The fate of cells is influenced by their microenvironment and many cell types undergo differentiation when stimulated by extracellular cues, such as soluble growth factors and the insoluble extracellular matrix (ECM). Stimulating differentiation by insoluble or "immobilized" cues is a particularly attractive method because it allows for the induction of differentiation in a spatially-defined cohort of cells within a larger subpopulation. To improve the design of de novo screening of such insoluble factors, we describe a methodology for producing high-density peptide microarrays suitable for extended cell culture and fluorescence microscopy. As a model, we used a murine mammary gland cell line (NMuMG) that undergoes epithelial to mesenchymal transition (EMT) in response to soluble transforming growth factor beta (TGF-β) and surface-immobilized peptides that target TGF-β receptors (TGFβRI/II). We repurposed a well-established DNA microarray printing technique to produce arrays of micropatterned surfaces that displayed TGFβRI/II-binding peptides and integrin binding peptides. Upon long-term culture on these arrays, only NMuMG cells residing on EMT-stimulating areas exhibited growth arrest and decreased E-cadherin expression. We believe that the methodology created in this report will aid the development of peptide-decorated surfaces that can locally stimulate defined cell surface receptors and control EMT and other well-characterized differentiation events.

Statement of Significance

Scope of work: This manuscript aims to accelerate the development of instructive biomaterials decorated with specific ligands that target cell-surface receptors and induce specific differentiation of cells upon contact. These materials can be used for practical applications, such as fabricating synthetic materials for large scale, stem cell culture, or investigating differentiation and asymmetric division in stem cells. Specifically, in this manuscript, we repurposed a DNA microarray printer to produce microarrays of peptide-terminated self-assembled monolayers (SAMs). To demonstrate the utility of these arrays in phenotypic assays with mammalian cells, we monitored the induction of epithelial to mesenchymal transition (EMT) in murine mammary epithelial cells using specific peptide ligands printed on these arrays. Novelty: We, and others, have published several strategies for producing peptide-based arrays suitable for long-term phenotypic assays. Many reports relied on patterning steps that made adaptation difficult. The use of a DNA microarray printer as the sole production tool simplified the production of peptide microarrays and increased the throughput of this technology. We confirmed that simplification in production did not compromise the performance of the array; it is still possible to study short-term adhesion, long-term growth, and complex phenotypic responses, such as EMT, in the cells. EMT was studied using immunofluorescent staining after four days of culture. Impact: This methodology will serve as a foundation for future screening of instructive biomaterials in our research group. As DNA printers are broadly available in academic institutions, we foresee rapid adaptation of this approach by academic researchers.
1. Introduction

In this report, we developed a platform based on an array of self-assembled monolayers (SAMs) of peptide alkanethiols (ATs) on gold, that permits the investigation of physiological responses in cells cultured on diverse ligands that bind cell surface receptors. As a model system, we used a well-established cell line that is known to undergo epithelial to mesenchymal transition (EMT) in response to extracellular ligand stimulation. EMT is an essential process that was first recognized in organ formation during embryonic development. This process is characterized by a shift in cell morphology from squamous, epithelial-like cells, to motile, spindle-like mesenchymal cells [1,2]. It promotes migration and increased invasiveness of cancer cells; carcinoma cells that undergo EMT become more motile and give rise to metastases [1–3]. The signaling pathway that drives EMT also controls the signaling that causes the formation and maintenance of highly tumorigenic cells, known as cancer stem cells (CSCs) [4–6]. CSCs, by definition, are a subpopulation of cells within a tumor that have a higher tumor forming capacity in xenograft models; CSCs were also proposed to be the cells that form metastases after EMT has occurred in vivo [3]. The non-stem and cancer stem cells interconvert reversibly via a process closely allied to EMT; this process is stochastic and only a small percentage of cancer cells undergo EMT-like events that lead to de-differentiation to putative CSCs [7,8]. To aid the investigation of EMT and its connection to differentiation/dedifferentiation events in a tumor, we developed a method that would permit the stimulation of EMT in cells in a spatially localized manner.

