Separation of distinct adhesion complexes and associated cytoskeleton by a micro-stencil-printing method

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Abbreviations: BPAG1e, bullous pemphigoid antigen 1e; CAD, computer-aided design; DABCO, 1,4-diazabicyclo[2.2.2]octane; DMEM, Dulbecco’s modified Eagle medium; FAK, focal adhesion kinase; μCP, microcontact printing; PBS, phosphate buffer saline; PDMS, poly(dimethylsiloxane); UV, ultra-violet

Adhesion between cells and the extracellular matrix is mediated by different types of transmembraneous proteins. Their associations to specific partners lead to the assembly of contacts such as focal adhesions and hemidesmosomes. The spatial overlap between both contacts within cells has however limited the study of each type of contact. Here we show that with “stampcils” focal contacts and hemidesmosomes can be spatially separated: cells are plated within the cavities of a stencil and the grids of the stencil serve as stamps for grafting an extracellular matrix protein—fibronectin. Cells engage new contacts on stamped zones leading to the segregation of adhesions and their associated cytoskeletons, i.e., actin and intermediate filaments of keratins. This new method should provide new insights into cell contacts compositions and dynamics.

Introduction

Separation of phases in soft matter physics is often studied for characterizing systems.1-3 It involves a variety of approaches, ranging from lipid chemistry to statistical mechanics. Beyond the understanding of the dynamics of polymers and lipids at the mesoscopic scale, these studies allow to isolate zones within vesicles.4-6 Mammalian cells can be viewed in a similar manner: they exhibit structures which are entangled, and this can lead to limitations in understanding the nucleation and growth of the associated structures. Here we applied these ideas on cells and we successfully separated two adhesion structures: “focal adhesions” and “hemidesmosomes,” and their associated cytoskeletons, which are naturally strongly entangled. For that purpose, we promoted cell motility and forced cells to engage motion on specific adhesions in the explored zone. The scope of this study is to show the separation of entangled structures while opening a new way for characterizing their molecular components and signaling pathways.

We now outline briefly basic elements of cell adhesion to present the rationale for the separation. Cells engage adhesion with their environment by assembling contacts.7 Their areas are typically about few micrometers squared, they nucleate, grow and disassemble rapidly within minutes. They consist schematically of three layers, (1) the extracellular matrix protein layer, (2) a specific adhesion complex of transmembraneous proteins and associated cytoplasm proteic partners and (3) a specific cytoskeleton. For example, focal contacts are composed of (1) fibronectin, (2) integrins associated with vinculin, paxillin, FAK, etc and (3) the actin stress fibers.8,9 Likewise, hemidesmosomes are structured of (1) laminin, (2) integrin associated with plectin and BPAG1e and (3) the intermediate filaments.10 Generally, it is the specific binding of an extracellular matrix protein to a transmembraneous adhesion complex which dictates the type of the adhesion contacts.

These distinct types of contacts do often co-localize. This spatial feature hinders their thorough characterizations, which makes it difficult to distinguish their respective components. In addition, this overlap most likely generates some interplay between proteins of the contacts and the associated cytoskeletons; signaling pathways can be also entangled for the same reasons. It seems therefore important to design an assay for disentangling both contacts spatially in order to be able to dissect their specificity.

For this purpose, it is needed to trigger cell motility on a new zone of the surface potentially allowing one type of contact to be assembled, i.e., focal contacts—and not hemidesmosomes—for promoting motion. Imposing a cell to go in a given direction was
The fibronectin solution Fig. illustrates the cell monolayer embedded inside a wall of fibronectin. We propose the so-called wound healing assay was designed to trigger such motion. A pipette or a syringe "scratches" a cell monolayer thus removing some rows of cells. Cells on both sides of the wound get polarized, they start to migrate to close the space devoid of cells. This approach has allowed to unravel key features of cell motility.

The scratch has however some limitations. Cells are detached in a non-controlled manner which potentially generates differences in results from experiments to experiments and between laboratories. In addition, the first row of migrating cells is damaged during the procedure. Also, the instrument can affect the surface in several ways: it can remove the extracellular matrix protein layer deposited by the cells or by the experimentalists on the scratched region; it can also generate some trenches within the surface by engraving the plastic or glass coverslip; this can also affect the migration.

