Sticking It to Conventional Models;

Micropatterned hiPSC Culture as a Novel Approach for Studying Complex Molecular and Cellular Systems

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Abstract

Studying complex molecular and cellular systems in humans presents a crucial challenge that demands specialized models for visualization and experimentation. While human induced pluripotent stem cells (hiPSCs) offer a valuable tool due to their ability to maintain a pluripotent state and differentiate upon direction, they can yield irreproducible results. To overcome this limitation, better-controlled and more sophisticated hiPSC differentiation models are needed. One promising approach to the study of tissue-scale cell fate patterning involves pre-patterned geometrical confinement (Warmflash et al., 2014; Etoc et al, 2016). In this study, we aimed to test a modified methodology for producing hiPSC micropatterned adhesive islands to investigate complex molecular systems, specifically intermediate filament protein vimentin and the effects of molecular chaperones. We were able to construct two CRISPR-Cas9 plasmids that targeted the human vimentin gene and showed that upon transfection into HeLa cells, they induced a vimentin-null phenotype. We then successfully adapted the Warmflash et al. protocol to produce adhesive islands in HeLa and hiPSC which constrained the cells using pre-patterned geometric constraints. This is a crucial step in studying complex molecular systems and genetic manipulations in differentiating human cells. Our modifications also enhanced the cost-effectiveness of our model potentially making it more applicable to a teaching setting (CU MCDB Skills Center) . In the long term, this approach has the potential to shed light on the role(s) of vimentin and the effects of molecular chaperones on hiPSC differentiation and development.

Keywords: human induced pluripotent stem cells; adhesive islands; micropatterning; intermediate filament proteins; vimentin; differentiation; chaperones.

Introduction

Studying complex molecular and cellular systems in vitro is a challenge due to their dynamic nature, the artificial nature of the environment, and the unexpected effects of various cellular and environmental factors. A critical aspect is whether the cells being studied can be induced into carrying out the complex behaviors that occur in situ, that is, within an intact organism. Controlling the differentiation of cultured cells can be difficult. Additionally, visualization and quantification of molecular and cellular systems is challenging, often requiring more specialized conditions rather than simple culture of established cell lines. Human induced pluripotent stem cells (hiPSCs) offer a unique model due to their ability to self-renew indefinitely and maintain a pluripotent state (Warmflash et al., 2012). These cells offer an in vitro perspective on development, and also present significant potential for the use of cell-based therapies in regenerative medicine. However, traditional methods of hiPSC culture typically lack controlled spatial organization and often fail to accurately reproduce cellular interactions present in vivo. The result can be haphazard differentiation and unnatural heterogeneity. A promising approach to the study of tissue-scale cell fate patterning involves the use of pre-patterned geometrical confinement (Warmflash et al., 2014; Etoc et al, 2016). In this model, hiPSCs are compelled to adhere selectively to pre-patterned regions (adhesive islands) in culture thereby geometrically constraining colony size, shape, and location, as well as cell-cell interactions. Confining cells to adhesive islands leads to more reproducible patterns of induced cellular differentiation of hiPSC. It also imposes control over cell-cell interactions and cell-extracellular matrix interactions, improves cell viability compared to standard hiPSC conditions, and provides better defined culture conditions. These more stringent conditions make hiPSC micropatterned islands a superior model for studying many complex molecular and cellular systems.

Our initial interest in these novel models emerged while investigating functions and phenotypes associated with Vimentin (VIM), a type III intermediate filament protein. Interestingly, vimentin as well as other intermediate filament proteins (IFp) can be knocked out and still produce viable and fertile mice. However, vimentin null mutations are mostly intolerant in humans, potentially indicating an important role (Toivola et al., 2010) (Klymkowsky, 2019). In mouse, numerous phenotypes associated with IF

