

Microcontact Printing on Human Tissue for Retinal Cell Transplantation

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Objectives: To demonstrate that microcontact printing, a modern materials fabrication technique, can be used to engineer the surface of human tissue and to show that inhibitory molecules can be used to pattern the growth of retinal pigment epithelial cells or iris pigment epithelial cells on human lens capsule for transplantation.

Methods: Photolithographic techniques were used to fabricate photoresist-coated silicon substrates into molds. Poly(dimethylsiloxane) stamps for microcontact printing were made from these molds. The poly(dimethylsiloxane) stamps were then used to “wet-transfer” growth inhibitory molecules to the surface of prepared human lens capsules that were obtained during cataract surgery. Human retinal pigment epithelial and rabbit iris pigment epithelial cells were grown on a lens capsule substrate in the presence and absence of a patterned array of inhibitory factors.

Results: We found that human lens capsule could be microprinted with a precision similar to that obtained on

glass or synthetic polymers. Retinal pigment epithelial cells and iris pigment epithelial cells cultured onto an untreated lens capsule showed spreading and formed into fusiform-appearing cells. In contrast, cells cultured on a lens capsule with a hexagonal micropattern of growth inhibitory molecules retained an epithelioid form within the inhibitory hexagons.

Conclusion: Inhibitory growth molecules can be micropatterned onto human lens capsule, and these micropatterns can control the organization of retinal pigment epithelial cells or iris pigment epithelial cells cultured onto the lens capsule surface.

Clinical Relevance: Microprinting on autologous human tissue may facilitate efforts to effectively organize cell cultures and transplantations for the replacement of vital ocular tissues such as the retinal pigment epithelium in age-related macular degeneration.

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ONE OF the major biotechnological goals of modern medicine is to introduce transplanted cells into defective tissue in a way that will replace or renew lost function. Examples include replacement of articular cartilage in arthritis,¹ regeneration of skin where it has been lost,² and retinal pigment epithelial (RPE) and iris pigment epithelial (IPE) cell transplantation in retinas affected by age-related macular degeneration (AMD). In particular, the transplantation of RPE³ or IPE⁴ cells to rescue diseased photoreceptors is one of the leading experimental therapies for AMD, the most common form of blindness in industrialized nations among individuals older than 65 years.⁵ The injection of a suspension of epithelial cells into the subretinal space has been shown to rescue some photoreceptors for a limited period.⁶ However, simply injecting the pig-

mented cells into the subretinal space is unlikely to resolve AMD because the cells fail to form well-ordered monolayers, and they may arrange themselves into multilayers with random orientations and phenotypic variability.⁷

Cellular patterning technology such as microcontact printing (soft lithography) can be used to place a matrix of growth or inhibitory factors on a substrate to modulate the shape, function, proliferation, differentiation, and overall viability of cells grown on that substrate.^{8,9} However, work to date has applied microcontact printing only to glass or synthetic substrates such as poly (DL-lactic coglycolic acid) films¹⁰ or other polymers. These synthetic substrates work well for culturing cells in vitro but have potential limitations in clinical applications because of the risk of inflammation or immune response.⁷ Particularly, in physiologically sensitive areas, such as the space beneath the photoreceptor layer in

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the retina, it may prove important to use autologous material. Thus, we sought to determine whether microcontact printing on an autologous human lens capsule would be feasible, and whether it could control the growth of native RPE and IPE cells.

METHODS

LENS CAPSULES

Human lens capsules were obtained following an anterior capsulorrhexis during cataract surgery under institutional review board approval. The capsules were stored in a phosphate-buffered saline solution at 4°C, and then soaked in a 0.05% trypsin-EDTA solution for 1 hour prior to use to remove native lens epithelial cells, followed by soaking for 30 minutes in a penicillin-streptomycin solution (6.25 mg/mL of penicillin; 10 mg/mL of streptomycin) to prevent bacterial contamination. All reagents were obtained from Life Technologies Incorporated, Rockville, Md. To prevent the lens capsules from curling up, they were carefully spread out on sterilized plastic coverglasses prior to use and then dried under a UV lamp for 3 hours. Rabbit lens capsules were obtained from New Zealand white and red rabbits during a lensectomy procedure and prepared as described earlier. The care of the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocol was approved by the Administrative Panel on Laboratory Animal Care at Stanford University, Stanford, Calif.

