisilazane (HMDS) prior to parylene vapor deposition. Moreover, application of other adhesion promoters, such as A-174 silane, to the surface prior to parylene deposition is not recommended as it creates an extremely strong bond between the parylene-C and the substrate, and thus the parylene stencil tends to tear apart in multiple places during peeling. Further details of the parylene stencil fabrication and other applications can be found in our recent paper.[34]

Adsorption of HA on parylene-C surfaces

Fluorescein-conjugated HA (100 μg mL^{-1}) was incubated for 1 h on various substrates including glass, parylene-C, PDMS, and polystyrene, as well as plasma treated parylene-C (PT-parylene) and PDMS (PT-PDMS). Plasma treated surfaces were exposed to air plasma for 5 min (Harrick Inc.). All surfaces were rinsed with distilled water after HA incubation and visualized using the Nikon TE 2000U. Fluorescent intensity distribution was quantified using the NIH-Image J software.

Cell adhesion on parylene-C stencils

NIH-3T3 cells in the appropriate media in the density of ∼780 cells mm^{-2} were incubated on parylene-C and parylene-C coated with various biomolecules. After 6 h, the surfaces were washed with PBS and the attached cells were incubated in a solution containing the NIH-3T3 media and 1 μg mL^{-1} of DAPI for 45 minutes. Several images were taken at random using the Nikon TE 2000U camera and spot advanced software. The cells in the image were counted using the ImageJ software.

Cell staining

To visualize various cell types in patterned co-cultures, cells were stained with fluorescently labeled dyes and tracked in culture: carboxyfluorescein diacetate succinimidyl ester (CFSE, green), PKH26 (red), and Cell Tracker Blue (Molecular Probes). To stain cells with PKH26 dye, cells (2 × 10^7 cells mL^{-1}) were suspended in the diluent-C solution and mixed with 4 × 10^{-6} M PKH26 dye in a 1 mL of diluent-C solution and incubated at 25 °C for 5 minutes. To stain with CFSE dye, cells were suspended in 10 μg mL^{-1} CFSE in PBS solution at a concentration of 1 × 10^7 cells mL^{-1} and incubated for 10 min at room temperature. Both staining reactions were quenched with addition of an equal volume of DMEM supplemented with 10% FBS. For staining with Cell Tracker Blue the cells were centrifuged and then resuspended in the pre-warmed working solution and incubated for 15 to 30 minutes under growth conditions appropriate for the particular cell type.

Generation of static patterned co-cultures

To create static patterned co-cultures PDMS was sterilized with ethanol and then incubated with FN (5 μg mL^{-1}) for 45 min. Microfabricated parylene-C stencils were then placed on the PDMS and incubated with a suspension of mES cells (∼5000 cells mm^{-2}) for 6 h. The surfaces were then rinsed with PBS to remove non-adherent cells. Parylene-C stencils were gently peeled from the PDMS surface to create mES cell micropatterns. FN (5 μg mL^{-1}) was dispensed on top of the micropatterned mES cells and incubated for 20 minutes. AML12 hepatocytes were then seeded (∼5000 cells mm^{-2}) and incubated for 6 h. AML12 cells adhered to the FN coated PDMS to generate co-cultures of mES cells surrounded by AML12 cells (Fig. 1).

Generation of dynamic patterned co-cultures

To create dynamic patterned co-cultures, the top surface of a parylene-C stencil was incubated with HA for 1 h, washed, and reversibly sealed on a FN-coated PDMS substrate (FN at a concentration of 5 μg mL^{-1} coated for 45 minutes). mES cells (∼5000 cells mm^{-2}) were then seeded for 6 h on parylene-C stencils. Cells selectively adhered to the FN-coated PDMS substrate through the holes in the microfabricated stencil (since parylene-C membranes were non-adhesive to cells due to the HA coating). To prepare the parylene-C surface for

seeding of the second cell type, collagen (500 µg mL^{-1}) was deposited on the HA coatings and incubated for 20 minutes. AML12 hepatocytes or NIH-3T3 fibroblasts ($\sim$5000 cells mm^{-2}) were then seeded on top of parylene-C/PDMS constructs. The adhesion of the second cell type on the collagen-coated parylene-C stencils generated patterned co-cultures. To co-culture the mES cells with a second cell type, the surface was again washed with PBS and parylene-C was gently peeled from the PDMS substrate. mES cell micro-patterns were left behind as the other cells were removed. The resulting structure was subsequently treated with FN (5 µg mL^{-1} for 20 minutes) and the third cell type (NIH-3T3 or AML12 hepatocytes) was seeded on top of the mES micropatterns. The combination of these two co-cultures in sequence forms a dynamic co-culture (Fig. 1).