mutations can be rescued through treatment with molecular chaperones (Toivola et al., 2010) suggesting that some of the phenotypic effects in IF-null animals are due to secondary effects. Therefore, an important question arises: what are the equivalent (chaperone-rescueable) phenotypes in humans associated with vimentin null mutations? Addressing these questions requires a human model system where we can determine whether there are differences in development/differentiation between wild type and vimentin-null cells and if so, can treatment with molecular chaperones or over-expression of protein chaperoneαβ-crystallin can rescue these defects. hiPSCs offer a good model system for such studies due to their ability to maintain a pluripotent state and differentiate into various cell types when directed. However, under standard culture conditions, differentiation can be slow and inconsistent. Warmflash et al. developed an hiPSC system that facilitates quick and uniform differentiation, unlike standard culture or organoids that tend to develop slowly, similar to in vivo conditions (Warmflash et al., 2014). Constrained cultures are well-suited for studying complex human molecular systems, and controlled rapid differentiation enables us to more accurately observe the phenotypic effects of IFp mutations and molecular chaperones on development. The primary goal of this study was to recreate the micropatterned adhesive island hiPSC system described by Warmflash et al. to provide an improved model for studying complex molecular and cellular systems, with potential, in the future, to investigate functions and phenotypes associated with Vimentin and other intermediate filament proteins. To achieve this goal, I had to master and troubleshoot multiple techniques including knowledge of standard hiPSC culture, the complex intricacies of constructing adhesive island surfaces, transfection, and immunofluorescence microscopy. Given the multitude of skills involved, this process makes a potentially useful skills set for adoption by the CU MCDB Skills Center.

Materials and Methods

PDMS Pattern Transfer of Micropillar Arrays

Connor Miksh of the Kristi S. Anseth Lab (University of Colorado Boulder) performed the PDMS pattern transfer of micropillar arrays to produce our PDMS micropatterned stamps. A micropatterned (micropillar) stamp was fabricated using a 500μm diameter x 500 μm spacing x 500 μm tall silicone micropillar array purchased from RMS as a master template (*Research Micro Stamps*, n.d.). The master template was plasma treated (Plasma Etch, PE-25) with 10cc/min of pure oxygen at 200.3 mTorr for 60 sec at 100W to remove contaminants. Following plasma treatment, the master template was surface-treated with a chemical vapor deposition of 5 μL Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma) in a vacuum chamber for 2 hours to prevent it from sticking to the PDMS negative mold. Lastly, the master was washed with deionized water to remove unbound Trichloro(1H,1H,2H,2H-perfluorooctyl)silane.

A 5:1 ratio of polydimethylsiloxane (PDMS) and its curing agent (Sylgard 184) were combined and degassed in a vacuum chamber for 10 min. A 6-well glass bottom plate (Cell-Vis) was plasma treated in the same manner as the master template. 3mL of the mixed PDMS was added to one well of the 6-well plate and the surface-treated master was placed feature side down (facing glass bottom) in the PDMS-filled well and was cured at 60℃ for four hours (Fig. 1A). After curing, the master was removed from the well leaving a negative pattern template within the well (Fig. 1B).

The PDMS negative within the 6-well was plasma treated to decontaminate the surface. It was also surface treated by the chemical vapor deposition of 10 μL Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma) in a vacuum chamber for 2 hours. A 5:1 ratio of polydimethylsiloxane (PDMS) and its curing agent (Sylgard 184) were combined and degassed in a vacuum chamber for 10 min and was added to the negative pattern template. The PDMS was cured at 60℃ for four hours. After curing, the pattern template was cut and removed resulting in the positive pattern (final stamp).

