ELECTRONIC SUPPLEMENTAL INFORMATION

Surface area of spreading and thickness of valve interstitial cell *in vitro*. We first measured the natural area of spreading of valve interstitial cells (VICs) in sparse *in vitro* culture conditions on different substrates, to determine the surface area of spreading of VICs as a function of substrate stiffness. These results were then used to design rectangular templates for subsequent soft lithography and microcontact printing experiments. Briefly, VICs were sparsely seeded onto collagen gels, polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, Midland MI) coated coverslip and tissue culture plastic at a density of 3000 cells/cm^2. After 24 and 48 hours, phase contrast images of at least 50 single VICs per substrate were captured using a regular phase contrast microscope. Surface area of spreading of the VICs was then computed using Image J software (National Institutes of Health, Bethesda MD). Unconfined compressive stiffnesses of the seeding substrates were measured using an Instron 5944 uniaxial apparatus (Instron Corp, Norwood MA) using standard testing protocols.[1] Average cell spreading areas ranged from 600-1800um^2 after 48 hours culture as a function of substrate stiffness (Fig. 1A, ESI†). Specifically, VIC surface area measurements were consistently about 1700μm^2 for a wide range of substrate moduli from 10kPa to 1MPa, 48 hours after seeding (Fig. 1A, ESI†). VIC thickness was statistically similar for the three aspect ratios (Fig. 1B, ESI†).

Verification of valve interstitial cell aspect ratio as a function of imposed mechanical strain. As we mentioned previously, we chose three width-to-length aspect ratios (ARs; 1:3, 1:5, 1:7) for the current study based on previously published data on cell shape in normotensive and hypertensive strain and pressure conditions.[2] In order to independently confirm this, we seeded VICs at a confluent density within chambers on a stretchable polydimethylsiloxane (PDMS) membrane imposed external cyclical strain at 0%, 10% and 20% for 48 hours.[3] Phase contrast images of cells were taken using an upright microscope and the cellular AR was measured by manual tracing using ImageJ software. Our results (Fig. 1C, ESI†) indicate that the chosen three ARs (1:3, 1:5, 1:7) are a reasonable representation of cells under 0%, 10% and 20% cyclic strain respectively. We further demonstrate (Fig. 1D, ESI†) that the steady-state VIC aspect ratios are achieved after approximately 12 hours of cyclic stretching, which is lower than our experimental duration of 48 hours.

Determination of optimum culture conditions to maintain single cell culture. VICs in suspension are significantly smaller in projected area than adhered cells (142.6±3.26μm vs.

944.59±3.26μm based on our measurements, $n>50$, $p<0.05$; Fig. 1D, ESI†). There is therefore a high possibility that more than one cell can adhere onto a rectangular fibronectin feature during initial seeding. In addition, cells can also divide while on a rectangular feature. Our first objective was therefore to determine the optimum culture conditions that would result in the highest yield of single cells on the coverslip, both on first seeding and over the course of the 24 to 48 hour experiment. We hypothesized that (I) feature surface area; (II) cell seeding density and (III) FBS concentration would have the highest effects on single cell yield, and tested these factors as follows.

(I) Effect of feature surface area. As mentioned earlier, cells are significantly smaller in suspension than when adhered. We therefore first tested single cell yield on a range of island surface areas based on the spread of VICs on PDMS. We hypothesized that the larger the surface area, the lower the single cell yield as a larger number of cells might fall on larger fibronectin features. Briefly, based on the surface area of spread of VICs (Fig. 1A, ESI†), cells were seeded on 1500, 1700, 1900 and 2000μm^2 features. After 48 hours, cells were fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield PA) and fluorescently stained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies, ThermoFisher, Waltham MA). Images were then obtained at more than 20 image fields of the coverslips. Percentage of single cells was quantified by dividing the number of counted single cells by the total number of cells in that specific image field. Our results indicated that single cell yield was significantly higher for 1500, 1700 and 1900μm^2 patterns compared to 2000μm^2 patterns (Fig. 2A, ESI†). Within these three surface areas, 1700μm^2 patterns tended to reliably yield the largest numbers of single cells. We thus proceeded to use a pattern surface area of 1700μm^2 for all subsequent experiments.