The most characterized extracellular stimulus that initiates EMT and enrichment of CSC-like cells, in multiple cancer cell lines, is transforming growth factor beta (TGF-β) [9]. The TGF-β signaling pathway is initiated by the dimerization of TGF-β serine/threonine kinase receptors (TGF(b)RI/II); this complex then phosphorylates intracellular Smad, leading to the translocation of phospho-Smad into the nucleus [1,10]. In vivo, TGF-β acts as both a soluble ligand and an extracellular matrix (ECM)-immobilized ligand [11]; immobilization of TGF-β on polymeric scaffolds in vitro is known to preserve its bioactivity and ability to elicit signaling in cells. Localized immobilization of proteins is a powerful tool in cell biology [12], but further minimization of proteins to functional short peptides can help generalize this technology to bypass issues such as residue-specific immobilization and inactivation of proteins. Specifically, Kiessling et al. previously demonstrated that EMT can be induced in large scale populations of mammary epithelial cells cultured on top of surface immobilized peptide analogs of TGF-β. The cells displayed signatures of EMT, such as down-regulation and internalization of E-cadherin, increase in smooth muscle actin expression, changes in cell morphology, and growth arrest [2].

The use of chemically-modified surfaces for investigating fundamental cell biology questions dates back to 1978, when Folkman et al. used surfaces of different hydrophobicity to show that the area to which a cell spreads determines its survival [13]. In the 1990s and early 2000’s, “binary” micropatterning of SAMs into areas that promoted and suppressed attachment of cells made it possible to probe aspects of cellular adhesion [14], migration [15], and division [16]. In the 2000’s, several groups reported technologies for co-patternning multiple chemically-defined SAMs for co-culturing different cell lines [17]; these arrays of SAMs empowered the discovery of peptide-modified substrates that control differentiation of human embryonic stem cells [18]. Patterning technologies can dissect spatially-defined phenotypic transformation in individual cells, such as branching morphogenesis [19], or asymmetric division [20]; technologies for this application should allow for the control of chemical composition, size, and shape of patterned areas to accommodate single or multiple cells. Although the technology for surface patterning has developed significantly in the past 20 years, many techniques have been perfected to only yield high-fidelity binary patterns; modern technologies for patterning multi-component surfaces relies on cumbersome serial processing, accurate alignment, and access to instrumentation not accessible outside of specialized laboratories [21]. The most scalable techniques for multi-component patterning of surfaces are those that repurpose well-developed, high-throughput screening instruments, such as robotic spotters or microarray printers. While these technologies have been applied to the fabrication of protein arrays [22–24], their application to the fabrication of peptide monolayers is limited [25,26].

In this report, we describe the use of a DNA microarray printer to pattern gold coated surfaces with micron-scaled islands of peptides that bind to TFG(b)RI/II and integrin receptors. The peptides are conjugated to an alkanethiol linker for the creation of SAMs on the gold-coated glass surfaces. In the future, this method can be used to investigate the heterogeneity of EMT-responses and CSC-dedifferentiation events in carcinoma cell lines in vitro (Fig. 1). Contrary to the popular method of using soft lithography for printing spatially defined SAMs, a DNA microarray printer provides an alternative, simple, robust, and reproducible method of generating arrays containing diverse surfaces. The size of the spots can be tuned within a range of 150–400 μm in diameter to create arrays for monitoring the growth of single or multiple cells (Fig 1D). In this report, we primarily use areas of 150 μm in diameter to accommodate multiple cells for the study of cell adhesion, spreading, proliferation, and EMT in spatially defined areas in one common media.

2. Results and discussion

2.1. Validating E-cadherin expression in NMuMG cells grown on large surfaces of peptide SAMs

We selected three peptides for the production of SAMs and microarrays of SAMs with NMuMG cells. Peptides LTGKNPFMFFHRN and MHRMPSFLPTTL were previously reported to cluster TGF-βRI/II...
and potentiate the effects of soluble TGF-β at low concentrations [18]. The peptide sequence GRGDS, which binds to cell integrin receptors, was included as a negative control. As a substrate for SAMs, we used coverslips sputtered with a layer of translucent gold to facilitate the analysis of these arrays via phase-contrast and fluorescent/confocal microscopy. NMuMG cells were monitored for the following hallmarks of EMT: decreases in E-cadherin levels, increases in smooth muscle actin levels, growth arrest, and morphological changes. Gold exhibited no deleterious effect on NMuMG cells and cells cultured on surfaces displaying GRGDS SAMs exhibited no changes in the basal level of E-cadherin expression.