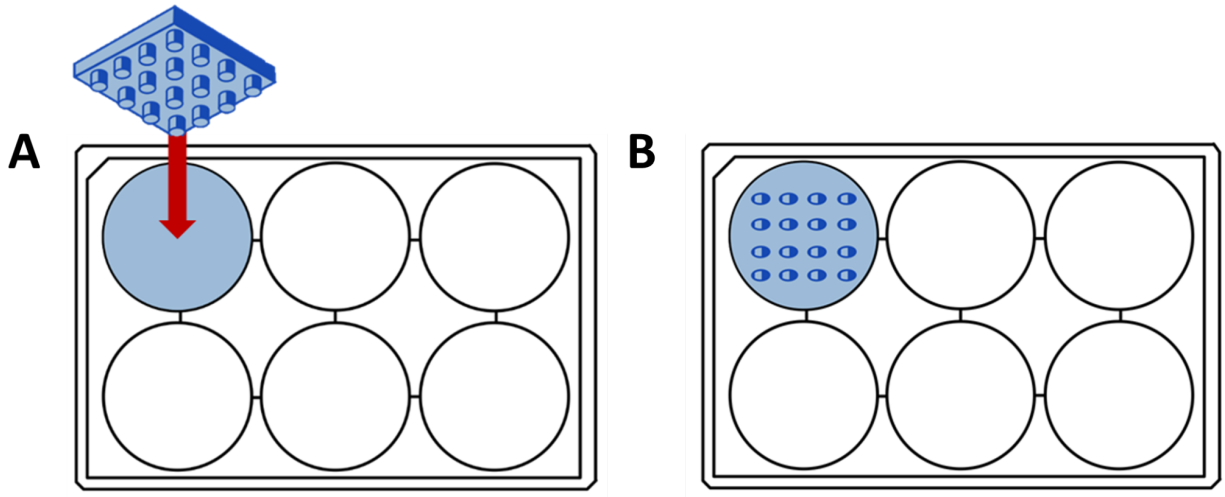

Figure 1. Casting PDMS Negative Patterns from the Master Template. (**A**) The negative pattern constructed to produce multiple PDMS stamps was made using the surface-treated/ plasma-treated silicone master stamp template and a plasma-treated glass-bottomed 6-well culture plate (Cell-Vis). The template was placed feature-side-down into a well containing a degassed mixture of polydimethylsiloxane (PDMS) and its curing agent (Sylgard 184) and cured at 60℃ for four hours. (**B**) After curing, the master template was cut and removed from the PDMS mold resulting in a negative pattern for stamp replication.

Glass Micropatterning

PDMS stamps were sonicated in 70% EtOH, then dried under ultraviolet (UV) in the BSC. Adhesive tape was placed on the feature side of the stamp to remove any residues. VWR micro cover glass (22 x 22mm) were dipped in 0.1 M NaOH and then flamed using 100% (200-proof) EtOH to sterilize. The stamps and cover glass were glow-discharged (negative setting for normal glow-discharge) for 2 min on high setting (coating current = 50mA) at 1x10-1 mbar to activate the surfaces (Quorum Emitech K100X). Immediately after, stamps were pressed feature-side down to the cover glass slips in 35mm diameter plastic culture dishes (Corning), custom-made 93g sterile weights were placed on top of the stamp to hold it in place, and 0.1 mg mL-1-poly-l-lysine-g-polyethylene glycol solution (PLL-PEG) was added to passivate the non-patterned regions on the cover glass. The PLL-PEG solution was left to

incubate until dry. Stamps were carefully removed with forceps and the culture dishes were rinsed once with PBS++ (phosphate buffer saline containing 0.5 mM $MgCl_2$ and 1.0 mM $CaCl_2$). Laminin-521 was diluted 1:20 in PBS++, and 1mL was added to each culture dish containing passivated cover glass and left to incubate overnight at 4°C. The following day, the cover glass was rinsed with PBS++ three times (five min. per) to remove excess unbound Laminin and aspirated. Cover glass slips were carefully removed from the culture dishes using tweezers and transferred to a 6-well tissue culture plate.

HPS cell lines and maintenance

In this study we used human-induced pluripotent stem cells (hiPSC) from a female aged 33 years (iC5-7).These lines were generated from deidentified specimens from Lonza's publicly available biorepository (C4, C5, C6, and C7 fibroblasts). Lonza tested to confirm these cells were negative for HIV, HepB, HepC, and mycoplasma. Reprogramming was performed by the University of Colorado Denver, School of Medicine, Stem Cell Biobank, and Disease Modeling Service Core via transfection of modified mRNAs and miRs using virus-free and non-integrating methods (Kogut et al., Nat Comm, 2018). The core also performed characterization assays; Karyotype analysis; mycoplasma testing; pluripotency testing; and tri-lineage differentiation.

hiPSC were cultured with mTESR1 plus media (STEMCELL technologies) on Laminin- coated adhesive islands on coverslips in 6-well culture dishes incubated at 37ºC and 5% CO2 (*MTeSR™ Plus Offers Enhanced Stability for Human ES and IPS Cell Maintenance, Now Manufactured Under CGMP*, n.d.) (*Laminin from Mouse Engelbreth-Holm-Swarm (EHS) Sarcoma 114956-81-9*, n.d.). Medium was replaced on a daily basis.

Thawing and Plating hiPSC

hiPSC (IC72) cryovials were quickly thawed in a 37°C water bath before being transferred to a 15mL tube with 6mL mTeSR Plus Media. The tube was centrifuged at 300 x g for five minutes at room temperature, the supernatant was removed, and the cells were resuspended in 2mL mTeSR Plus Media

with 2uL of 5mM ROCK inhibitor (CU Stem Cell Research and Technology Resource Center). 1mL of cell suspension was added to each well containing the pre-patterned cover glass and was allowed to settle for 15 min in the incubator before adding 1mL mTeSR Plus media and allowing cells to settle for another 10 min in the incubator. Excess media was removed leaving just enough to cover the cover glass and an additional 2mL mTeSR Plus media was added per well until the following day.

