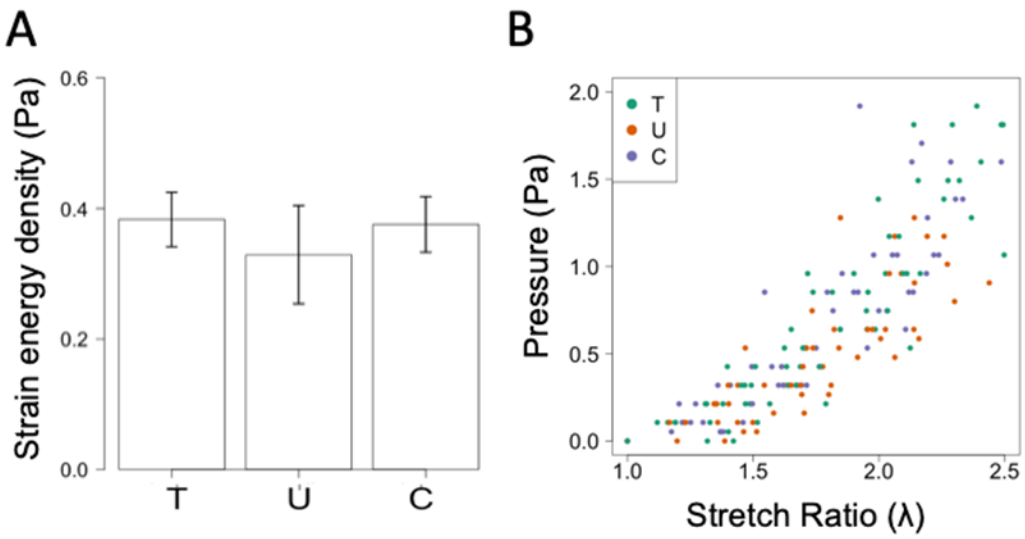

Figure S1. Osmotic stress does not alter tissue stiffness. (A) Micropipette aspiration measurements of strain energy density, (B) Mechanical response curves for the cushions tested n = 5-6 cushions per condition per 3 independent experiments, SEM shown.