Results and discussion

One of the key components of fabricating patterned co-cultures is to be able to engineer the surface properties of microstencils. Previously, we have demonstrated that layer-by-layer self assembly of ionically charged biopolymers on HA coated surfaces can be used to reversibly change surface properties from cell repulsive to cell adhesive to form patterned co-cultures.[11,23] We hypothesized that by combining this approach with mechanically robust, microfabricated parylene-C stencils, patterned co-cultures could be generated with controlled spatial and temporal resolution. In our approach, static co-cultures can be fabricated by seeding primary cells in the open holes of the microstencils, and then seeding the support cells on the regions beneath the stencil once it has been removed. Alternatively, dynamic patterned co-cultures can be generated by seeding the primary cell type in the open holes of the stencils and seeding the support cells on the surface of the stencil. By removing the parylene-C stencil, the support cells can be removed while maintaining the primary cell type. Subsequently, a secondary support cell type can be co-cultured with the first cell type. The sequential order of these static co-cultures forms a dynamic co-culture in which ES cells are exposed to two different cell types

HA adsorption on parylene-C

To engineer the surface properties of parylene-C microstencils, we used layer-by-layer deposition of HA and collagen. We examined the adsorption of HA on parylene-C in comparison with other substrates. To examine the degree of HA adsorption, fluorescent HA was incubated on a variety of surfaces and the degree of fluorescence was analyzed. As shown in Fig. 2A, HA adsorbed to parylene-C at comparable levels to other commonly used substrates such as PDMS, glass, and polystyrene. In addition, consistent with previously published reports,[11,35] it was found that plasma treated substrates were more hydrophilic, and had increased HA adsorption compared to the untreated substrates. Specifically, contact angles of PDMS and parylene-C decrease from $\sim$110° and $\sim$75°, respectively, to <20° upon plasma treatment. In our case, plasma treatment of parylene-C increased the adsorption of HA nearly two fold ($p < 0.01$). This increase may be attributed to hydrogen bonding between the
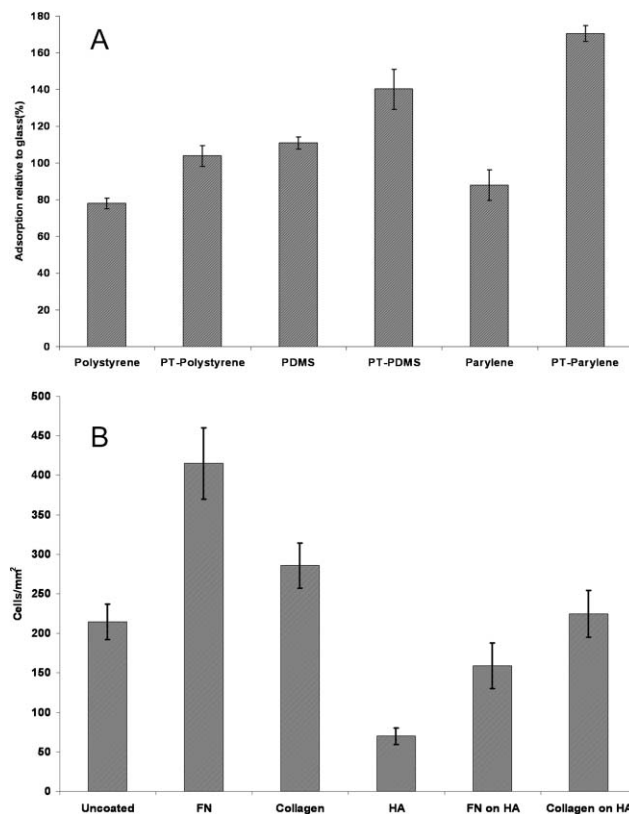