We validated that the level of E-cadherin expression in NMuMG cells cultured on large areas SAMs displaying LTGKNFPMFHRN and MHRMPSFLPTTL changed significantly when compared to cells cultured on GRGDS. After four days of culture, E-cadherin levels, visualized via immunofluorescent staining, were observed to be the highest in NMuMG cells grown on GRGDS and the lowest in NMuMG cells grown on LTGKNFPMFHRN (Supplementary Fig. S1). We also monitored these surfaces daily using phase-contrast microscopy to check for morphological changes (Supplementary Fig. S2A). After four days, the cells cultured on SAMs of LTGKNFPMFHRN and MHRMPSFLPTTL, and in media treated with soluble TFG-β lost epithelial morphology and gained a spindle-like appearance (Supplementary Fig. S2B).

2.2. Optimization of printing SAMs

For all microarray experiments, we used a pattern consisting of 4 identical quadrants of 9 x 9 pattern (Fig. 1B). To integrate commercially available, small footprint microscope cover slides (18 x 18 mm) with the DNA microarray spotter, we used custom-made stainless steel slide holders for printing (Fig. 1C). In all cell-based experiments in this manuscript, we used Telechem SMP3 Stealth Micro Spotting Pins at 0 ms contact time to produce peptide-modified spots of 150 μm in diameter. To demonstrate the range of spot sizes that can be generated on the arrays, we printed spots with a diameter of 400 μm using SNS15 Stealth Solid Pins at 0 ms contact time (Fig. 1D). Shortly after printing, we immersed the arrays into the solution of glucamine-alkanethiol to form a protein- and cell-repelling background between the spots [27]. The best printing results were achieved on freshly-deposited gold-coated surfaces (Supplementary Fig. S3). We attempted to reuse gold-coated surfaces by stripping off the SAMs with a detergent and plasma cleaning; upon reprinting the arrays, we noticed that the alignment of the printed spots highly varied on reused surfaces. In addition to printing peptide-AT, we also printed rows of KCN solution to produce a convenient visual reference of etched gold (Fig. 2A) [28,29].

We spiked the solutions of peptide-alkanethiols used for printing with fluorescein to visualize the locations of the printed areas before seeding cells (e.g. Fig. 2B). We observed minor variations in the intensity of fluorescence, but these variations should not be interpreted as non-uniformity in SAMs, as printing deposits an excess amount of alkanethiols for the formation of SAMs. To confirm the functional uniformity of the monolayers, we produced three independent arrays and allowed for short term adhesion of NMuMG cells (24 h) to minimize any variability from long-term growth (Supplementary Fig. S4). Counting the number of cells per spot yielded only minor variations in the number of cells adhered to each peptide spot in three independent arrays of 36 replicates on the same array.

2.3. Optimization of cell concentrations for short-term growth

The optimal cell concentration for short-term growth experiments (0–5 days) should initially yield 1–5 cells adhering per area to allow cells to divide in the areas during the subsequent growth period. We optimized conditions for NMuMG cells by seeding a suspension of 10,000, 25,000, or 50,000 cells in 3 mL to an array in a 6-well plate. After one hour of incubation, we rinsed off the non-bound cells and supplemented the arrays with fresh NMuMG
media. Fig. 2C describes the number of cells on the arrays after 24 h of culture. We found the optimal density of cells for short-term growth to be 3,300 cells/mL, or 10,000 cells/well (Fig. 2C).

2.4. E-cadherin expression of cells on printed SAMs

We cultured NMuMG cells on microarrays that displayed LTGKNFPMFHRN, MHRMPSFLPTTL, and GRGDS to identify conditions that induce EMT in cells in spatially defined areas. We anticipated EMT to be induced in cells grown on islands of SAMs with LTGKNFPMFHRN and MHRMPSFLPTTL, but not on parallel islands of GRGDS. For one to three days, cells exhibited high levels of E-cadherin on all three peptides. On the fourth day, we detected decreased levels of E-cadherin in cells growing on LTGKNFPMFHRN and MHRMPSFLPTTL, whereas cells growing on GRGDS did not show any decrease in E-cadherin expression (Fig. 3).