An alternative method to wound healing was designed to remove cells in a controlled manner: the stencil consists in placing cells in holes surrounded by microfabricated structures. By removing the structure, cells can migrate, thus mimicking the traditional wound healing method. However, cells migrate on a surface depleted from a specific coating; it is then probably covered non-specifically by proteins of the serum after removal of the structure.

Microcontact printing (µCP) allows the controlled patterning of biomolecules on which cells can be deposited. We propose to combine the stencil approach together with the stamping method. Cells are placed into the holes of the stencil while the lower side of the stencil itself stamps proteins of the extracellular matrix, here fibronectin, in order to promote focal contacts formation and thereby cell motility. Because of its dual role, we name stampcil the microfabricated tool. When the stampcil is removed, cells start migrating on a surface coated with fibronectin. We are able to show that focal contacts are then separated from hemidesmosomes, and their associated cytoskeletons are also segregated.

Results and Discussion

We illustrate the preparation of the stampcil on Figure 1. Briefly a master is prepared to generate a grid of typical dimensions with cavities of 900 × 900 × 200 μm³ in volume separated by walls of 100 μm in width. This particular width was selected because it matches the width of a pipette-induced wound on a confluent cell monolayer. Liquid PDMS is poured onto the template and pressing; it is then probably covered non-specifically by proteins of the serum after removal of the structure.

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Figure 1. Schematic of the stampcil fabrication procedure: (A) SU-8 mold; (B) pouring of PDMS onto the template and pressing; (C) curing and peeling-off of the stampcil; (D) inking with 10 μg ml⁻¹ fibronectin (see Fig. S1); (E) stamping onto the surface; (F) incubating with cells; (G) releasing of the stampcil.
in migrating cells. It is worth noting that this approach is distinct from experiments where adhesions such as focal contacts and adherens junctions are separated; our contacts are naturally physically mixed and we triggered their separations through a new approach combining induced motility and specific adhesions. In addition, other approaches would allow the separation of entangled contacts by suppressing contacts targeting integrins through siRNA or by blocking contact formation through antibodies. However, such approaches would not promote a local control provided by our stampcil approach, which allows normal entanglement within cells.

In addition, this approach could be extended to any combinations of contact types, because it relies on the proven formation of biological organelles upon stamping of a variety of proteins. The key feature is just to ink the stampcil with a specific protein of the extracellular matrix and to match the cell type so that the expected contacts can be formed. This could also work for studying adherens junctions by inking cadherins on the stampcils: it would allow us to study the interplay between focal contacts and cell-cell contacts on planar surfaces. Other developments such as imposing a topography on the stamped regions of the stampcil could generate also constraints on the migration with potential applications. Further studies will demonstrate the extent of general applicability of our method. Finally, because the mechanism of segregation is based on a specific ligand-receptor recognition, the separation of adhesions and cytoskeletons should be observed on any cell lines.

If phase separation in vesicles is often associated with interaction energies between lipids, segregation of adhesion structures relies on other rules. The binding of the ligands to the associated receptors dictates the nature of the future cellular structure. Our study illustrates this rule: cells migrate and the exclusive binding of integrin to fibronectin triggers only one type of contacts to be assembled. The induced migration is needed since these cells are not migrating without free space being generated (either by scratch or by the stampcil removal), and the saturation of the new zone under exploration allows unique assembly of the cellular structure. We anticipate that such simple rules for phase separation in cells should have broad applications in a variety of situations in vivo and in vitro.

**Materials and Methods**

**Chemicals and reagents.** Poly(dimethylsiloxane) (PDMS) (Sylgard 184) was obtained from Dow Corning; SU-8 2025 and SU-8 developer were purchased from Microchem Corp.