Indirect Immunohistochemistry

hiPSC island containing coverslips are removed from 6-well cultures and briefly washed in Tris Buffered Saline (TBS). The coverslips were then placed in 100% cold methanol for twenty minutes at 20ºC to fix the cells. After fixing the cells, the coverslips were carefully washed by dipping into TBS three times, carefully clearing away excess TBS between washes. The coverslips were then placed into a custom-constructed staining-rack box and incubated for ~20 minutes at room temperature with ~40µL of 1:100 dilution of the mouse monoclonal anti-Vimentin antibody AMF-17b (Developmental Studies Hybridoma Bank). The coverslips were carefully washed by dipping into TBS three times, carefully clearing away excess TBS between washes, and then incubated with ~40µL anti-Alexa 568-conjugated goat anti-mouse-Ig secondary antibody (1:400) (Research Organics) at room temperature for 20 min. The coverslips were carefully washed by dipping them into TBS three times, carefully clearing away excess TBS between washes. Once dry, one drop of mounting media (UltraCruz Mounting Medium with DAPI, Lot #L0709) was added to a glass slide and the coverslip was carefully placed on top (cell side facing the glass).

Imaging

Imaging was performed using Carl Zeiss IM 35 Inverted Microscope for fluorescence images and Nikon TMS Inverted Microscope for in vitro images. Photographs were taken using an iPhone lens adapter (Microscope Lens Cell Phone Adapter, purchased from Amazon).

Results

HeLa cells exhibit GFP-labeled high specificity Cas9 marker indicative of successful transfection.

To create HeLa cells carrying a vimentin-null mutation and begin our exploration of the micropatterned adhesive island system, we first needed to develop a reagent capable of inducing a vimentin-null mutation (vimentin knockout). Two previous undergraduates in the lab, Maggie Trout and Caleb Gammon, working with the advice of Bilge Birsoy, designed two guide RNA sequences and subcloned them into the pCAG-eCas9-GFP-U6-gRNA plasmid (purchased from Addgene). Once confirmed by DNA sequencing, VIM1 and VIM2 plasmid DNA was tested by transfection into HeLa cells. HeLa cells (obtained from Nausica Arnoult's Lab (University of Colorado, Boulder) were transfected with VIM1 or VIM2 plasmid DNA using Fugene 6 Transfection Reagent (Roche, Catalog #11815091001). Successful transfection results in a double-stranded break at the targeted location, triggering the cell's natural repair mechanisms, potentially leading to the insertion, deletion, or replacement of genetic material. Direct Fluorescence arising from the expression encodes GFP-tagged eCas9 protein was visualized using a Zeiss IM35 microscope with a fluorescein (green) filter set. Imaging revealed the presence of green fluorescent cells; the plasmid-encoded eCas9-GFP protein was found to have entered, and concentrated in the nucleus of the transfected cells (Fig. 2A). Transfection rates were relatively low, as relatively few cells expressed GFP (Fig. 2A).

