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HETEROGENEITY IN CELL DISTRIBUTION DUE TO COLLECTIVE ORGANIZATION LEADS TO LOCALIZED HIGHER STRESSES.

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INTRODUCTION

Collective cell behavior drives many crucial biological phenomena including morphogenesis, tissue repair, and cancer metastasis. These behaviors arise from cell interactions in addition to individual cell behavior [1], as it has been shown that an individual cell's behavior differs when in communication with other cells [2]. Previous studies on collective cell behavior have focused mainly on epithelial monolayers showing that cells are relatively uniform throughout the monolayer and cells are extruded when the layer become dense. Further, when cultured on protein islands which provide geometrical constraint, non-uniform patterns of proliferation more on edges, and cell alignment emerge due to gradients in mechanical stresses and contact guidance at the edges [3, 4]. Cell-cell transmission and build-up of stresses within these monolayers and constrained cell aggregates have been estimated with computational models since they cannot be directly measured. The models suggest symmetric stress distribution in circular aggregates, higher on the edges and lower in the center yet they assume uniform cell properties.

In our observations of valvular interstitial cells (VICs), we noticed more heterogeneity of cell distribution than reported with other types of cells on micro contact printed islands regardless of the shape. VICs crowd in the middle, yet, in contrast to epithelial cells, they do not extrude as single cells. Rather densification occurs at the center of aggregates which leads to apoptosis, likely driven by collective cell behavior and reduction of stress in the center which is predicted by our computational models when radially varying modulus or contractility are included [5]. We have also observed heterogeneity in the cell distribution and formation of multicellular bands spanning the 200 μm circular 2D aggregates [5]. Further, rather than high tractions around the edge of each pattern, we observed hotspot tractions associated with the cells spanning the aggregates.

We hypothesize that the heterogeneity observed in traction force maps is due to the formation of bands with more elongated cells at the endpoints and the formation of local hyperconfluent regions with rounded cells within constrained cell aggregates. To quantify the mechanical state of the cells within the aggregates, we measure the collective forces that cells apply to the substrate using traction force microscopy (TFM). Further, we utilize relatively large 400 μm diameter microcontact printed circular islands to increase the homogeneity of the cell behaviors and minimize the ability of single cells to span the pattern.

METHODS

Substrate preparation

Circular collagen patterns of 400 μm were printed onto 19.2 kPa polyacrylamide (PA) gels. We used the size of micropatterns of 400 μm to minimize single cell spanning of the pattens and increase the homogeneity of the collective behavior i.e., obtain a more clear differentiation between edge and center of the cell islands.

The surface of each substrate was sandwiched between glass coverslips coated with fluorescent micro-beads to transfer fluorescent displacement tracking beads to the surface of the gel which were prepared per [5]. To analyze the displacement of the beads, a custom MATLAB code was used[5].

Direct microcontact printing

To form microcontact printed protein islands, collagen was directly printed on the previously described PA gels per [5].

Cells and media

Porcine aortic VICs, isolated per [6] from a heart obtained from a local abattoir (Blood Farm, Groton, MA), were chosen as a primary fibroblastic cell type; porcine VICs are commonly used for CAVD research due to the similarity to human VICs. Cells were cultured in DMEM with 10% FBS and 1% Antibiotic/Antifungal at 37 °C in 10% CO2.

Experimental design

VICs were seeded at passage 3-6 at 12,500 cells per cm^2 [7] on microcontact printed substrates. 24 hours post-seeding, the aggregates

were imaged with phase contrast and red fluorescent channels; then utilizing trypsin, the cells were detached from the substrate and a red fluorescent image was captured again. Two red fluorescent images, with cells attached and after cells detachment, were analyzed using the customized MATLAB code, to calculate the displacement on the surface of the PA gel.

RESULTS

Heterogeneity of cell distribution in microcontact printed aggregates

Despite the large size of the protein islands, we observed heterogeneity in cells distribution and formation of multicellular bands spanning across the protein island diameter. Figure 1 shows a four-day time-series images in which a clear multi-cellular band is formed. From observations of many aggregates, it appears that banding occurs over time when cells have more affinity with lining up with each other than forming symmetric rings due to contact guidance around the edges. The bands do not disappear, rather they strengthen over time. This banding is not predicted by existing computational models and the mechanisms driving this behavior are unclear.