Fig. 2 (A) The adsorption of fluorescein HA on different substrates normalized to glass control (100%). HA generally adsorbs better to hydrophilic surfaces compared to the hydrophobic surfaces. Plasma treatment enhanced the hydrophilicity of the surfaces which increased the adsorption compared to the untreated substrates. (B) Cell adhesion on a parylene-C stencil coated with FN, HA and collagen.

hydrophilic moieties in HA with silanol, carboxylic acid or hydroxyl groups on the hydrophilic substrates.[36] It is noteworthy that previous experiments have found that HA films remained stable for at least 7 days in PBS.[36] However, it is believed that in the presence of cell culture medium and serum, the non-adhesive properties of HA films deteriorate as a result of interactions of HA molecules with the proteins in solution.[37] Yet, based on the short exposure time of HA films to the proteins in the media, the transient properties of these films are not expected to influence our results. To switch the surface properties of HA coated surfaces to cell adhesive, we used collagen coatings. Collagen has been found to adsorb on HA films and to switch the surface properties to cell adhesive despite its weakly cationic properties.[23]

Cell adhesion on surface modified parylene-C stencils

To examine cell adhesion on surface modified parylene-C stencils, NIH-3T3 cells were seeded onto various surfaces, and, after 6 hours, the number of adherent cells was counted. As shown in Fig. 2B, parylene-C surfaces that were coated with FN and collagen had improved cell adhesion properties while HA coated surfaces inhibited cell adhesion.[37–39] This is because surface immobilized HA can minimize protein mediated cell adhesion and non-specific protein adsorption due to its hydrophilic nature.[38] In addition, by adsorbing

collagen on HA, the surfaces were switched from cell-repellent to cell-adhesive. Collagen treatment on HA resulted in greater cell adhesion in comparison to FN on HA, which is consistent with previous reports.[23] The reason for the increased adsorption of the collagen may be attributed to the positive charge of the collagen.[23] The adsorption of the collagen over a HA coat plateau in ~2 minutes suggested little conformational change.[23] FN adsorbed over the HA undergoes a larger conformational change which affected its biological activity and decreased the cell adhesion.[40] Based on these results, we used collagen to change the HA-coated parylene-C stencils to cell adhesive.

Generation of static co-cultures

To generate static patterned co-cultures, a two step process was developed (Fig. 1). In the first step, a reversibly sealed parylene-C stencil was used to localize the primary cells to specific regions of a substrate; while in the second step the stencil was removed and the support cells were seeded in the regions surrounding the micropatterns of the primary cells. For the first step, we used microfabricated parylene-C stencils that contained holes ranging from 40 μm to 200 μm to generate cell micropatterns. These holes can be fabricated down to a diameter of 3 μm, with the diameter and cell type dictating approximately how many cells settle into each hole. For example, we were able to form a single-cell array of NIH-3T3 fibroblasts using 40 μm diameter holes. To demonstrate the applicability of the reversibly sealing parylene-C stencils for fabricating static patterned co-cultures, the stencil was placed on a FN coated substrate and seeded with the first cell type as shown in Fig. 3A. For the second step in the process, the surfaces were washed with PBS to remove non-adherent cells and the parylene-C stencil was gently removed, which revealed micropatterns of the primary cell type as displayed in Fig. 3B. The second cell type was next seeded on the substrate surface and adhered to the regions surrounding the first cell type (Fig. 3C). Because cues from surrounding cells influence cell behavior, cells in co-culture with support (i.e. feeder) cells better preserve their phenotype.[41,42] For example, hepatocytes co-cultured with fibroblasts have been shown to produce liver specific enzymes in proportion to the density of fibroblasts.[16] Thus, static co-cultures generated using this approach can be useful in providing tissue like environments for drug discovery assays and for improved tissue culture systems. Previously, PDMS stencils were used to generate patterned co-cultures;[18] however, since PDMS stencils are mechanically weak and difficult to handle, parylene-C stencils may be an improved technique to fabricate patterned co-cultures.