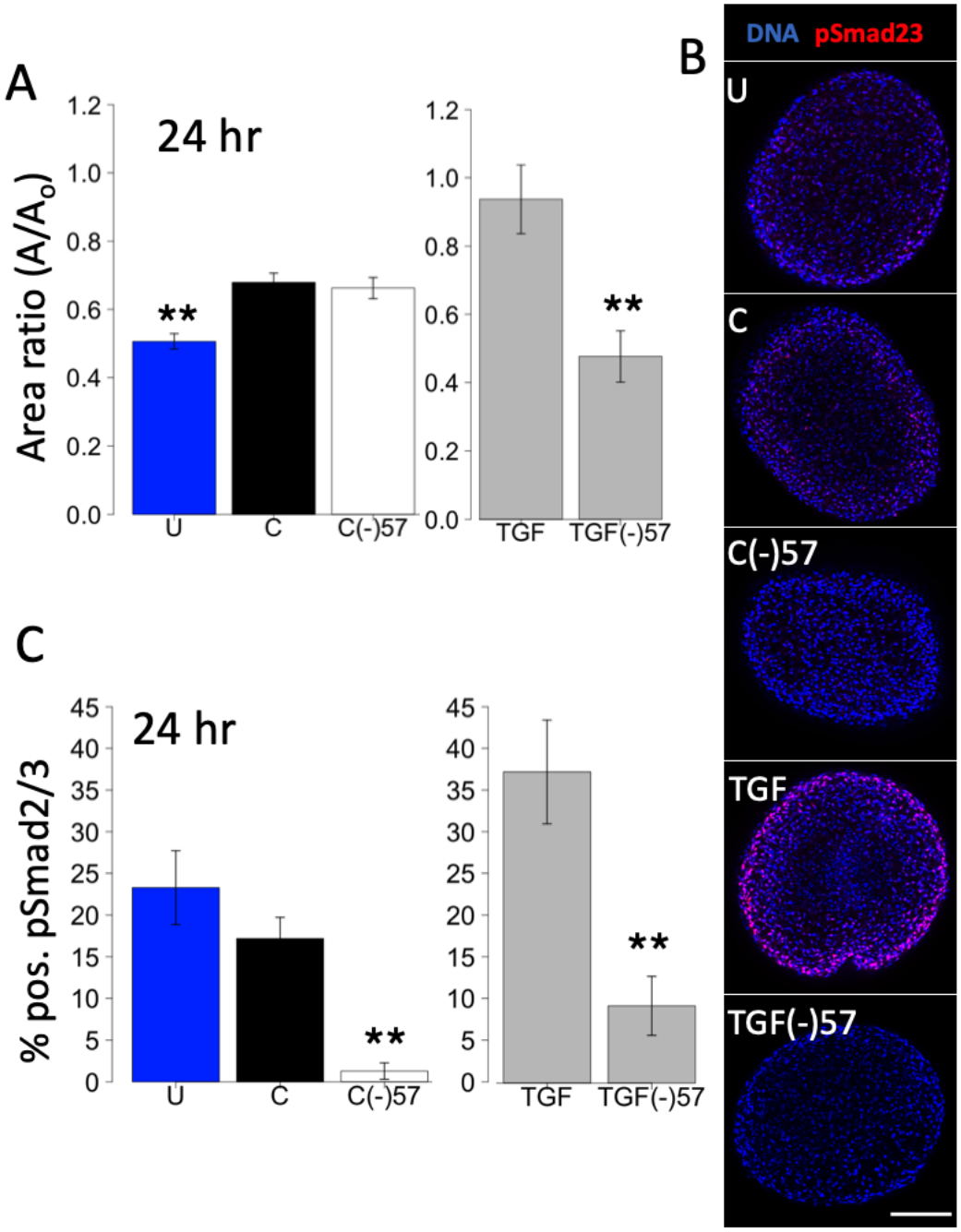

Figure S2. Osmotic stress compaction phenotype is pSmad2/3 independent. (A) Compaction trend at 24hrs ** $p < 0.005$ between conditions (B) Number of cells positive for pSmad2/3 at 24hrs (D) Whole mount IF images of 24 hr. cushions stained for pSmad2/3, scale bar 100uM, n=6-8 cushions per conditions, SD shown.