FABRICATION OF MICROCONTACT PRINTING STAMPS

Poly(dimethylsiloxane) (PDMS) stamps were prepared as described in the literature.⁸ Briefly, a chrome mask with the desired micrometer patterns was fabricated at the Stanford Nanofabrication Facility using conventional photolithography. The mask was used to pattern a 7- μ m-thick, photoresist-coated silicon wafer. Poly(dimethylsiloxane) in a 10:1 mixture of elastomer to curing agent (Sylgard 184; Dow Corning Corporation, Midland, Mich) was poured onto the patterned silicon wafer and cured at 100°C. After 1 hour, the PDMS stamp (mold) was removed from the patterned silicon wafer. Poly(dimethylsiloxane) stamps (1 cm²) were placed in a plasma cleaner-sterilizer (PDC-32G; Harrick Scientific Corporation, Ossining, NY) for 1 minute at 100 W to obtain a hydrophilic surface. To stamp the lens capsule surface, the PDMS stamp was placed carefully onto a thin layer of either 5% mucilage (Elmer's Products Incorporated, Columbus, Ohio) in distilled water or 2% polyvinyl alcohol (PVA) in distilled water. In both cases, the solution was supplemented with 0.1 mg/mL of fluorescein for imaging. Immediately after contact, the PDMS stamp was then removed from the thin layer of solution and placed in contact with the lens capsule to "wet transfer" the solution. A 40-g weight was placed on top of the stamp for 30 minutes after which the microprinted lens capsule was sterilized under a UV lamp prior to cell culture. After stamping, the lens capsule remained dry and adhered to the plastic coverglass.

CELL CULTURE

Human RPE cells (ARPE-19; American Type Culture Collection, Manassas, Va) were maintained in a combination of Dulbecco modified Eagle minimum essential medium and Ham F-12 nutrient mixture supplemented with 10% fetal bovine serum at 37°C in an atmosphere containing 6.5% carbon dioxide. The cells were removed from 100-mm tissue culture dishes using

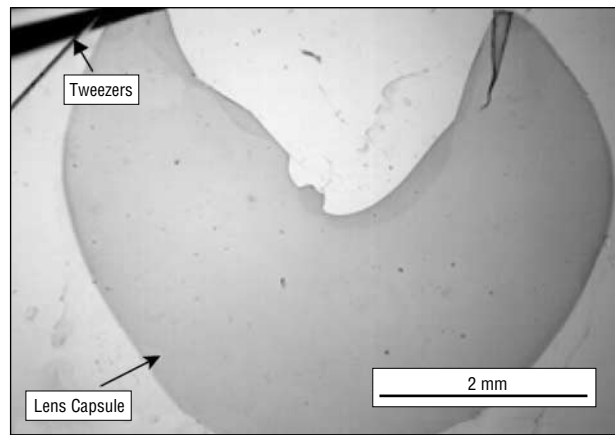


Figure 1. Light micrograph of human lens capsule stained with 0.04% trypan blue and dried onto a plastic coverglass. Surgical tweezers are visible in the top left corner.

a 0.05% trypsin-EDTA solution and were cultured weekly at a 1:10 ratio. Retinal pigment epithelial cells were cultured onto lens capsules at 1×10^6 cells/mL. Iris pigment epithelial cells were harvested and isolated from New Zealand white and red rabbits following the enzyme-assisted microdissection procedure described by Hu et al.¹¹ The primary culture was maintained in a Ham F-12 nutrient mixture with L-glutamine supplemented with 20% fetal bovine serum and 50 μ g/mL of gentamicin sulfate. The media was changed every 3 days. Primary cultures of IPE cells were cultured onto lens capsules at 1.6×10^5 cells/mL. All cell culture reagents were obtained from Life Technologies Incorporated.