Generation of dynamic co-cultures

To generate dynamic patterned co-cultures, a parylene-C stencil, coated with HA on its top surface, was reversibly sealed on a FN treated PDMS slab (Fig. 1). mES cells seeded on the stencil/PDMS construct selectively adhered to the FN-coated PDMS substrate through the holes of the micropatterned parylene-C stencil. Non-adhered cells were removed by rinsing the surface with PBS. As seen in Fig. 4A, HA coating on the surface of the parylene-C stencil minimized cell
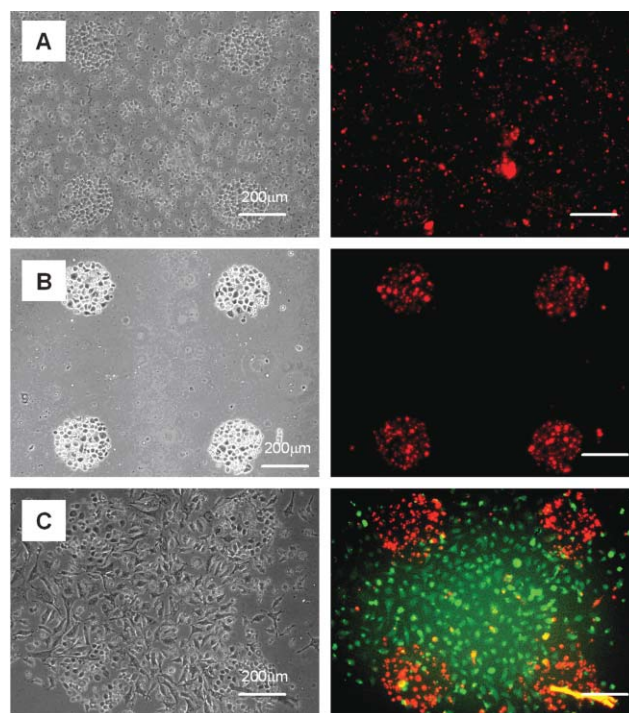

Fig. 3 Light micrograph (left) and the corresponding fluorescent (right) images of the steps in the formation of static co-cultures using parylene-C stencils. (A) The stencil was placed on a FN coated-PDMS surface and seeded with mES cells. (B) Upon removal of the parylene-C stencil a cell micropattern was formed. (C) Subsequently, AML12 cells were seeded on the exposed regions of the substrate.

adhesion. To switch the surface properties of the parylene-C stencil to cell adhesive, collagen (500 μg mL^{-1}) was deposited on the HA coated parylene-C stencil. A secondary cell type, such as AML12 hepatocytes, were then seeded on the stencil, which adhered to the collagen coated regions on the parylene-C stencil as displayed in Fig. 4B. To expose the primary cells to another cell type, the parylene-C stencil was gently peeled from the PDMS surface, leaving behind patterns of mES cells as shown in Fig. 4C. The underlying substrate was then coated with FN, and the third cell type, NIH-3T3 fibroblasts were then seeded around the mES cell micropatterns, as shown in Fig. 4D.