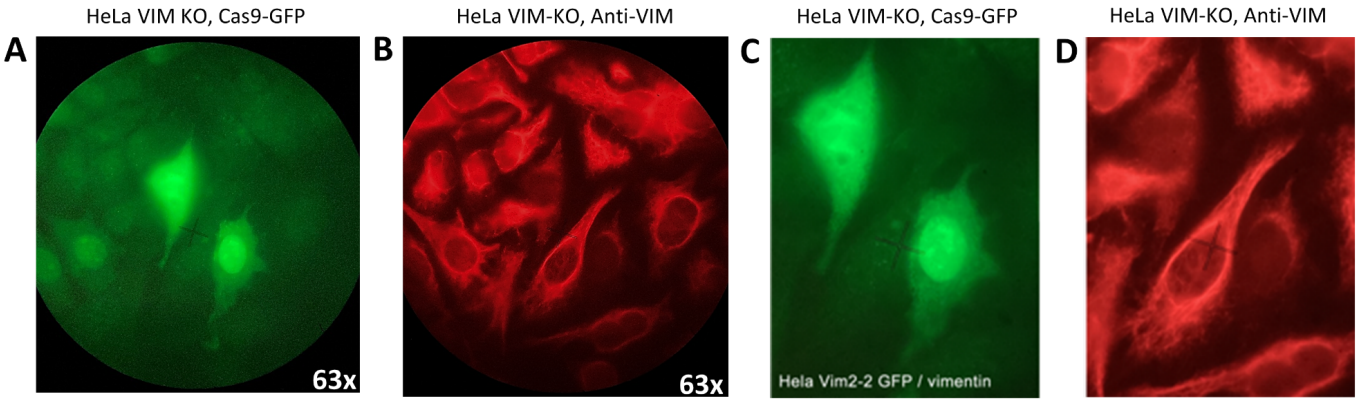

Figure 2. Fluorescence Microscopy of Vimentin Knockout in HeLa with GFP-labeled Cas9.

Immunofluorescence microscopy of fixed cells was performed using a primary mouse monoclonal-Anti-Vimentin (Anti-VIM) antibody and a secondary Alexa 568-labeled rabbit-anti-Mouse immunoglobulin antibody. (**C**) Cas9-GFP expressing HeLa cells (green) indicate that Cas9 entered the nucleus of the cell suggesting that our plasmid is effective in transfecting HeLa cells. (**D**) In the two visible Cas9-GFP expressing cells, the level of vimentin appears to be significantly lower or absent compared to the wild type (i.e. untransfected neighboring cells). This correlation between the cells expressing GFP and the cells not expressing vimentin is more clearly shown in these cropped images.

Transfection of HeLa cells was sufficient in knocking-out Vimentin.

Following successful transfection of HeLa cells, fluorescence microscopy indicated the absence of vimentin expression in the transfected cells, as evidenced by the lack of anti-vimentin staining in those cells specifically (Fig. 3B). It is also notable that despite low transfection rates (Fig. 2A), all cells that expressed GFP (a marker for successful transfection) also appeared to lack vimentin expression (Fig. 2C).

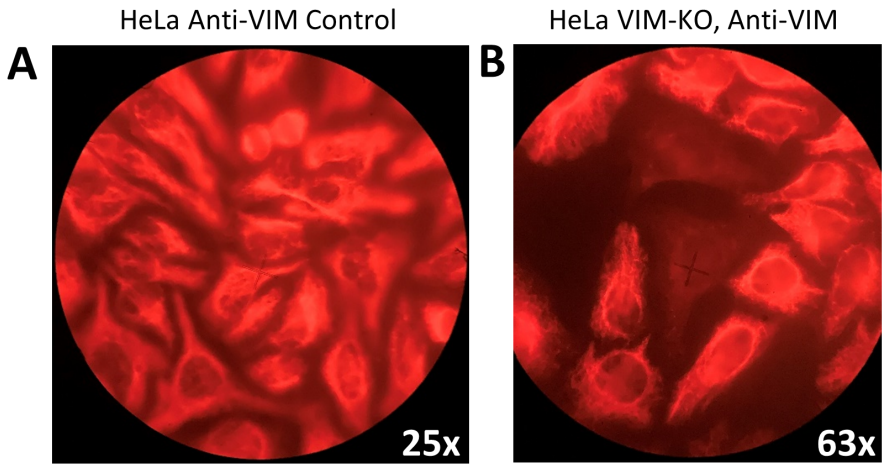

Figure 3. Fluorescence Microscopy of Vimentin Knockout HeLa versus Control HeLa.

Immunofluorescence microscopy was performed using a primary antibody, Anti-Vimentin (Anti-VIM) 1:400 made in mouse, and a secondary antibody, Anti-Mouse made in rabbit 1:400. Both were labeled with Alexa Fluor® 568 (AF568, Alexa 568). The bright orange indicates VIM-positive cells whereas the darker muted cells are negative for VIM.

Geometric confinement is sufficient in controlling adhesion of HeLa cells.

Given that the transfection reagent appeared to induce vimentin-null mutations in HeLa cells, we proceeded to investigate island formation in these cells. Unlike hiPSCs, HeLa cells are capable of adhering to glass cover slip surfaces without the need for a coating agent, as they naturally produce extracellular matrix (ECM) proteins such as fibronectin and laminin, which promote cell attachment and growth (Masters, 2002). Therefore, the passivating agent (PLL-PEG) and the PDMS stamp are the only constraints that govern geometric adhesion in HeLa cells. These constraints proved adequate in controlling HeLa cell adhesion to a simple micropatterned surface, as demonstrated by Coomassie Brilliant Blue staining of cell bodies (Fig. 4B) and fixed-cell microscopy (Fig. 4A).