MICROSCOPY

Lens capsule (**Figure 1**) was imaged with a digital camera (Cool Pix 900; Nikon Corporation, Tokyo, Japan) attached to an inverted microscope. All fluorescently stamped lens capsules were imaged using an inverted microscope (Eclipse TE300; Nikon Corporation) with a xenon light source (75 W) and a digital camera (Orca-ER; Hamamatsu Corporation, Bridgewater, NJ). Both patterned and unpatterned cells were imaged on the inverted microscope using a Hoffman modulation contrast condenser. Scanning electron microscopic (SEM) images were obtained by fixing the cells in 5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4 for 3 hours. They were washed in sodium cacodylate (0.1M, pH 7.4) and postfixed in 1% osmium tetroxide for 3 hours at 4°C and then dehydrated. Thick sections (1 μ m) for light microscopy were cut on an ultramicrotome and stained with toluidine blue. Thin sections (150 nm) were prepared from selected areas and stained with uranyl acetate-lead citrate for transmission electron microscopy. All electron microscopy materials were from EM Sciences, Fort Washington, Pa.

RESULTS

Figure 1 shows a typical piece of lens capsule that can be obtained after a routine anterior capsulorrhexis as part of a cataract surgery on a human. Microcontact printing onto native human lens capsule is difficult because the lens capsule is a delicate membrane that tends to curl at the edges and roll up. To resolve this problem, we flattened the lens capsules onto plastic coverglasses and dried them in a sterile environment; this produced smooth, flat surfaces for microcontact printing.