(II) Effect of seeding density. As mentioned earlier, each substrate coverslip had approximately 12,500 single cell island features. We thus hypothesized that the closer the seeding density was to this number of features, the higher the yield of single cells. We further postulated that a seeding number lower than the total number of features would increase the chance of single cell islands on the coverslip. We therefore tested seeding densities from 5,000 – 15,000 cells per coverslip (corresponding to approximately 500 - 1500 cells/cm^2 seeding density). After 48 hours, cells were fixed with 4% PFA and fluorescently stained with DAPI and quantified as before. Seeding densities from 500 to 1000 cells/cm^2 consistently produced approximately 80% of single

cell yield. We observed that the number of multiple cells per feature increased if we increased the seeding density as seen in the density of 1200-1500 cells/ cm^2. Therefore, we used a seeding density of 1000 cells/ cm^2 for the remaining experiments reported in this paper (Fig. 2B, ESI†).

(III) Effect of FBS concentration. As increasing FBS concentration has a strong proliferative effect on cells,[4] we hypothesized that lowering the concentration of FBS during the course of culture would prevent VICs from dividing while on a rectangle. Three concentrations of FBS (0%, 2% and 10%) were tested by exchanging the culture medium at 4 hours and 24 hours after seeding. All other components of the medium remained unchanged. After 48 hours, cells were fixed with 4% PFA and fluorescently stained with DAPI and quantified as before. Our results indicated that low FBS-containing medium produced high number of single cells (Fig. 2 C-E, ESI†). The best culture condition was to feed cells with 0% FBS-containing medium for the first 4 hours. This condition was postulated to restrict cells to attach solely to fibronectin patterns. After 4 hours, 2% FBS-containing medium was exchanged to both remove non-adhered cells and provide enough nutrients for cells to grow. 24 hours later, cells were fed with 0% FBS-containing medium for growth arrest. From this point, we kept using 1700 μm^2 patterns as well as the seeding density of 1000 cells/ cm^2. Taken together, we consistently obtained an average of 80% single cells per coverslip. This result was especially significant as it was currently the only study that reported the specific yield of single cells and the methodology to achieve it. Other studies on single cells *in vitro* either did not report the exact number of single cell that they achieved or reported very low numbers of approximately smaller than 10%.[5]