HaCaT cells (obtained from the German Cancer Research center, DKFZ) were cultivated in DMEM 10% fetal calf serum supplemented with gentamicin (40 μg ml⁻¹). The following reagents were used: anti-integrin α6 (clone GoH3, BD Bioscience PharMingen), anti-vinculin (clone VIN-11-5, Sigma-Aldrich), anti-paxillin (clone Z035, Zymed), anti-keratin14 (Convance), phalloidin Alexa Fluor 488 (Molecular Probes), anti-laminin 332 (Epiligrin, clone P3H9, Chemicon) and fibronectin-rhodamine (Cytoskeleton). All secondary antibodies were obtained from Molecular Probes.

**Fabrication of the stampcil.** The stampcil consisted of an array of 10 × 10 square-shaped wells of 900 × 900 μm² in internal area with a separation between wells of 100 μm. It was fabricated using PDMS and standard soft-lithography techniques. First, a photolithography mask was designed with a CAD software (AutoCAD, Autodesk, Inc.) and purchased from Selba SA. A mold of SU-8 photoresist (MicroChem Corp.), 200 μm in height, was fabricated by first spin-coating SU-8 photoresist onto a silicon wafer (Siltronix) and patterned by UV exposure (MJBi contact mask aligner; SUSS MicroTec). Finally, the mold was developed by using SU-8 developer (MicroChem Corp.) (Fig. 1A). Then, a 1:10 (w/w) mixture of cross-linker and Sylgard 184 silicone elastomer (Dow Corning Corp) was degassed under vacuum and a small drop poured onto the SU-8 template. A thin film (125 μm) of polystyrene (Goodfellow) was placed onto the PDMS drop and carefully pressed against it in a custom-made four-screw metal press (Fig. 1B). Finally, the whole set-up was cured at 65°C for 4 h. The stampcil was then carefully peeled off (Figs. 1C and 2A) and UV-sterilized prior to use.

**Scratch assay.** Cells were seeded on a 12 mm coverslip. The confluent monolayer was scratched using a blunt glass microneedle. Cells were left to migrate for 2 to 4 h and fixed.

**Stampcil assay.** The stampcil was placed face-up onto a parafilm sheet and a PDMS frame matching its external dimensions was deposited on top of it thereby forming a cavity (see Fig. S1). The stamping side of the stampcil was then

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*Figure 2. (A) Optical image of the PDMS stampcil (scale bar: 1 mm). (B) Fibronectin network (in red) patterned with the stampcil. Scale bar: 500 μm. (C) Fluorescent image after releasing the stampcil of 1 cavity filled with cells and stained for actin (in green). Scale bar: 200 μm.*
incubated for 1 h with fibronectin-rhodamine (10 μg ml⁻¹) and dried for 30 min (Fig. 1D). Fibronectin was stamped on a 18 × 18 mm² coverslip placed into a Petri dish by applying pressure for 10 min (Figs. 1E and 2B).

Cells were then seeded into the cavities of the stampcil and incubated for 2 h at 37°C in 5% CO₂ (Fig. 1F). Medium was then added into the dish holding the coverslip and cells were left to grow for 2–3 d. Then, the stampcil was removed and the medium was replaced with fresh medium supplemented with 1% BSA. Cells were left to migrate for 1 h and fixed (Figs. 1G and 2C). Note that migration time was lower compared with the scratching assay, because in the latter case the first row of cells

**Figure 3.** The stampcil segregates the focal adhesions/hemidesmosomes adhesions and the actin/intermediate filaments cytoskeletons. (A) The stampcil and the HaCaT cells were removed. Laminin (green) was stained directly on the coverslip stamped with fibronectine-rhodamine (red). Scale bar: 20 μm. (B) HaCaT cells were left to migrate for 4 h after wounding the monolayer and then fixed and stained for hemidesmosomes (ITGA6, green) and focal adhesions (paxillin, red). Scale bar: 10 μm. (C) After removing the stampcil, cells were left to migrate for 1 h and then fixed and stained for hemidesmosomes (ITGA6, green) and focal adhesions (vinculin, red). Scale bar: 10 μm. The dashed line indicates the FN border. (D) After wounding the monolayer, cells were left to migrate for 4 h and then fixed and stained for actin (phalloidin, green) and intermediate filaments (keratin 14, red). Scale bar: 10 μm. (E) After removing the stampcil, cells were left to migrate for one hour fixed and stained for actin (phalloidin, green) and intermediate filaments (keratin 14, red). The dashed line indicates the fibronectin border. White arrowheads indicate actin (lower) and intermediate filaments (upper) segregation. Scale bar: 10 μm.
needed more time to recover from the wound and migrate through the layer of dead cells whereas cells started to migrate immediately after the stamp release. No major difference was observed between 1 h and 4 h when using both assays.