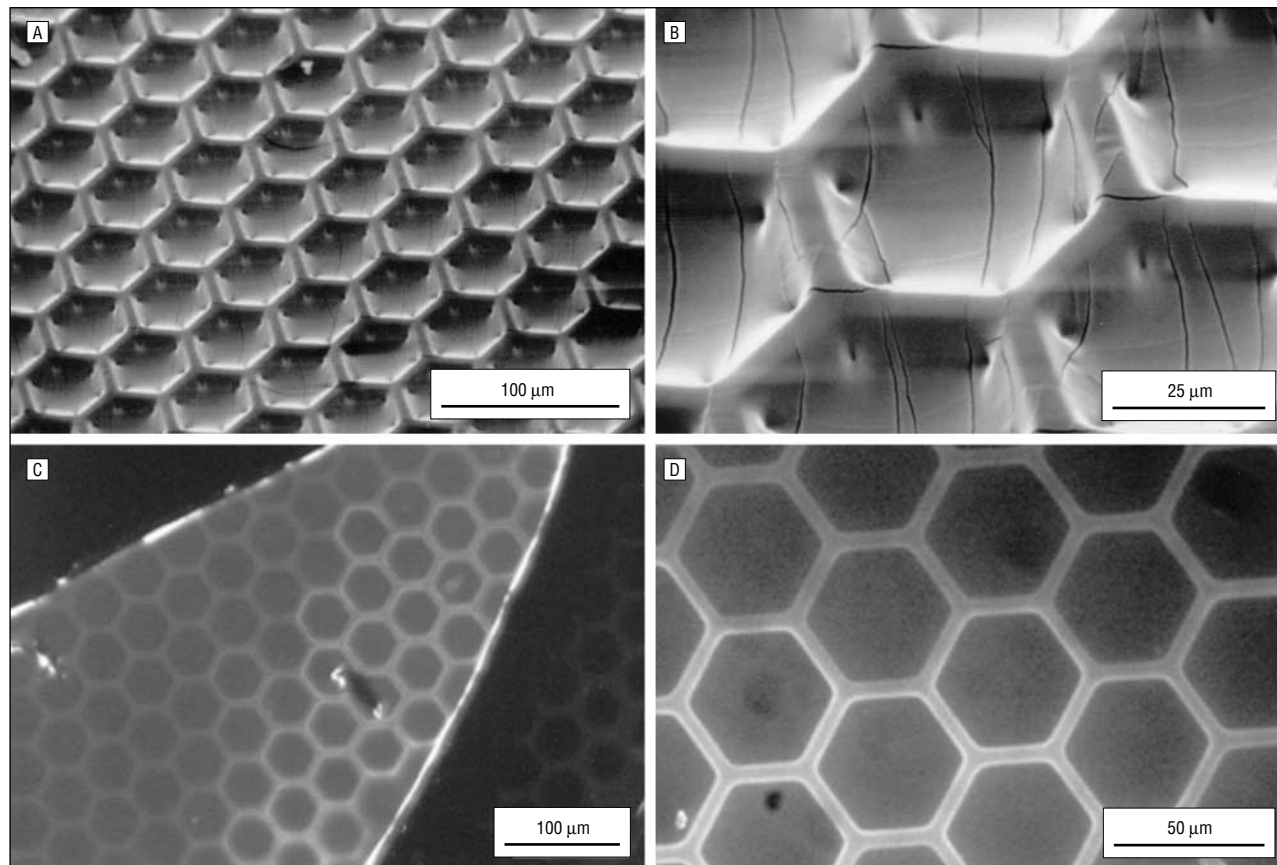


Figure 2. A and B, Scanning electron microscopic view of a poly(dimethylsiloxane) stamp of a hexagonal grid with hexagons with 50- μm diameters and 5- μm widths of the printed line. The structural height of the poly(dimethylsiloxane) stamp is 7 μm from the base (at the dimples) to face. The cracks are an artifact of preparation for scanning electron microscopy. C and D, Fluorescent images of a human lens capsule printed using this poly(dimethylsiloxane) stamp with a combined mixture of 2% polyvinyl alcohol and 0.1 mg/mL of fluorescein. The bright areas show microprinting of the fluorescein solution.

One use for microcontact printing has been to pattern extracellular matrix molecules to promote cell attachment to inorganic surfaces, like glass, that by themselves do not allow cell growth. Since native lens capsule naturally promotes epithelial cell growth, our goal with microcontact printing was to create a pattern of inhibitory molecules that would limit unwanted spreading, growth, or attachment of cells. Polyvinyl alcohol is a biocompatible polymer that can inhibit cell attachment by producing a barrier to cell-adhesion proteins. When we coated portions of lens capsule with PVA, we found that it attached firmly to the surface of lens capsule without any additional chemical linkages, and cells did not adhere to the coated areas.

An example of microprinting onto the human lens capsule is illustrated in **Figure 2**. Figure 2A and B show an elastomeric microcontact printing stamp fabricated from PDMS with a relief pattern of hexagons with 50- μm diameters. The hexagonal shape was designed to mimic that of RPE cells in the human macula. Figure 2C and D show human lens capsule microstamped with a hexagonal array of PVA using this stamp.

Figure 3 and **Figure 4** compare the behavior of epithelial cells cultured onto human lens capsule with and without a microprinted, 25- μm -diameter, hexagonal pattern of PVA. Figure 3A shows a culture of primary rabbit IPE cells on unpatterned rabbit lens cap-

sule, and Figure 3B shows an SEM of human RPE cells on unpatterned human capsule. In the absence of any inhibitory pattern, both RPE and IPE cells grow in irregular and uncontrolled directions and tend to spread and flatten out (Figure 3A and B). In contrast, RPE (Figure 4A) and IPE cells grown on lens capsule that had been microprinted with a hexagonal array of PVA stayed confined within each hexagon and retained a globular or epithelioid shape. The SEM and transmission electron microscopy images of RPE cells cultured in this way (Figure 4B and C) show that they are cuboidal, conform to the hexagonal pattern, and have greater microvilli than the unpatterned cells. To evaluate the health of the cultured cells on micropatterned lens capsule (Figure 4C), we examined the cells with transmission electron microscopy after 14 days. We observed ultrastructural features that are consistent with healthy and viable cells, including an intact plasma membrane, a uniform nuclear membrane with nucleopores, polysomes, intact mitochondria, as well as an extracellular matrix at the basal interface between the cell and the underlying lens capsule.

It is also important to examine how long microprinted PVA will maintain adherence to autologous tissue. We found that RPE cells on microprinted lens capsule after 24 days in culture maintained the same precise pattern after this 24 days, were still viable, and retained their cuboidal structure.