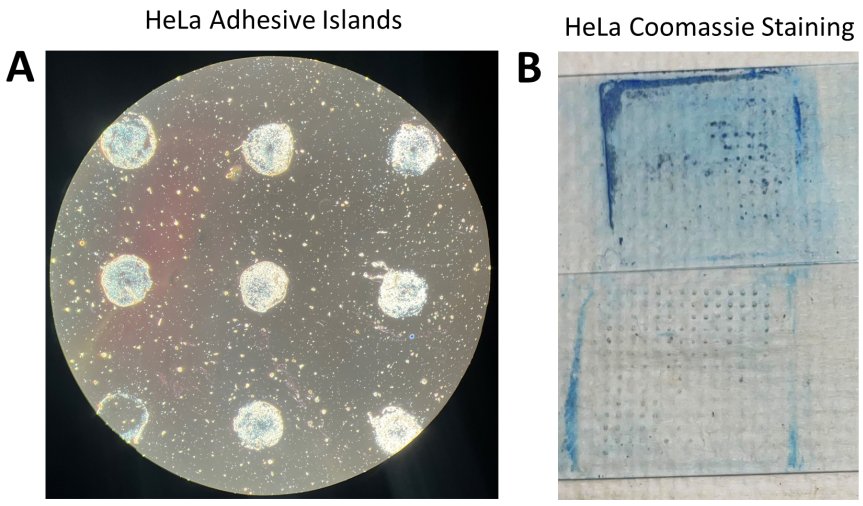

Figure 4. Micropatterned HeLa Adhesive Islands. (**A**) Fixed-cell 4x imaging was taken through Nikon TMS Inverted Microscope with an iphone adapter for image capture. HeLa cells were plated onto pre-patterned islands on glass coverslips without Laminin coating, using standard HeLa passaging protocols. Glass Micropatterning was done the same way as for hiPSC, however no Laminin coating was required (**B**) Coomassie Brilliant Blue staining of HeLa cell bodies was done using standard methodology reveals cell adhesion to the pre-patterned surface.

Geometric confinement is sufficient in triggering hiPSC self-organizing patterning.

Having demonstrated the effectiveness of the passivating agent (PLL-PEG) in concert with the PDMS stamp in creating HeLa cell adhesive islands, we proceeded with hiPSC cultures. The glass coverslip surface was both passivated with PLL-PEG to prevent cellular adhesion and geometrically pre-patterned regions were coated with laminin to facilitate adhesion. Live cell imaging of hiPSC taken after 15 min of incubation at 37℃ directly after plating revealed that geometric confinement was sufficient in creating hiPSC adhesive islands (Fig. 5B). Successfully creating these islands required a large amount of time-intensive experimentation. After much trial and error, Aryeh Warmflash provided us with advice on plating hiPSCs that included incubating the cells directly after plating before adding culture media to facilitate adhesion. After this adjustment, the system is now ready to characterize hiPSC behaviors.