The precision of this dynamic co-culture method has not been quantified in this study; however, many aspects of it have been verified in previous research involving layer-by-layer deposition.[11,23] If the primary cell type has collagen-specific cell-surface receptors, collagen may be bound by the cells[43,44] during collagen incubation. However, this is unlikely to switch the non-cell adhesive property of the primary cell monolayer because the amount of collagen bound by these receptors is too small to allow integrins of the support cells to establish focal contacts. Nevertheless, independent of collagen concentration, adhesion of the second or third cell types on top of the primary cells was observed to a small extent. Although this may vary with the degree of contact-inhibition exhibited by the cell types in study, we do not anticipate this to be a limitation. In previous co-culture studies using collagen to control the pattern of two cell types, the secondary cells did not adhere
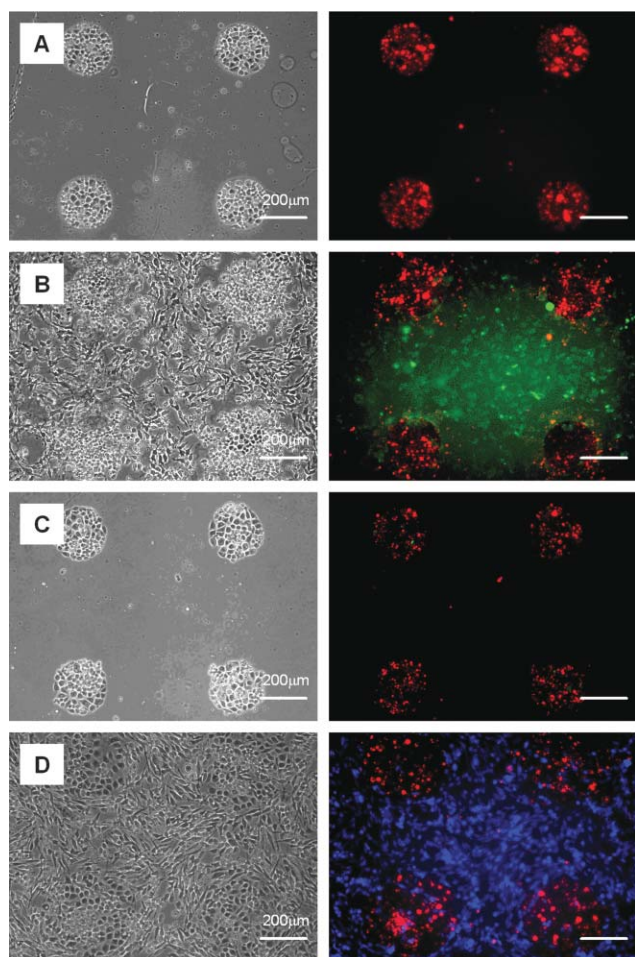
Fig. 4 Light micrograph (left) and the corresponding fluorescent (right) images of the steps in the formation of dynamic co-cultures using parylene-C stencils. (A) An HA coated parylene-C stencil was reversibly sealed on FN treated PDMS and seeded with mES cells. (B) The patterned co-cultures of mES cells and AML12 hepatocytes. (C) To generate dynamic co-cultures, the stencil was gently peeled away, leaving the mES cells. (D) After depositing a layer of FN, a third cell type (NIH-3T3) was seeded on the exposed surface.

on top of the primary cells so long as the primary cells had formed a confluent monolayer.[11,23,45,46]

The ability to generate dynamic co-cultures is potentially useful for studying stem cell differentiation and for generating improved tissue culture systems. For example, in the demonstrated system, mES cells interacted with a defined cell type for a particular period of time followed by exposure to another cell type. We have generated various dynamic co-cultures in various sequences using the three cell types NIH-3T3, AML12, mES cells. The interaction of the mES cells with the AML12 cells would likely produce changes in the mES cells at the molecular level. These conditioned mES cells might exhibit different behavior when exposed to the next cell type (NIH-3T3). Most of the interaction would be through paracrine signaling of soluble molecules such as lineage-specific growth factors. However, along the borders of the patterned regions, juxtacrine signaling will occur between cells in physical contact; this cell–cell contact between different cell types will occur to a greater extent after removal of the parylene-C stencil in

the dynamic co-culture when the two different cell monolayers are on the same plane. Furthermore, the duration of exposure to each cell type and the sequence of the cell types interacting with the mES cells can be varied, making the model a versatile tool in studying the dynamics of cell–cell interactions.

Growth and stability of the patterned cells and co-cultures

To analyze the stability of the cell micropatterns generated using parylene-C stencils, micropatterned mES cells, either alone or in co-culture, were tracked for 5 days. Initially, we analyzed the stability of mES cell micropatterns surrounded by HA coated surfaces. In these studies, the stencil was maintained on the surface and the media was replaced every day. As it can be seen from Fig. 5, micropatterned mES cells maintained their morphology for at least 3 days (Figs. 5A–C). However, the patterns degenerated by day 5 (Fig. 5D). These results are in agreement with HA coated surfaces generated on other polymer systems.[47]