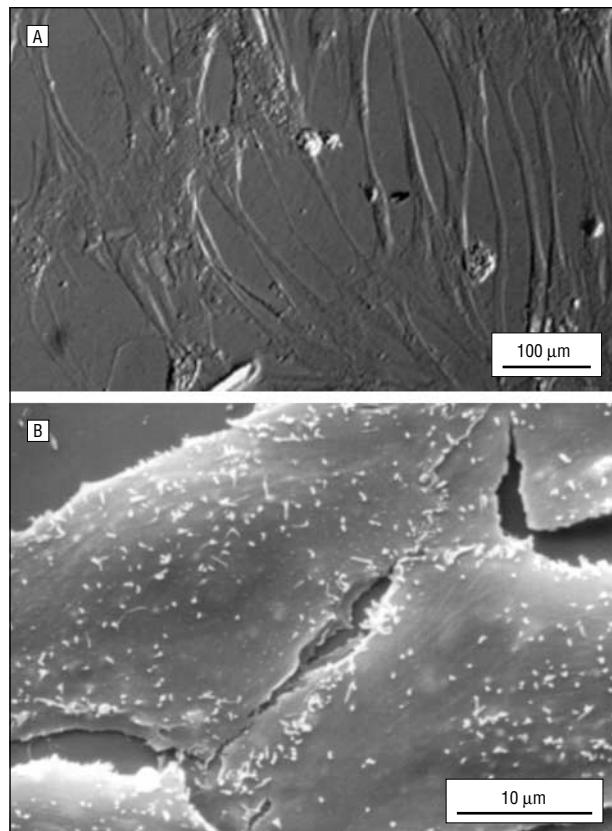


Figure 3. Retinal pigment epithelial cells and iris pigment epithelial cells grown on unpatterned native lens capsule. A, Hoffman modulation contrast micrograph of an unmodified rabbit lens capsule with rabbit iris pigment epithelial cells cultured for 7 days. Similar results were found when the iris pigment epithelial cells were cultured onto a human lens capsule. B, Scanning electron microscopic view of retinal pigment epithelial cells after 2 days of culture on unmodified human lens capsule.

COMMENT

Retinal pigment epithelial and IPE cell transplantation have emerged as important new therapeutic options for AMD because of the damage to the well-ordered RPE cell layer.⁷ The Bruch membrane, the 2- μm -thick basement membrane of both the RPE and choriocapillaris, is also diseased in AMD.¹² It has been suggested that diseased Bruch membrane prevents epithelioid growth of cells and, thus, may limit proper reattachment of transplanted RPE or IPE cells.¹³ Moreover, Bruch membrane becomes thickened and cracked, eventually allowing the in-growth of neovascular vessels.¹⁴

To address these challenges, transplantation of the RPE cells on biological and synthetic substrates including lens capsules and biopolymers has been proposed. Of these, human lens capsule has emerged as an excellent candidate for subretinal transplantation because it is a readily obtainable autologous tissue that already functions as an endogenous basement membrane for lens epithelial cells.^{15,16} It consists mainly of large glycoproteins including collagen type IV, laminin, and fibronectin.¹⁷ Even though lens capsule is the thickest basement membrane in the body, it measures only approximately 15 μm in thickness at its anterior pole.¹⁸ However, as shown by our data and by others, one problem with all growth fa-