Supplemental Figures

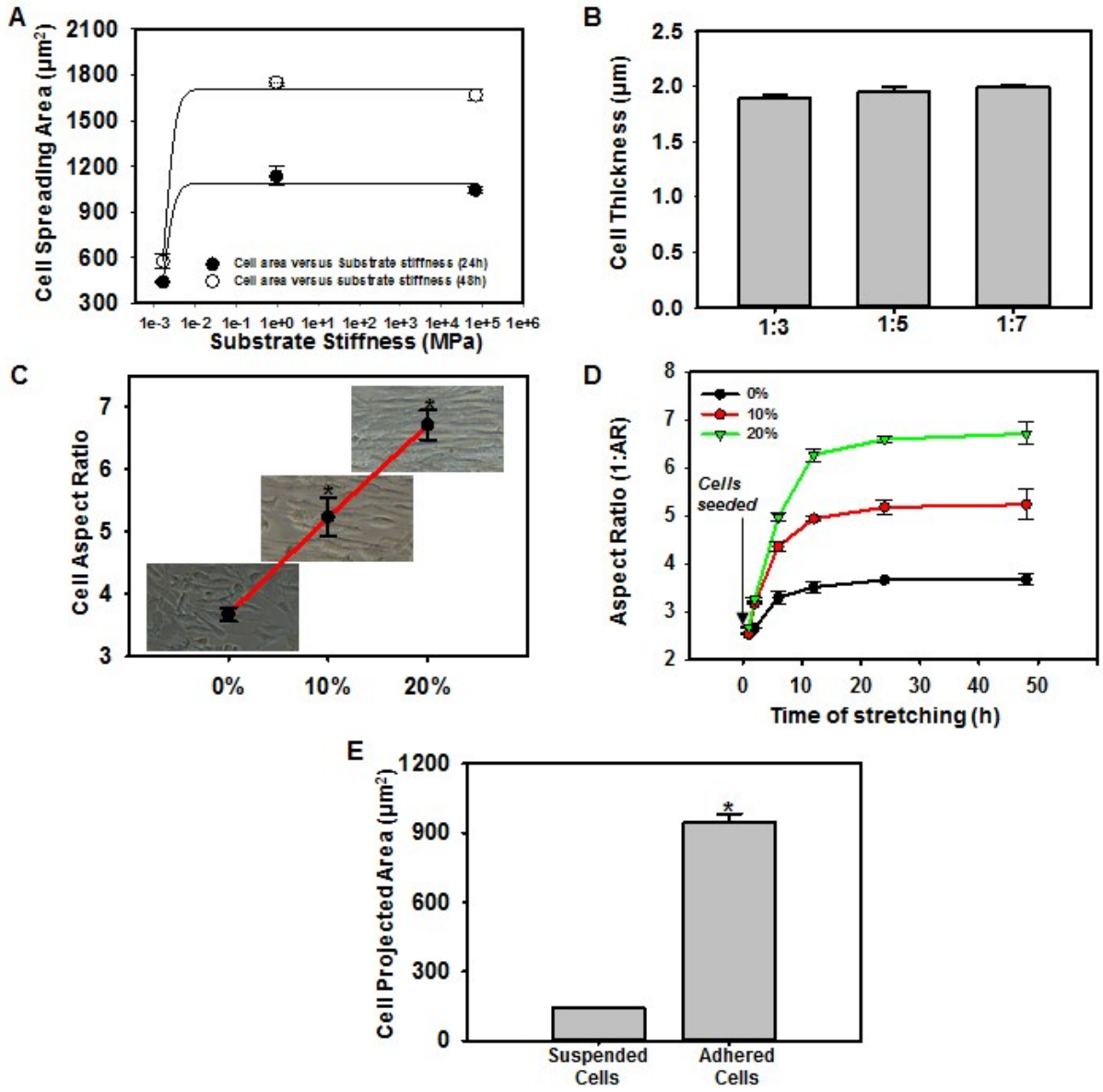

Figure 1: (A) Variation of VIC spreading area as a function of substrate stiffness measured after 24 and 48 hours post-seeding of cells. (B) VIC cell thickness data. (C) VIC AR measured on cyclically stretched cells. (D) Temporal evolution of VIC AR as a function of 0%, 10%, or 20% cyclic stretch. (E) Projected area of cells in suspension and adherent cells (* $p<0.05$).