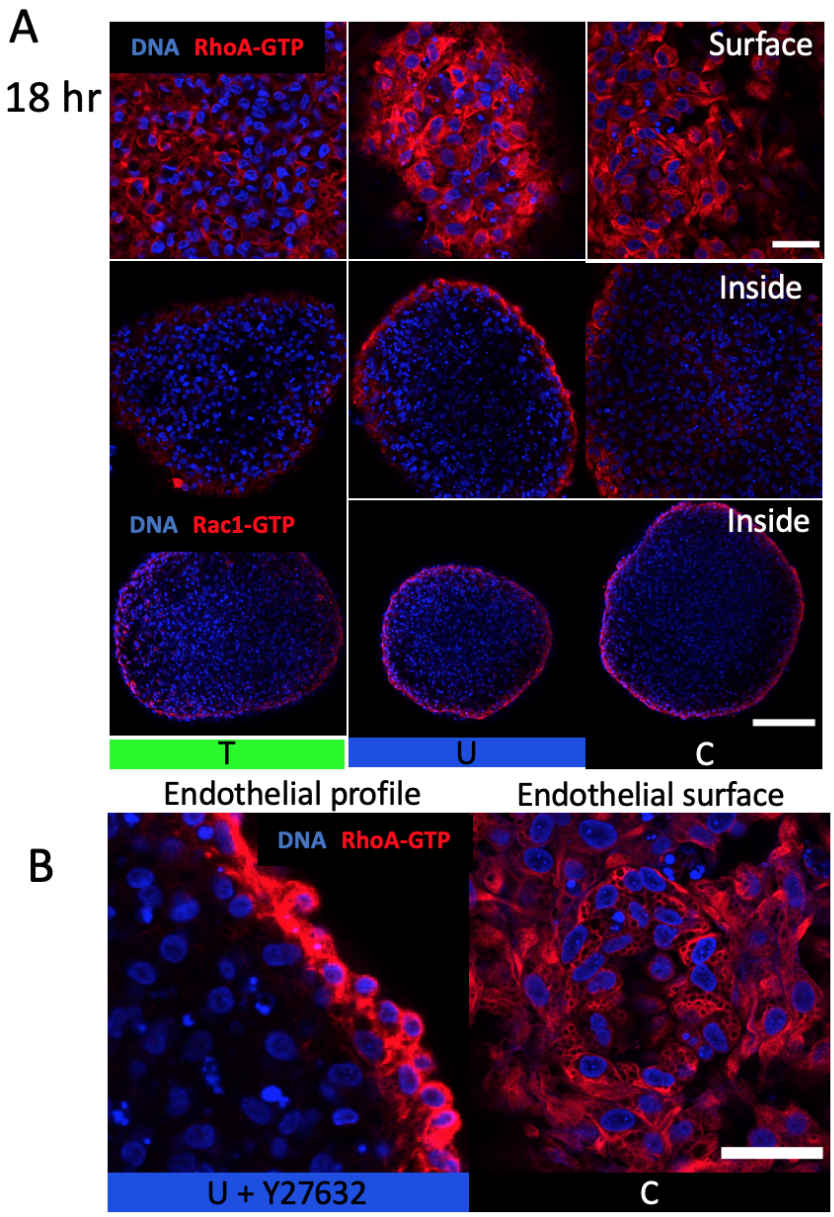

Figure S3. Osmotic stress affects endothelial RhoA-GTP patterning not activity. (A) RhoA-GTP localization on cushion surface and 30 microns inside and Rac1-GTP inside (B) Disruption of apical-basal polarity at endothelium with ROCK inhibition (C) Honeycomb like pattern in RhoA-GTP stain associated with compressive stress

Evaluation of 2D and 3D compaction

Cushion area can be measured from a single image of a live cushion and captures the relationship between treatment conditions without requiring measurements orthogonal to the imaging plane. Figure S4 demonstrates that using either an area ratio (Fig. S4A) or volume ratio (Fig. S4B), the relationship between conditioned medias is preserved.

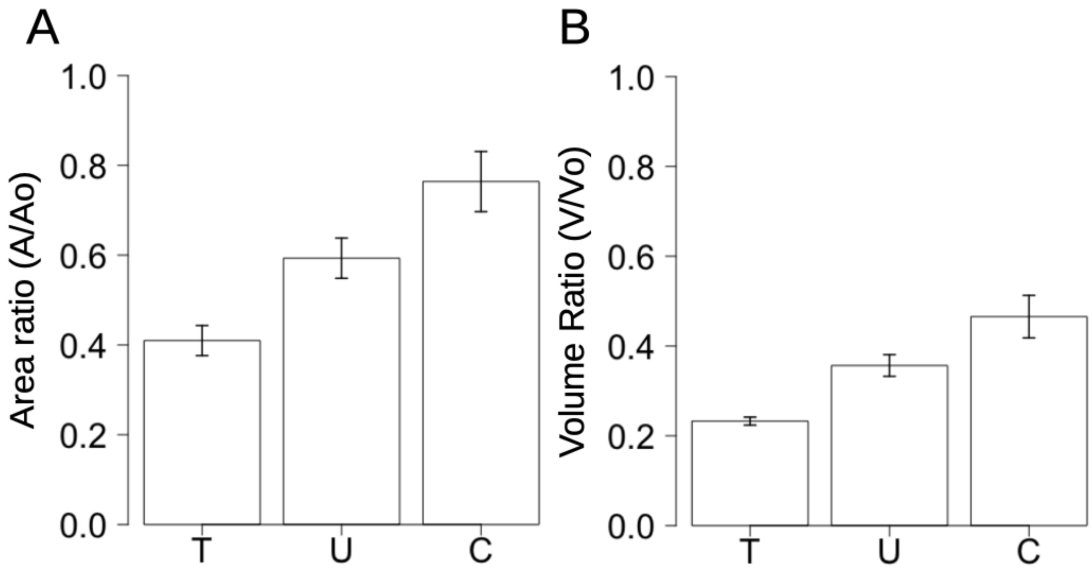

Figure S4. Area and volumetric compaction of HH25 cushions yield the same relationship between conditioned medias. (A) Area ratios comparing cushion areas at 0hrs and 24hrs. (B) Volume ratios comparing cushion volumes at 0hrs and 24hrs. T = Tensile stress, U = Unloaded, C = Compressive stress.