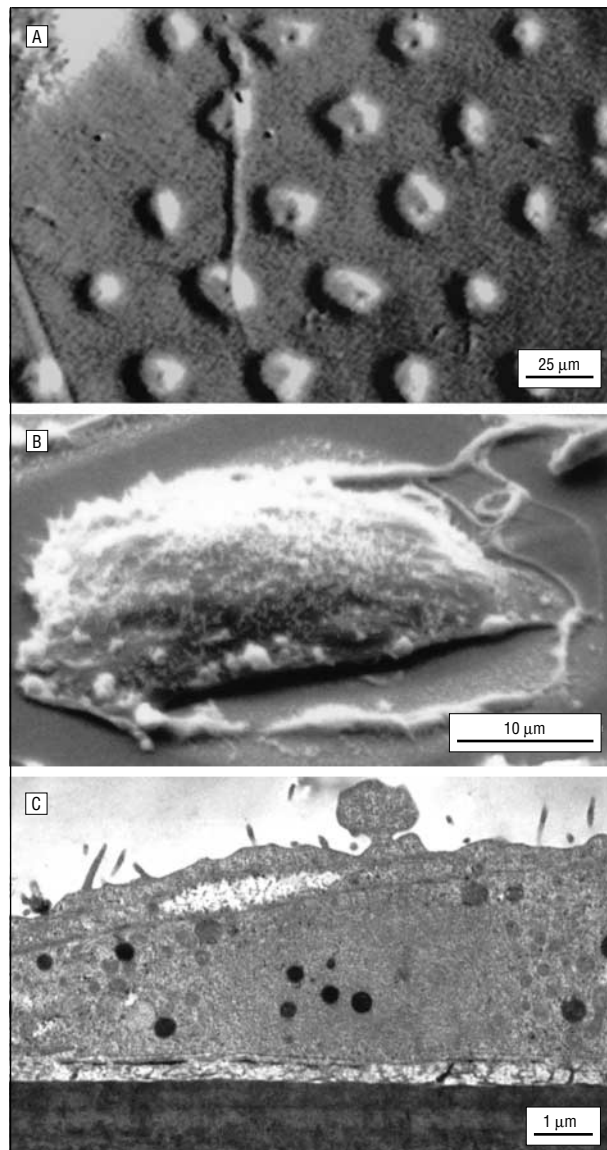


Figure 4. Retinal pigment epithelial cells grown on micropatterned lens capsules. A, Photomicrograph of retinal pigment epithelial cells cultured for 1 day on a human lens capsule stamped with a micropattern of polyvinyl alcohol in a 25- μm array. Cells remained viable and in this pattern formation for as long as 24 days. B, Scanning electron microscopic view of a micropatterned retinal pigment epithelial cell on a human lens capsule after 14 days in culture. C, Transmission electron microscopic view of retinal pigment epithelial cells cultured for 14 days on a hexagonally patterned lens capsule (original magnification $\times 30000$). In this figure, 2 cells are patterned within the hexagon and the junction between them is visible in the photomicrograph.

cilitating substrates is the uncontrolled nature of cell growth and cell differentiation. Several groups have found that unpatterned cells not only take on fusiform shapes but often lose polarity, phenotype, and differentiated features.¹⁶ We believe that microcontact printing might resolve this problem by preventing uncontrolled cell growth and directing a precise organization of cells on the substrate. Microcontact printing is a powerful microfabrication method that can engineer a surface with chemically active patterns or topologic features with sizes ranging 30 nm to 100 μm .⁸

Although microcontact printing has been described in the literature for glass and polymer surfaces,⁸

to our knowledge, its use on intrinsic biological materials had not been demonstrated previously. We show that microcontact printing can be performed successfully on autologous human tissue. Moreover, RPE and IPE cells plated on a lens capsule that has first been microprinted with PVA (Figure 4A) stay discretely apart on initial cell attachment and remain in these precise formations for as long as 4 weeks in culture. We are working toward modifying our PDMS stamping patterns and the longevity of the inhibitors to guide the arrangement of cells to form eventually a transporting epithelial layer. Others have shown that with certain cell lines, it is possible to form a tight layer of epithelial cells in culture by depositing large numbers of cells and growing them in a low calcium medium¹⁹ that inhibits cell-cell and cell-substrate spreading. However, a low calcium medium can be damaging to cells, and large numbers of cells may not be readily available in clinical situations such as the harvesting of IPE cells from an iridectomy sample¹¹ or the separation of RPE cells from an autologous peripheral biopsy specimen.²⁰⁻²² Microcontact printing, in contrast, would allow epithelial cultures to be grown from a limited sample of these cells while maintaining native phenotype and allowing great flexibility and reproducibility in defining cell size and shape.

CONCLUSIONS

We have shown that the surface of autologous human tissue (lens capsule) can be engineered by microcontact printing to serve as a replacement basement membrane for attaching and organizing cultures of epithelial cells. The use of autologous tissue, such as a patient's own anterior lens capsule, offers several advantages over synthetic culture substrates because it can safely be implanted in delicate areas such as the subretinal space without inducing an immune rejection.¹⁶ Our group is investigating these micropatterned membranes for transplantation of RPE and IPE cells as a treatment for AMD; however, microprinted human tissue such as lens capsule or other biological membranes may prove applicable as a universal membrane substrate for other ocular diseases or in tissue engineering applications elsewhere in the body.

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