Fabrication of Dynamic Surfaces for *in vitro* Study of 'Epithelial Mesenchymal Transition' Progression

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Abstract:

A dynamic extra cellular matrix (ECM) mimicking surface is introduced that allows tuning of surface bound compounds by ultraviolet irradiation. This is accomplished by synthesizing a photocleavable variant of cyclic Arginine-Glycine-Aspartate (cRGD), a known promoter of integrin-based cell adhesion [1,2], and manipulating its concentration in the presence of cells. Surfaces were fabricated from thin film deposited titanium and gold on borosilicate glass that was reacted with an ethylene glycol (EG_6) diluted mixture to create a heterogeneous monolayer. The resultant surfaces were cultured with Madin Darby Canine Kidney (MDCK) cells and irradiated to observe changes in phenotype and immunofluorescence. The design results in the ability to actively observe progression of epithelial mesenchymal transition (EMT)-like phenotype changes in the MDCK cell line.

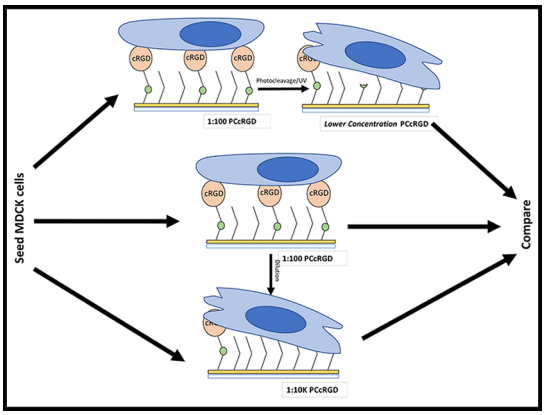

Figure 1: Strategy for observing and confirming EMT progression with dynamic PCcRGD surfaces.

Introduction:

EMT is a well-studied morphological change in epithelial cell lines where adherent, clustering cell populations transition into a migrating, mesenchymal phenotype due to changes in ECM adherence controlled by mechanical and biochemical cues [1]. This process has been shown to play a role in the progression of metastatic cancer, as it enables the movement of cancer cells throughout the body and away from the initial tumor site. The importance of one ECM component, cRGD, has been demonstrated previously with high surface densities yielding clustered cells and low densities resulting in EMT-like phenotypes.

The goal is to synthesize a photocleavable variant of surface-bound cRGD (PCcRGD) and demonstrate the ability to alter the overall cRGD concentration in the presence of MDCK cells and observe the progression of phenotype changes with real-time immunofluorescence imaging.

Summary of Research:

PCcRGD was synthesized by reacting cRGD with photocleavable PEG (PCP) in tetrahydrofuran (THF) with triethylamine (ET_3N) acting as a catalyst. This solution was reacted over 24 hours and then evaporated before being characterized by nuclear magnetic resonance (NMR) spectroscopy. Once the structure was confirmed, a 50 micromolar stock in methanol (MeOH) was made to fabricate surfaces. Pre-prepared borosilicate glass wafer layered with a 5 nm titanium nitride (TiN) mid-layer and a 20 nm gold (Au) top-layer were ozone cleaned and then reacted with 20 microliters of 1:100 and 1:10K PCcRGD:EG_6 dilution solutions (50 micromolar each) overnight in a sealed vessel. Surfaces were cleaned with MeOH and dried with nitrogen (N_2) before being placed in phosphate-buffered saline (PBS) in a 24-well plate.

The 1:100 dilution surfaces were irradiated with the gold surface facing down in PBS using 365 nm ultraviolet radiation for a dosage of 2.4 joules before being cultured with 2.0×10^4 MDCK cells per well (3.5 cm diameter circle). Cells were incubated at 37°C and 5% carbon dioxide (CO_2) for eight hours before being fixed, permeabilized, and stained for immunofluorescence of actin, phospho-myosin, and nucleus. This procedure created "pre-irradiation" surfaces, and a similar procedure was used for "post-irradiation" surfaces, where 2.0×10^4 MDCK cells per well were cultured onto each surface and allowed to adhere in an incubator

for three hours before being irradiated for a 2.4 joule dosage and then incubated for an additional five hours. Phase contrast and immunofluorescence imaging were done on a Zeiss single-channel microscope. Images were taken at 40x magnification with a 100-500 millisecond exposure time and then merged.

For live imaging of actin dynamics, MDCK cells were cultured at 1.1 × 10^4 cell per well in a 24-well plate and incubated overnight before being incubated for 12 hours with a solution of 2.5 microliters lipofectamine and 0.5 micrograms LifeAct-GFP DNA in 400 microliters MEM basic media per well. The 1:100 dilution surfaces were cultured with transfected cells, incubated for three hours, and then irradiated with a 2.4 joule dosage. Time-lapse imaging was done on an Olympus upright, single-channel microscope.