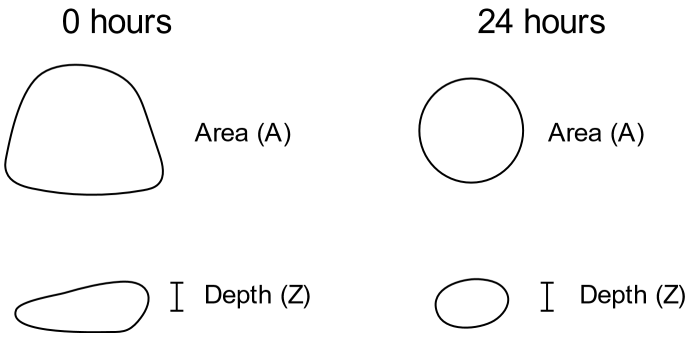

Figure S5. Schematic of HH25 cushion morphologies at 0 and 24 hours of culture

To approximate the volume of live cushions, cushion height was measured by comparing the microscope Z-position when focused on the top-most surface of the cushion and the centerline of the cushion. Volumetric compaction of HH25 cushions was calculated using two different approximations for volume at t = 0 hours and t = 24 hours. At the initial time point, the cushions are flatter and their upper volume was approximated as their 2D area (A) multiplied by their calculated thickness (Z) as represented in Figure S4:

$$\frac{V_o}{2} = A_o Z_o$$

At the 24-hour time point, however, cushions form an ellipsoid. While the volume of an ellipsoid is given by $V = \frac{4}{3}\pi abc$, where a, b, and c represent the axial radii, compacted HH25 cushions have relatively similar radii on their x- and y-planes under a microscope. As a result, the formula can be simplified to:

$$V_f = \frac{4}{3}\pi abc = \frac{4}{3}\pi r^2 Z_f$$

$$\frac{V_f}{2} = \frac{2}{3} A_f Z_f$$

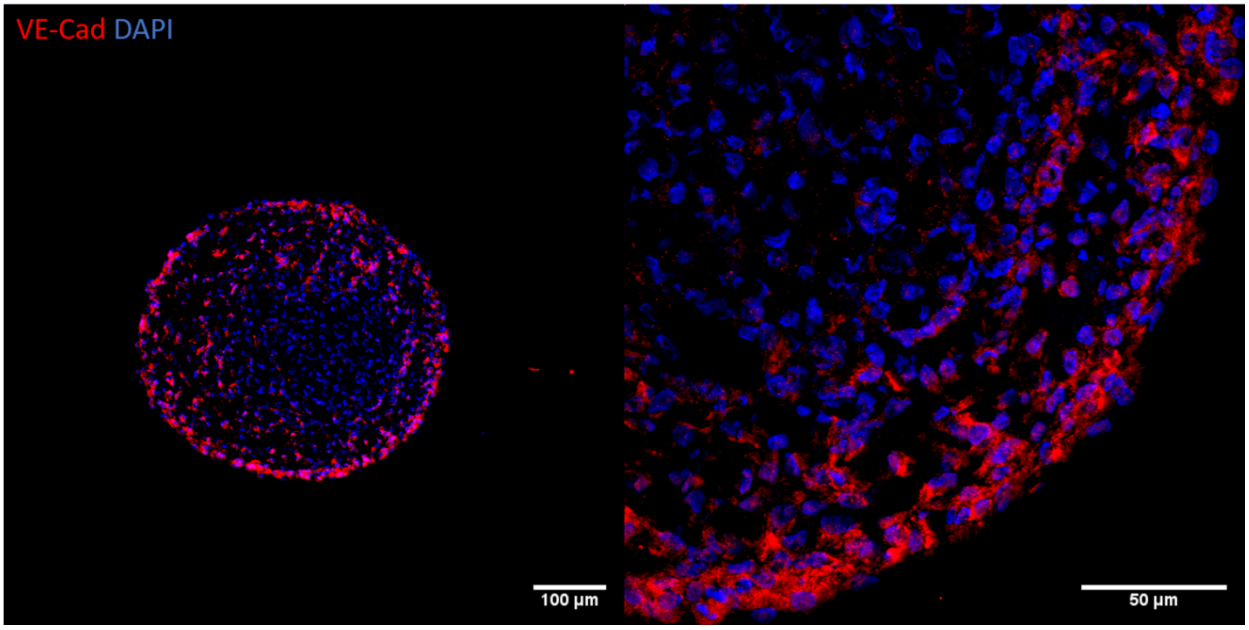

Figure S6. VE-Cadherin labeling shows most endothelial cells remained on the surface of cushions.

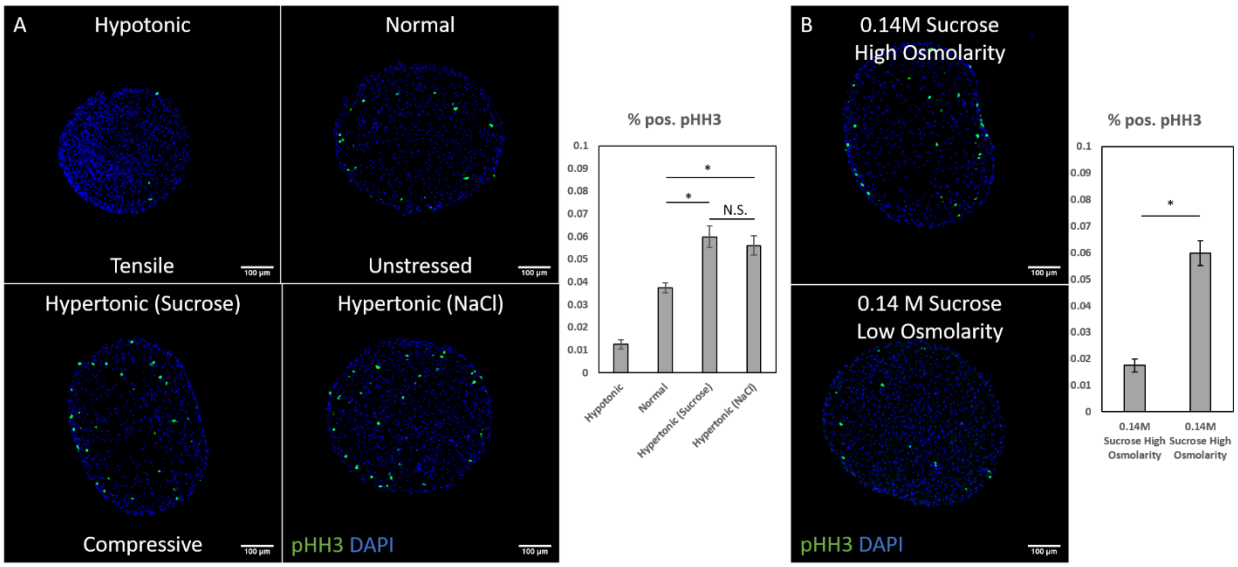

Figure S7. Addition of sucrose did not have a significant impact on cell proliferation. (A) Representative IF images of pHH3 in HH25 cushions cultured under osmotic stress for 24 hours, and quantification of pHH3 positive cells, n = 4-5, SD shown. (B) Representative IF images of pHH3 in HH25 cushions cultured in media with the same amount of sucrose added but different osmolarity for 24 hours, and quantification of pHH3 positive cells, n = 4-5, SD shown.

Table S1. Cytokines and inhibitors

Target	Name	Concentration	Provider	Dosage reference
ROCK	Y-27632	10 μM	Cell Signaling	(Gould et al., 2016)
Alk2/3	LDN193189	1 μM	Sigma	(Ahsan et al., 2016; Boergermann et al., 2010)
Alk 5/7	SB431542	2.6 μM	Sigma	(Buskohl et al., 2012a,b)
MEK	U0126	10 μM	Sigma	(Aubin et al., 2004)
NM-Myosin II	(+-) Blebbistatin	1 μM-10 μM	VWR BioVision	Manufacture solubility and empirical titration