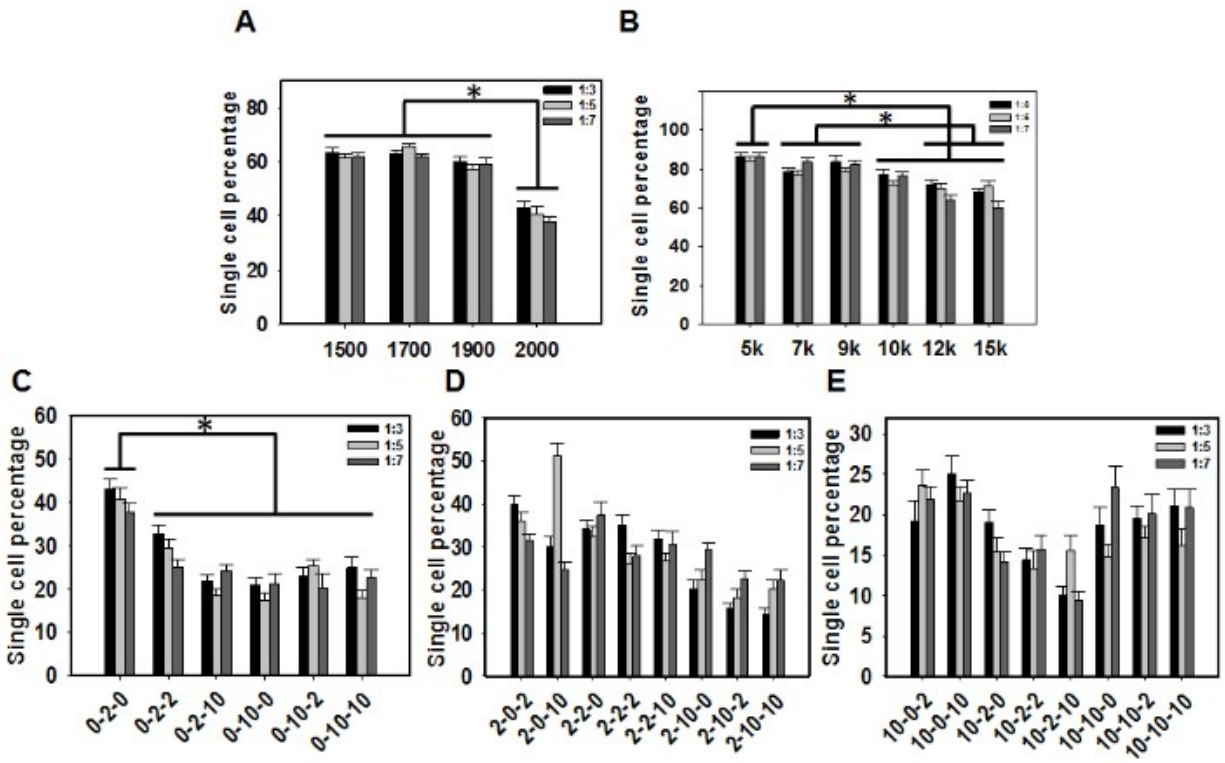

Figure 2: Factors affected single cell yield. (A) Percentage of single cells as a function of patterned fibronectin surface area. (B) Percentage of single cells at different seeding densities. (C-E) Percentage of single cells as a function of FBS percentage in media grouped according to concentration at seeding (n>50 individual cells, * $p<0.05$).

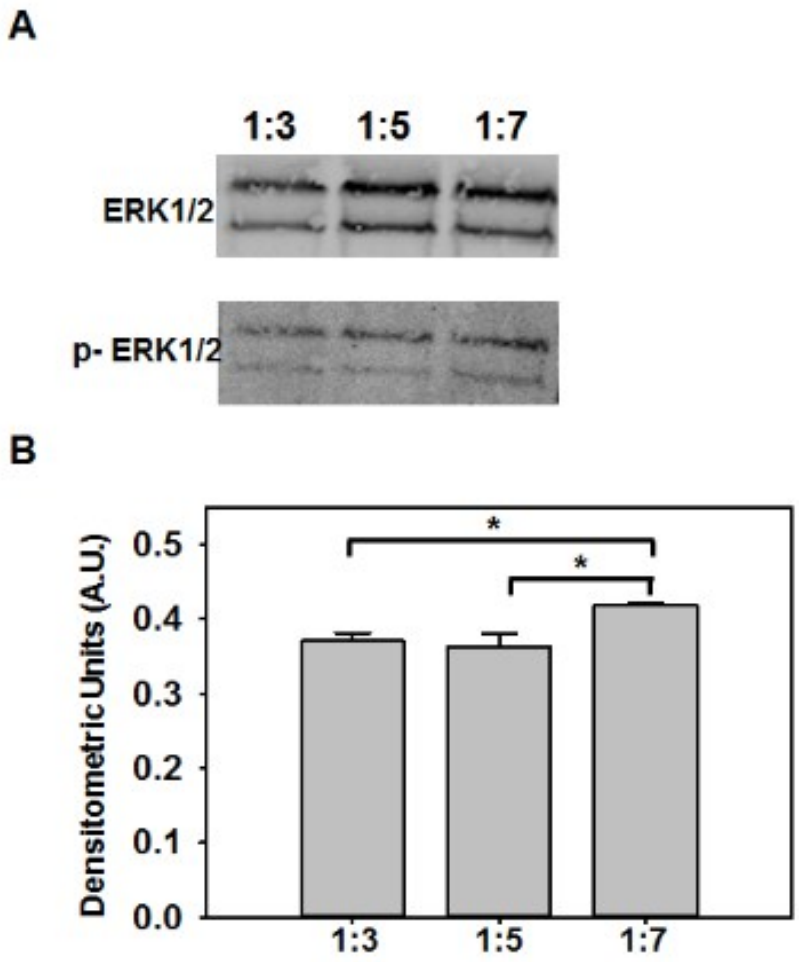

Figure 3: (A) Representative western blot scan for ERK1/2 phosphorylation analysis. (B) Densitometric analysis of ERK1/2 phosphorylation western blots.* $p<0.05$.

Supplemental References

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