Results and Conclusions:

Analysis of pre-irradiation images shown in Figure 2 show results similar to those seen with traditional 1:10k cRGD surfaces [2] with degradation of the cortical actin surrounding cell clusters, formation of actin stress fibers across cells, and gaps in phospho-myosin at cell-cell junctions. Observation of the visual morphology of cells on surfaces with phase contrast imaging provided further evidence that PCcRGD surfaces behave similarly to cRGD surfaces fabricated by dilution. Post-irradiated surfaces showed identical changes in actin and phospho-myosin to a lesser degree, which infers that the surface is capable of changing cRGD density in the presence of cells, though the concentration of surface-bound PCcRGD after irradiation is between the 1:100 and 1:10k cRGD controls. Live imaging showed an increase in membrane ruffling in cells following exposure to irradiation, which is a result of actin formation in cytoplasmic protrusions found in migratory cells [3].

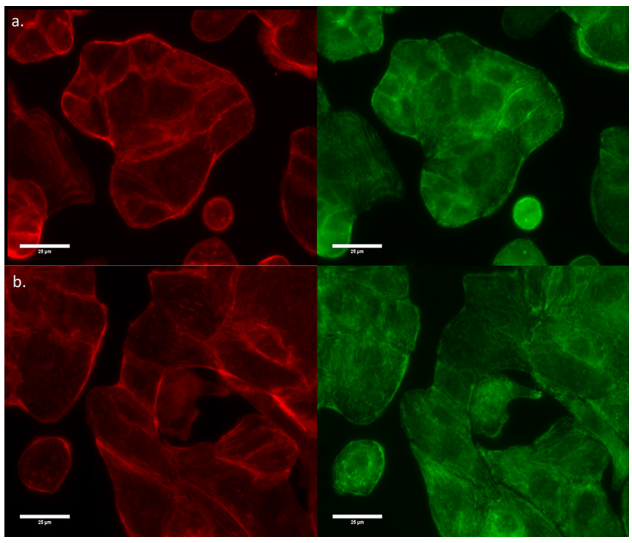

Figure 2: Immunofluorescence of MDCK cells on (a) 1:100 PCcRGD surface and (b) 2.4 J UV-irradiated 1:100 PCcRGD surface. Actin shown in red and phopsho-myosin shown in green.

Future Work:

The ability to observe progression of cell morphology is crucial for understanding the inner workings of EMT, however the issue of unknown concentration of surface-bound PCcRGD before and after irradiation makes comparison difficult. Future work will focus on developing procedures to do this, as well as exploring the cleavage rate of PCcRGD relative to irradiation dose. There is evidence that an additional cleavage reaction is occurring under specific experiment conditions shown in Figure 4, and this complicates the process of comparing to static cRGD surfaces. This cleavage mechanism must be identified and either controlled or used to reach the desired surface density.

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References:

[1] de Rooij, J., et al. (2005). "Integrin-dependent actomyosin contraction regulates epithelial cell scattering." J Cell Biol 171(1): 153-164.

[2] Marlar, S., et al. (2016). "Reduced adhesive ligand density in engineered extracellular matrices induces an epithelial-mesenchymal-like transition." Acta Biomaterialia 39: 106-113.

[3] Cheresh, D. A., Leng, J., and Klemke, R. L. (1999). Regulation of Cell Contraction and Membrane Ruffling by Distinct Signals in Migratory Cells. The Journal of Cell Biology, 146(5), 1107-1116.

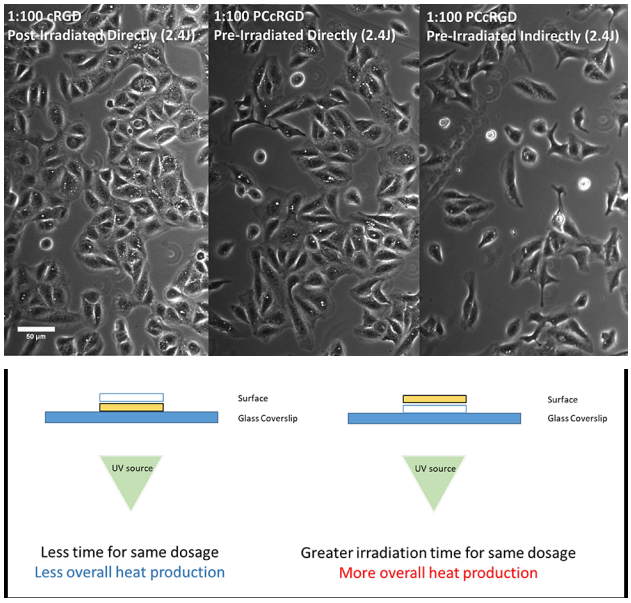

Figure 3, top: Morphology differences in MDCK cells based on orientation during UV irradiation. Figure 4, bottom: Orientation of samples during surface irradiation.