2.5. Monitoring growth rate and cell morphology

To detect growth arrest, we counted the number of cells growing on individual peptide-modified areas after two, three, and four days in 35–40 replicates. We modified the original 9 × 9 pattern to an asymmetrical pattern with distinct spaces for patterning LTGKNFPMFHRN, MHRMPSFLPTTL, GRGDS, and KCN (Fig. 2A). This allowed for easier visual identification of peptide islands (Fig. 2B). On the second day, NMuMG cells adhered to only 50% of LTGKNFPMFHRN or MHRMPSFLPTTL modified areas, whereas cells...
adhered to 75% of the GRGDS modified areas. On the fourth day, dense populations of cells were found on over 60% of GRGDS modified areas. In contrast, both LTGKNFPMFHRN and MHRMPSFLPTTL areas contained biphasic populations; dense populations were observed in approximately two-thirds of the areas, and sparse populations were observed on one third of the areas (Supplementary Fig. S5).

Changes in cell morphology were difficult to deconvolute due to the combination of both growth and packing of cells in an area of limited size. NMuMG cells growing on GRGDS had a significantly smaller spreading area by day 4 than cells growing on LTGKNFPMFHRN and MHRMPSFLPTTL (Supplementary Fig. S6). For all three peptides, the size of individual cells doubled over the course of four days; cells spread from an average area of 1988 \( \mu \text{m}^2 \) on day 2 to 3745 \( \mu \text{m}^2 \) on day 4. Visual inspection showed that NMuMG cells grown on GRGDS islands yielded dense colonies resulting from rapid cell growth. Thus, the average area of a cell on a constrained growth island was small. Cell colonies were less dense on LTGKNFPMFHRN and MHRMPSFLPTTL islands due to reduced growth and division, yielding, on average, larger cells that spread through the entire area. The observed reciprocal relationship between cell number and cell area arises because cells grow and spread on the constant area, \( S \). Individual cells are capable of spreading over the entire area, exhibiting cells spreading an area as large as \( S \). As cells divide to produce \( N \) daughter cells, the areas of each individual cell is reduced to \( \sim \sqrt{S/N} \).

2.6. Culturing MDA-MB-231 and NMuMG cells on peptides found through phage display

In vitro display technologies, such as phage-[30], bacteria-[31] and cell-surface displays, are powerful for de novo discovery of ligands that target cell surface receptors and control differentiation of cells [32]. We previously developed a series of peptides that bind to MDA-MB-231 cells [33] and further demonstrated that many of these peptides can support adhesion of closely and distally related epithelial tumor cell lines (manuscript in review). We sought to test whether the identified peptides support adhesion of normal epithelial cells, and whether adhesion of cells to these peptides can induce EMT. Here, we show that peptide arrays can be used as a medium-throughput phenotypic screening method for validating peptides discovered by phage display. We previously identified the peptides STASYTR, GKPMPPM, AMSSRSL, IPAPLRS, and HAIYPRH by phage-display library selection against intact MDA-MB-231 basal breast cancer cells. Cellulose peptide arrays, created using the SPOT peptide synthesis method, validated the ability of these peptides to support short-term adhesion of MDA-MB-231 GFP cells [33]. When printed on the SAMs-array, all peptides supported short-term adhesion of MDA-MB-231 cells as well as their growth over the course of three days (Fig. 4A). NMuMG cells adhered to four of the five peptides. As these peptides bind to one of the receptors on mesenchymal-like cancer cells, we investigated whether any of these peptides can stimulate EMT in epithelial cells.

Over the course of 4 days, we observed a decrease in E-cadherin expression in NMuMG cells on surfaces that displayed IPAPLRS- and GKPMPPM-terminated SAMs to levels comparable to those observed on control TGF\(\beta\)-binding peptides (LTGKNFPMFHRN and MHRMPSFLPTTL). In contrast, the level of immunostaining with E-cadherin antibody in cells growing on HAIYPRH and AMSSRSL modified areas remained similar to that observed on cells cultured atop areas of GRGDS (Fig. 4B).