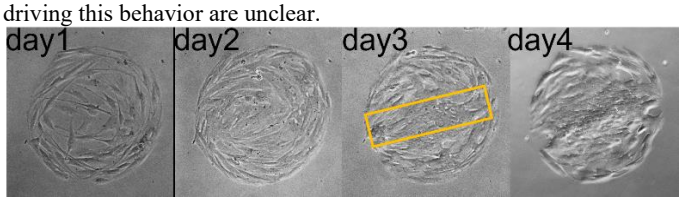

Figure 1. Phase images of an aggregate of 400 µm over 4 days, showing the heterogeneity of the cell distribution and formation of band crossing over the aggregate surface, yellow rectangle showing the cells alignment and band formation.

Hyper-confluency and banding changes the mechanical state of the cells in aggregate

Rather than generate high traction stresses uniformly around the circumference of the patter as is observed for epithelial cells, the calculated traction forces for the VICs is significantly higher at the endpoints of the bands (Fig. 2).

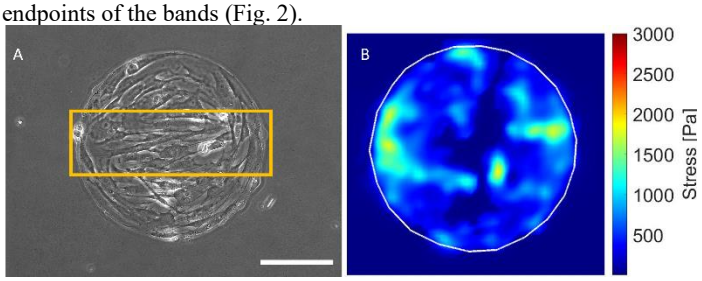

Figure 2. TFM demonstrates that the cells apply higher forces to the substrate at the endpoints of the bands, shown by yellow rectangle, collectively. A) Phase image of a sparse aggregate. B) TFM analysis of the aggregate in sparse stage. Scale bar = 200 µm.

Further, to quantify the forces cells apply to the substrate in local hyper-confluent areas, we have imaged a hyper-confluent aggregate, with a clear local hyper-confluent region, Figure 3. In the TFM image, Fig 3B, it is evident that the cells in hyper-confluent regions, even though very high in number and density, apply significantly lower forces to the substrate compared to the endpoints of bandings and the areas with elongated cells.

DISCUSSION

Multicellular banding creates heterogenous stress patterns

We hypothesized that with the larger protein islands, we would observe more uniform traction force patterns with high stress on the outer circumference and low tractions in the center. Indeed, single cells were no longer able to span the islands; however, we still observed substantial heterogeneity in the traction force maps. To our surprise, large multicellular bands formed across the patterns rather than forming radially symmetric patterns with predominantly circumferentially cell orientation. We observed that the traction stresses were clearly higher at the endpoints of the bands. Interestingly, in the center of the bands the cell density is very high, which is correlated with low stress and increased rates of apoptosis.

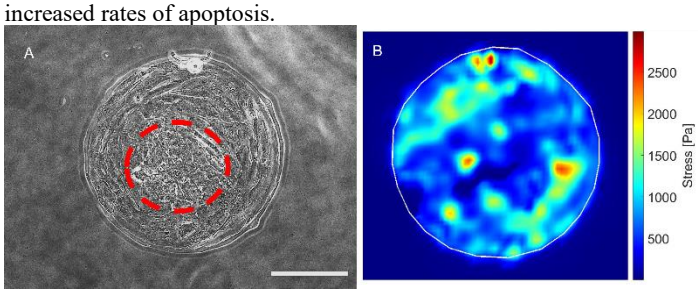

Figure 3. TFM shows that in hyper-confluent regions, shown by red dotted circle, the cells apply significantly lower forces to the substrate collectively. A) Phase image of a hyper-confluent aggregate. B) TFM analysis of the aggregate in hyper-confluent stage. Scale bar = 200 µm.

Asymmetric mechanical state

Previous studies have shown that cells require a homeostatic tension to survive; for example, states of low cell stress initiate apoptosis [8, 9]. Furthermore, indicators of high cell tension are localized to the edges of aggregates [10], while indicators of low cell tension are found in central regions [5]. Here we showed that these signals are not symmetric with primary mesenchymal cells and change with heterogeneity in confluency levels; we showed heterogeneity in cell distribution, which leads to asymmetric traction forces, which are higher at the two endpoints and not uniform around the edges.

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