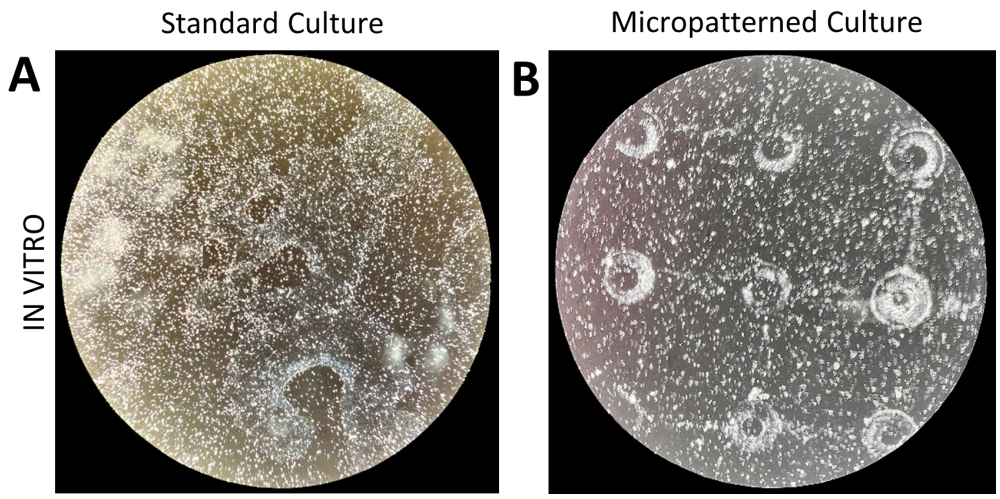

Figure 5. Standard hiPSC Culture versus Micropatterned hiPSC culture in Vitro at 4x. Live cell imaging was taken through Nikon TMS Inverted Microscope with an iphone adapter for image capture. (**A**) hiPSCs grown under standard culture conditions on Matrigel-coated 6-well culture plates form colonies that vary in shapes and sizes. (**B**) hiPSCs grown on pre-patterned Laminin coated surfaces adhere exclusively to those surfaces resulting in colonies that are geometrically constrained.

Discussion

We have produced a reproducible human model system using hiPSC that can be utilized for investigating complex molecular systems, IFp functions and phenotypes, genetic manipulations, effects of molecular chaperones, and more. I have made necessary alterations to the system developed by Warmfalsh et al. to adapt the system to the scale and financial limitations of the Klymkowsky Lab. This also ensures cost-effectiveness making it suitable for a teaching setting such as the CU MCDB Skills Center. To further enhance cost effectiveness, we suggest incorporating the negligible-cost B8 hiPSC media (Kuo et al., 2020) instead of MTeSR1 culture media into our model. This media represents only 3% of the cost of standard media and maintains hiPSC pluripotency and potential for differentiation. Our modifications to the Warmflash et al. model include changes in the construction of the PDMS stamp, the culture surface for hiPSCs, and the surface treatment, etc. Our stamps were constructed in the Kristi S. Anseth Lab at the University of Colorado, Boulder by Connor Miksch. Instead of standard soft lithography techniques, our stamps were produced from a silicone master template stamp purchased from RMS. The ability to reproduce multiple stamps from the master in house also contributes to cost effectiveness. We also used VWR micro cover glass (22 x 22mm) instead of 35-mm-diameter custom-made glass-bottomed culture dishes. Glass coverslips were flamed and glow discharged to treat the surface instead of plasma treatment. During passivation of the coverglass, we used custom-made 93g sterile weights to weigh down the PDMS stamps and allowed the PLL-PEG to completely dry before stamp removal. We hypothesize that the slightly abnormal hiPSC adhesive islands resulted from weighing the stamp during passivation (Fig. 5B). Despite small inconsistencies in hiPSC adhesion, the modifications we made to the original model by Warmflash et al. made it possible to produce adhesive islands within the confines of our facilities and resources.

Replicating this model system was challenging given the requirement for modifications to the Warmflash et al. method. Repeated experimentation achieved relatively consistent hiPSC adhesive islands, which directed our focus towards optimizing the system rather than studying molecular systems. With our success in achieving micropatterned adhesive islands in hiPSCs, we propose a continuation of

the study to investigate the effects of IFps and molecular chaperones on development/differentiation. To do this, we would create a vimentin-null hiPSC line using our plasmid, and confirm vimentin was knocked out using Western Blot Technique or gene sequencing. It would be interesting to study how hiPSCs generate spatially ordered germ layers by inducing differentiation with Bone morphogenetic protein 4 (BMP4). Warmflash et al. demonstrated that when exposed to BMP4, their micropatterned colonies reproducibly differentiate into three germ layers (like in gastrulation in vivo); an outer trophectoderm-like ring, surrounding ring of mesendoderm cells expressing primitive-streak markers, with an inner region of ectodermal cells (Warmflash et al., 2014). Cell fates are determined relative to the perimeter of the colony. It would be interesting to observe the results of staining for pluripotency markers like OCT4, SOX2, and NANOG before and after BMP4 treatment. Markers like these and more were used by Warmfalsh and colleagues to assess cell-fate patterning in response to differentiation using their micropatterned adhesive island model. Replicating their differentiation protocol involving BMP4 treatment to induce germ layer formation would be an appropriate application of our hiPSC micropatterned model. We could use our VIM-null hiPSC line and a standard hiPSC cell line in concert with our micropatterned adhesive islands and observe the phenotypic effects of knocking out vimentin on germ layer development and test whether molecular chaperones like heat shock protein 90 (HSP90), which has a known role in protein misfolding, can rescue development (Lackie et al., 2017).

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