**Immunofluorescence.** Cells were fixed in paraformaldehyde 4% in PBS for 10 min, permeabilized with 0.2% Triton in PBS for 10 min and blocked with sodium borohydride (0.5 mg ml⁻¹) in PBS for 10 min. Treated cells on coverslips were then incubated for 1 h with primary antibodies, washed in PBS and incubated for 30 min with secondary antibodies. Finally, coverslips were washed and mounted in Mowiol-DABCO buffer. All images were acquired on a Leica TCS SP2 confocal microscope using a 63× NA 1.4 Plan Apochromat HCX oil objective controlled by the LCS software (Leica Microsystems).

**Conclusions**

Using the stamping method, we showed that a segregation of different types of contacts and the corresponding cytoskeletons can be induced. The grids of a stencil were used as stamps for grafting fibronectin on the surface. Keratinocytes were plated within its cavities and allowed to migrate on the fibronectin surface. A separation of distinct adhesion complexes (focal contacts and hemidesmosomes) and associated cytoskeleton (actin and intermediate filaments of keratins) was observed, in contrast with conventional cell migration methods, such as scratching-assay where no separation was observed. This micro-stencil printing method should provide new insights into cell-contact composition and dynamics. Future studies will be instrumental in assessing the benefit of using this controlled assay which trigger and control cell motility on controlled ligands.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Supplemental Material**

Supplemental materials may be found here: www.landesbioscience.com/journals/celladhesion/article/22198

**References**

1. Chaikin PM, Lubensky TC. Principles of Condensed Matter Physics. Cambridge: Cambridge University Press, 1995.
2. de Gennes P-G. Scaling Concepts in Polymer Physics. Ithaca: Cornell University Press, 1979.
3. Safian SA. Statistical Thermodynamics of Surfaces, Interfaces, and Membranes. Reading: Westview Press, 2003.
4. Li Y, Lipowsky R, Dimova R. Membrane nanotubes induced by aqueous phase separation and stabilized by spontaneous curvature. Proc Natl Acad Sci U S A 2011; 108:4731-6; PMID:21383120; http://dx.doi.org/10.1073/pnas.1015892108
5. Muldana HS, Chiang HH, Butler PJ. Tuning membrane phase separation using nonaplipid amphiphiles. Biophys J 2012; 102:489-97; PMID:22325271; http://dx.doi.org/10.1016/j.bpj.2012.03.033
6. Tsafrir I, Caspi Y, Guedeau-Boudeville M-A, Arzi T, Stavans J. Budding and tubulation in highly oblate membrane phase separation using nonaplipid amphiphiles. Phys Rev Lett 2003; 91:138102; PMID:14525338; http://dx.doi.org/10.1103/PhysRevLett.91.138102
7. Geiger B, Bershadsky A. Assembly and mechanosensing function of focal contacts. Curr Opin Cell Biol 2001; 13:584-92; PMID:11544027; http://dx.doi.org/10.1016/S0955-0674(00)00255-6
8. Kanchanawong P, Shtengel G, Pasapera AM, Ramko EB, Davidson MW, Hess HF, et al. Nanoscale membrane phase separation using nonaplipid amphiphiles. Biophys J 2009; 96:1261-9; PMID:19808122; http://dx.doi.org/10.1016/j.bpj.2008.08.045
9. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. Science 2003; 302:1704-9; PMID:14657486; http://dx.doi.org/10.1126/science.1092053
10. Alon A, Meerson B, Tu Y. Microstencils: a new tool for cell biology. Small 2009; 5:3012-22; PMID:19657379; http://dx.doi.org/10.1002/smll.200901163