We also inspected the increase of smooth muscle actin (SMA) expression in NMuMG cells grown on peptide arrays displaying TGF\(\beta\)RI/II binding, phage derived, and integrin binding peptides for 4 days. We observed the highest SMA expression in cells cultured on LTGKNFPMFHRN, but no increased expression of SMA on surfaces modified with IPAPLRS when compared to cells grown on areas modified with GRGDS.

3. Conclusion

In phenotypic cell-based assays, the performance of microarray-printed peptide arrays is similar to the performance of previously...
reported peptide arrays [15,31]. These arrays integrate seamlessly with standard cell culture, imaging, and immunofluorescence technologies. Regular arrangement of peptide areas and visual references, such as a grid of etched, gold-free zones, facilitate both automatic analysis and daily manual monitoring of the assays. We believe that the major benefit of the microarray peptide SAMs described in this report, when compared to previous reports, is simplicity in fabrication and scalability in production. Specifically, it is possible to scale up both the number of different peptides printed on the surface and the number of arrays printed in a single session. Most DNA microarray printers are designed for high-speed production of up to a hundred arrays at once. One of the remaining limitations is the synthesis of peptide-alkanethiols (AT); every peptide-AT must be purified and characterized independently. Although the direct synthesis of peptides on the surface could potentially yield larger number of peptides and bypass the need for purification, there is a clear compromise between the number of peptides and quality of the array. In our experience, even an optimized method of synthesizing short peptides on a surface can yield products with less than 50% purity [34]. Another limitation is the analysis of the arrays; this problem can be avoided by using specialized, high-content analysis hardware and advanced image recognition software. An alternative solution is imaging of the arrays on a fluorescent gel or DNA array scanner. In conclusion, we believe that this printed SAM-array will be an important tool in validation of small-molecule and peptide ligands that induce differentiation and phenotypic transformations in cells.

4. Materials and methods

4.1. Synthesis of peptides on an alkanethiol linker

The alkanethiol linker was synthesized and conjugated to polystyrene monomethoxyxtryl chloride (MMT-Cl) resin in preparation for Fmoc solid phase peptide synthesis. The peptides LTGKNFPFMHRN, MHRMPSLPPTLT, STASYTR, GKPMPPM, AMSSRSL, IPAPLRS, and HAIYPRH were synthesized on AT-modified resin to make peptide-terminated alkanethiols as described in our previous publication [19,29]. Amino acids were purchased from Chempep. The peptides were purified using high performance liquid chromatography (HPLC) and verified through matrix-assisted laser desorption/ionization (MALDI) and liquid chromatography mass spectrometry (LCMS). Peptides were stored at –20 °C as lyophilized powder and as 1 mM solutions in Milli-Q water.

4.2. Making and storing gold chips

99.99% pure gold (Gold Maple Leaf pure bullion coin) was evaporated onto 18 × 18 mm glass cover slides using a thermal evaporation system from Torr International Inc., model THEUPG, to create the platform for self-assembled monolayers. Immediately before deposition, the glass cover slides were soaked in piranha solution (1 H₂O₂:3 H₂SO₄) for 20 min, extensively rinsed with milli-Q H₂O, and dried with argon gas. Warning: Piranha solution is extremely corrosive and potentially explosive when in contact with organic matter. 5 mm of chromium was first evaporated onto the glass surfaces, followed by 20 nm of gold. The gold surfaces were stored in a clean polystyrene Petri dish for up to 3 weeks.

4.3. Microarray printing of peptides and KCN onto gold surfaces

All peptides used for printing were dissolved in Milli-Q water at 1 mM concentration. We supplemented each solution with 0.2 mM fluorescein; this concentration was necessary to visualize the location of each spot. The KCN solution used for etching gold was made by dissolving NaOH and KCN in milli-Q water to yield a 1 mM of KCN in 1 M NaOH solution. Warning: this solution is extremely toxic and corrosive. The three solutions were printed onto the gold surfaces using the DNA microarray printer in the Microarray and Proteomics Facility (MAF) at the University of Alberta. Telechem SMP3 pins from Arrayit and 0 ms contact time were used to deposit the solutions onto the surface.

