Synergistic Effect of Adhesive Ligand Density and Soluble Factor TGF-β on Epithelial Mesenchymal Transition Progression

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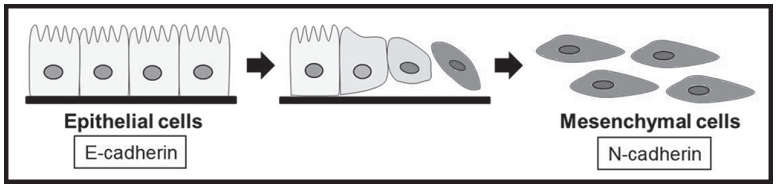


Figure 1: Visual schematic of EMT progression.

Introduction:

Characterized by a change in cellular phenotype and an adjustment in protein expression, the epithelial mesenchymal transition (EMT) describes the transformation of epithelial cells into a mesenchymal cell type. This transition is involved in the initial stages of cancer metastasis and enhances the migratory capacities and evasiveness of cancer cells [1]. In this study, we investigated the impact of two factors—surface density of an extracellular matrix protein-derived ligand and soluble transforming growth factor-β1 (TGF-β1)—on the progression of EMT. Gold surfaces functionalized in various ratios of adhesive ligand cRGD to bio-inert ethylene glycol (EG) were seeded with epithelial cells. This technique allows for simultaneous comparison of different stages of EMT in which morphological differences and changes in protein expression can be recorded. By investigating EMT in smaller, discrete steps, we can more accurately analyze the underlying mechanisms that drive this transformation and that ultimately drive cancer metastasis. For these experiments, Madine-Darby canine kidney (MDCK) cells were used.

Materials and Methods:

To study EMT progression, a five-day procedure was employed. On day one, gold (Au) 5 × 5 mm^2 surfaces were cleaned via sonication in acetone and UV-ozone treatment. The bare Au surfaces were then coated with various concentrations of cyclic [Arg-Gly-Asp-D-Phe-Lys], or cRGD, in EG overnight.

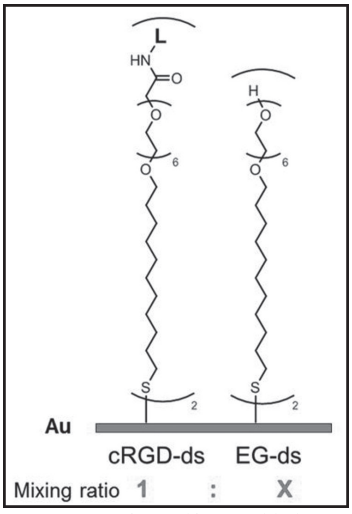
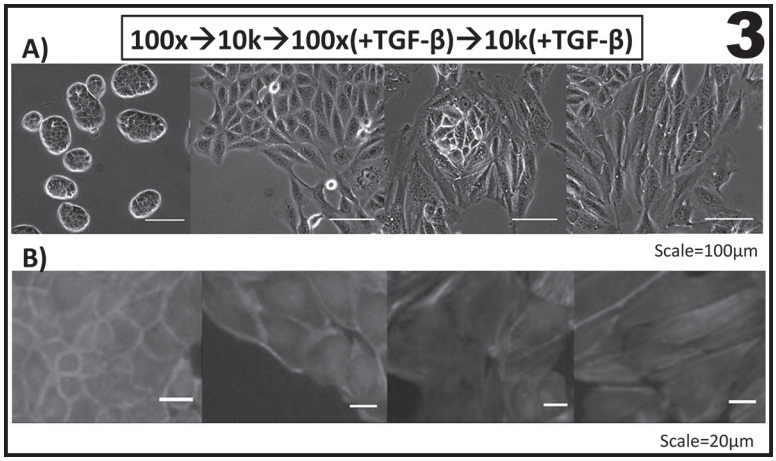


Figure 2: Surface self-assembled monolayers on a gold surface with "L" representing cRGD.

Figure 2 illustrates the structure of cRGD, represented by L, alongside EG bound to a Au surface. Because cRGD binds to $\alpha_v\beta_3$-integrin receptors on the MDCK cell membrane, it renders the Au surface adhesive to MDCK cells. Since EG repels protein absorption, MDCK cells attach to the surface exclusively by integrin binding to surface cRGD peptides. Adjusting the mixing ratio of adhesive ligand cRGD to protein-repellent ligand EG on the surface allows for effective tuning of surface adhesiveness to cells. Two mixing ratios of cRGD:EG were used in these experiments—1:100 (100×) and 1:10,000 (10k); 100× represents a high-density cRGD surface, while 10k represents a low-density cRGD surface.