The stability of co-cultures of mES cells with AML12 cells were also studied (Fig. 5E–H). It was found that mES cells displaced the surrounding AML12 cells over a period of 5 days. Although the pattern integrity was well maintained for 1 day
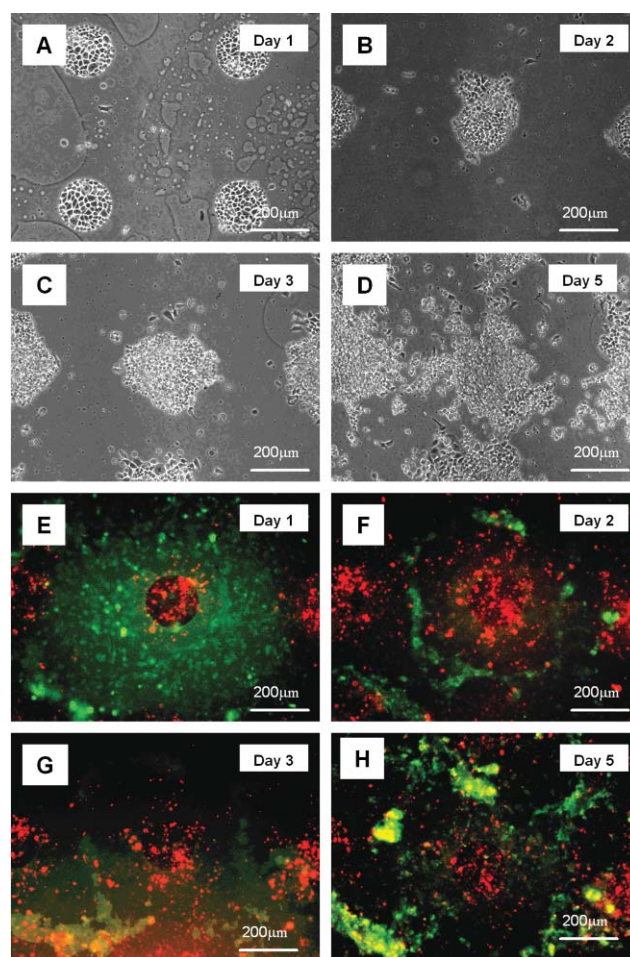

Fig. 5 The stability of mES cell patterns and mES/AML12 co-cultures. (A–D) The mES cell micropatterns degenerated over a period of 5 days. (E–H) In mES/AML12 co-cultures, the mES cells rapidly proliferated and spread to displace the surrounding AML12 cells.

after the initiation of the cultures, mES cells migrated to the surrounding parylene regions and removed the AML12 cells soon after. Cell growth inhibitors that may prevent proliferation are being investigated, but it is hypothesized that these will interfere with the experiments for which the technique was designed. The stability of micropatterns is a function of a number of parameters, such as the rate of proliferation, the mechanical strength of homotypic and heterotypic cell–cell interactions, and cell-substrate interactions. Therefore, we believe that the stability of the cultures will depend on the types of cells seeded, and their adhesion to each other and to the substrate. However, due to the nature of cell culture and proliferation, it is unlikely that this or any other spatially defined co-culture technique will be able to accommodate extended biological studies lasting longer than a week.

Our studies suggest that microfabricated parylene-C stencils are a potentially powerful method of fabricating patterned co-cultures. The mechanical stability and robustness, as well as the cell compatibility of these membranes make them suitable for cell culture and may be advantageous relative to PDMS stencils. In addition, the ability to fabricate and stack thin parylene-C stencils on each other can be used to generate dynamic co-cultures to control the dynamic interaction of more than three cell types by stacking multiple layers of stencils on each other, the removal of each can be used to control cell–cell interaction in a dynamic manner. Ongoing studies in our laboratory are focused on the fabrication and development of these stacked parylene-C layers, as well as the investigation of the biological processes controlling the dynamics of stem cell differentiation.

Conclusions

In conclusion, we have developed a method based on the use of mechanically robust, microfabricated parylene-C stencils to create patterned co-cultures using two approaches. In the first approach, static co-cultures were fabricated to control the degree of homotypic and heterotypic cell–cell interactions, while in the second approach a technique was generated to control the temporal sequence of the cell–cell interactions in patterned co-cultures. To our knowledge, this is the first report of using such microfabricated stencils for controlling the dynamics of patterned co-cultures. Thus, the use of microfabricated, biocompatible and mechanically robust stencils is a potentially versatile and inexpensive method of studying the degree as well as the dynamics of cell–cell interactions in tissue culture. These technologies provide new opportunities in tissue engineering, drug discovery and biological research.

Acknowledgements

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