After printing, the surface of the gold slide was placed on top of a solution of glucamine-AT to form a cell-repellent layer in between the printed areas. Specifically, 30 μL of the solution was deposited in a sterile Petri dish and the array was inverted onto the glucamine solution, forming a uniform layer of liquid sandwiched in between the array and the surface of the Petri dish. A wet napkin was placed on the lid of the Petri dish and the lid was sealed with Parafilm to prevent evaporation of the glucamine-AT solution. This blocking process was allowed to proceed for 12–24 hours at room temperature.

Prior to cell-adhesion studies, the arrays were sterilized with 95% ethanol three times for 20 min. The arrays were transferred to a sterile 6-well plate in a laminar flow hood and rinsed 3 times with 1× PBS and 3 times with MEM to eliminate traces of ethanol.

4.4. Cell culture conditions of NMuMG cells

NMuMG cells were maintained in DMEM supplemented with 10% FBS, non-essential amino acids, GlutaMax™, and 10 μg/mL of human insulin. For cell adhesion and growth experiments on peptide arrays, the cells were detached from the culture surface with trypsin (5 min), rinsed, and resuspended with growth medium, and seeded onto the arrays in six well plates. The arrays were incubated at 37 °C with 5% CO₂ for 2 h before the media was replaced with growth media supplemented with 1 ng/mL of soluble TGF-β1. The cells were cultured for 4 days at 37 °C with 5% CO₂.

4.5. Immunofluorescent staining

Cells on the gold surfaces were fixed with 2% paraformaldehyde (PFA) in PBS and stored at 4 °C for a minimum of 30 min. PFA was aspirated from the wells and the cell membrane was permeabilized using 0.1% Triton X-100 in PBS for 15 min. The arrays were incubated in a blocking buffer (10% calf bovine serum [CBS] and 10% Triton X-100 in PBS) for 1 h. For E-cadherin staining, the arrays were incubated with monoclonal rat anti-uvomorulin/E-cadherin (Sigma), diluted 1:200 in PBS, for 24 h at 4 °C. The samples were rinsed 3× 5 min with PBS and incubated with Alexa Fluor 488 conjugated rabbit anti-rat (Invitrogen) at 1:500 dilution at 4 °C. The samples were then rinsed 3× with PBS and incubated with Alexa Fluor 488 Donkey Anti Rabbit IgG (Invitrogen) at 1:500 dilution for 1 h at room temperature. For smooth muscle actin (SMA) staining, the arrays were incubated with rabbit anti-alpha smooth muscle actin (Abcam), diluted to 5 mg/mL, for 24 h at 4 °C. The samples were then rinsed 3× 5 min with PBS and incubated with Alexa Fluor 488 Donkey Anti Rabbit IgG (Invitrogen) at 1:500 dilution for 1 h at room temperature. All arrays were then rinsed 3× 5 min with 1× PBS and incubated with DAPI (1 mg/mL in 1× PBS) for 30 min at room temperature to stain the cells’ nuclei. The arrays were imaged using a confocal fluorescence microscope (Zeiss LSM 700) at 20× objective.

4.6. Area analysis and cell count

Zen software was used for image processing. Cell peripheries were traced to define the area to which each cell spread. The cell count was performed by manually counting the number of nuclei stained with DAPI dye, per peptide area. Multi-layered, over populated peptide spots were excluded from data analysis.
Boxplot graphs were created using Origin 8.6 software (OriginLab, Northampton, MA, USA), which show relative increase of cell number or area over time.

Acknowledgments

This work was supported by the Canadian Institute of Health Research (CIHR) Bridge Funding. E.L. acknowledges Alberta Innovates Health Solutions (AIHS) for the Summer Studentship; A.S. acknowledges Mitacs Globalink Research Internship. J.W. acknowledges the Undergraduate Research Initiative (URI) for the summer studentship. Infrastructure support was provided by Canadian Foundation for Innovation (CFI). Microarray printing was provided by Troy Locke in the Microarray and Proteomics Facility (MAF) at the University of Alberta.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2016.01.028.

References