On day two, approximately 5 × 10^4 MDCK cells per 10 cm^2 were seeded onto each of the cRGD-coated surfaces in serum-free medium to preempt any unwanted interaction of serum proteins with cell attachment at the beginning. Three hours after initial cell seeding, serum-free medium was aspirated and replaced with complete medium. On the following day, complete medium was supplemented with TGF-β at 10 ng/mL to induce EMT in two of the four samples. After two days of incubation with adhesive ligand cRGD and soluble growth factor TGF-β, MDCK

cells were fixed to the gold surfaces with a 1:1 methanol:acetone solution. From there, primary antibodies bound to membrane proteins E-cadherin and N-cadherin. Fluorescently-tagged secondary antibodies then bound to the primary antibodies. These secondary antibodies fluoresce upon exposure to UV light to aid in visualization of membrane proteins. This process of immunofluorescent staining was utilized to study the presence of cadherin proteins in MDCK cells on day five. Localization of E-cadherin or N-cadherin to the MDCK membrane indicates an epithelial or mesenchymal phenotype, respectively.



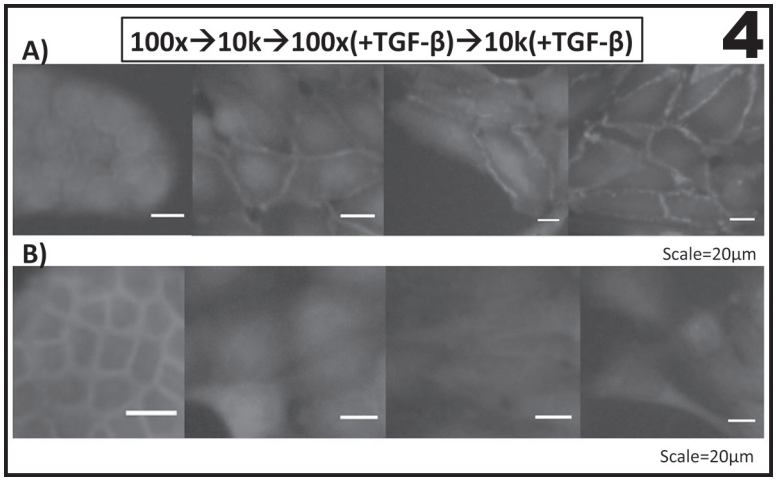


***Figure 3**, top: (A) Phase contrast images at day five. (B) Actin stained MDCK cells at day five. **Figure 4**, bottom: (A) N-cadherin and (B) E-cadherin stained MDCK cells at day five.*

Results and Discussion:

Both the adhesive ligand cRGD, and the soluble factor, TGF-β, played a role in controlling EMT progression in MDCK cells. Figures 3 and 4 show EMT progression from left to right with a decrease in cRGD concentration from 100× to 10k and with the addition of TGF-β. At high cRGD densities and no TGF-β, cells form tight clusters with a localization of F-actin in the cortical region of the cells. As cRGD density is decreased and TGF-β is added, cells begin to elongate, move away from the tightly-bound clusters, and organize F-actin into intracellular fibers. Finally at low cRGD and the presence of TGF-β, cells are completely elongated and F-actin is almost completely intracellular. This elongation and migration of cells coupled with the reorganization of actin within the cells allowed us to evaluate the EMT progression into four discrete steps.

To confirm this interpretation, protein localization of E-cadherin, an epithelial marker, and N-cadherin, a mesenchymal marker, was compared. At high cRGD densities and no TGF-β, cells localized E-cadherin proteins to the membrane while at low cRGD densities and the presence of TGF-β, cells localized N-cadherin to the cell membrane. Membrane localization of E-cadherin was less apparent than localization of N-cadherin perhaps because cells on 100× surfaces formed tight aggregates. Moreover, these trends in protein localization to the membrane support the conclusion that EMT progression was separated into four discrete steps.

Conclusions:

Lowering cRGD surface density and adding TGF-β gives a controlled progression of EMT in MDCK cells. Adjusting the concentration of TGF-β and surface density of cRGD allows for analysis of underlying mechanisms at various degrees of EMT progression, which may give rise to novel metastatic therapeutic techniques. Future work for this project includes the quantification of gene expression via PCR or western blot to determine specifically the degree of EMT progression of each sample.

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References:

[1] Kalluri, Raghu, et al. J. Clinical Invest., 119, 6, 1420-1428 (2009).