Biophysical Letter

Osmotic Regulation Is Required for Cancer Cell Survival under Solid Stress

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ABSTRACT   For a solid tumor to grow, it must be able to support the compressive stress that is generated as it presses against the surrounding tissue. Although the literature suggests a role for the cytoskeleton in counteracting these stresses, there has been no systematic evaluation of which filaments are responsible or to what degree. Here, using a three-dimensional spheroid model, we show that cytoskeletal filaments do not actively support compressive loads in breast, ovarian, and prostate cancer. However, modulation of tonicity can induce alterations in spheroid size. We find that under compression, tumor cells actively efflux sodium to decrease their intracellular tonicity, and that this is reversible by blockade of sodium channel NHE1. Moreover, although polymerized actin does not actively support the compressive load, it is required for sodium efflux. Compression-induced cell death is increased by both sodium blockade and actin depolymerization, whereas increased actin polymerization offers protective effects and increases sodium efflux. Taken together, these results demonstrate that cancer cells modulate their tonicity to survive under compressive solid stress.

Solid tumors grow under stress from the local tissue. This growth results in further accumulation of compressive stress in the tumor. To remain viable and grow in vivo, the tumor cells must be able to survive under these compressive stresses, which correspond to mechanical loads of 10–100 mmHg (1,2). Previous work has shown that this solid stress prevents the growth of cancer cell spheroids (1). Moreover, as this stress accumulates in vivo, it can lead to further adverse effects such as blood vessel compression, hindering drug delivery (2). Despite the importance of solid stress in tumor growth, however, few studies have explored how cancer cells actually support this stress.

Most of the literature suggests that the ability of cells to resist deformation largely emanates from the cytoskeleton (3). A series of studies have demonstrated that actin, microtubules, and intermediate filaments all contribute to support external compressive stresses (4–6). In accord with these studies, in a genetically engineered model of the epithelial-mesenchymal transition, we recently found that mesenchymal cells could support less stress than their epithelial counterparts, which correlated with decreased polymerized actin and cytoplasmic stiffness (7). Here, we show that compressive stress is not actively supported by cytoskeletal filaments, but induces NHE1-dependent sodium efflux from the tumor cells. Although actin polymerization did not actively support solid stress, depolymerization of actin mitigated the ability of cells to efflux sodium. Taken together, these results show that regulation of intracellular tonicity is required for cells to maintain viability under compressive stress.

Supporters of solid stress

To analyze how cancer cells support compressive-solid stress, we cultured tumor spheroids from single MCF7 breast cancer cells in inert agarose gels, resulting in the accumulation of solid stress (1,8). Next, to elucidate the roles of cytoskeletal filaments in supporting this stress, we treated the spheroids with a variety of cytoskeleton-stabilizing and -destabilizing molecules, and tracked changes in spheroid cross-sectional areas using live-cell microscopy (for details, see Supporting Materials and Methods in the Supporting Material). Unexpectedly, depolymerization of actin filaments using cytochalasin D resulted in an increase in spheroid size, whereas increasing actin polymerization with Jasplakinolide had no effect, suggesting that actin was not primarily responsible for supporting solid stress (Fig. 1 A). Modulation of microtubule polymerization did not alter spheroid diameter (Fig. 1 B), and depolymerization of intermediate filaments with 4 mM of acrylamide (9) produced a slight increase in spheroid area (Fig. 1 C). Since none of these treatments produced significant decreases in spheroid area, indicating that they actively supported stress, we hypothesized that the stress may instead be supported by osmotic pressure. Decreasing media tonicity caused spheroid swelling, whereas increasing the media tonicity
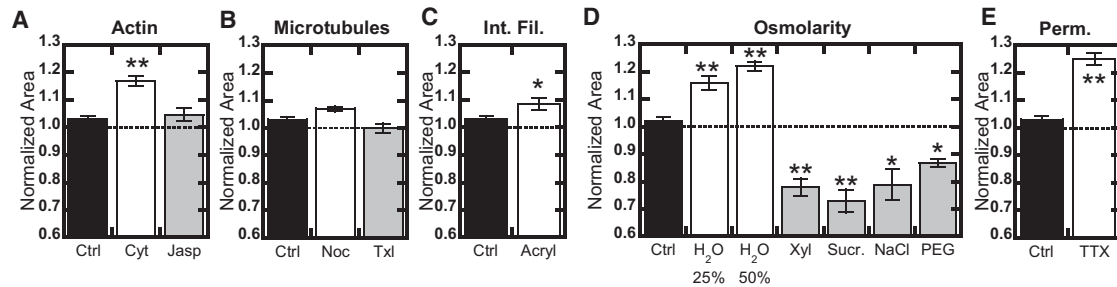
FIGURE 1 Change in spheroid area after 6 h of treatment, with all values normalized to the initial area before treatment. (*A*) Depolymerization of actin with cytochalasin D (Cyt) and enhanced polymerization with Jasplakinolide (Jasp). (*B*) Microtubule depolymerization with nocodazole (Noc) and stabilization with Taxol (Txl). (*C*) Depolymerization of intermediate filaments with acrylamide. (*D*) Altering media osmolarity with water (hypoosmotic) or 250 mM xylose, sucrose, NaCl, or PEG400 (hyperosmotic). (*E*) Spheroid permeabilization with 0.0025% Triton-X100 (TTX). Values given are mean $\pm$ SE; *$p < 0.05$, **$p < 0.01$.

with a variety of solutes caused spheroids to shrink (Fig. 1 *D*). In addition to breast cancer MCF7 cells, this phenomenon was also observed in ovarian cancer OVCAR3 cells and prostate cancer DU145 cells (Fig. S1), suggesting it is conserved across other solid tumor models.

To verify that these changes were due to solid stress and were not an artifact of the spheroid culture, we generated equal-sized spheroids by anchorage-independent growth in solution for 1 week before embedding them in agarose overnight for analysis in the absence of significant accumulated stress. These unstressed spheroids showed larger fold changes in response to tonicity, presumably because there was no initial stress, allowing for easier expansion against the gel. However, in contrast to the stressed spheroids, the unstressed spheroids collapsed after actin depolymerization (Fig. S2). This result shows that actin polymerization is required for cell structure under conditions of minimal solid stress, but primarily controls the ability of cells to efflux sodium under higher levels of solid stress.

Finally, to ascertain whether cells in steady-state stressed spheroids have higher or lower tonicity than their surrounding media, we permeabilized cells with 0.0025% Triton-X100. After permeabilization, the spheroids increased in area by >25% (Fig. 1 *E*). Based on osmotic models of

cell size, this implies that cells within stressed spheroids have lower tonicity than their surrounding media, such that after permeabilization, extracellular ions enter the cells, inducing an inward water flux (and consequently spheroid swelling) to equilibrate (10).

Sodium efflux under compressive solid stress

To ascertain how the cells decreased their intracellular tonicity, we utilized a 2D model of solid stress. Here, instead of allowing spheroids to accumulate solid stress from growth, we used weights to directly apply a controlled 5 mmHg stress to a monolayer of cells (11), on the same order of magnitude as the stresses generated by spheroids (9.8 $\pm$ 0.3 mmHg). Quantification of intracellular sodium with CoroNa Green revealed that the cells effluxed sodium after mechanical compression (Fig. 2 *A*).

Although blockade of sodium channel NKKC1 did not produce a statistically significant difference in sodium efflux under stress, inhibition of the sodium-hydrogen channel NHE1 with ethyl-isopropyl amiloride (EIPA) did significantly block sodium efflux ($p < 0.05$; Fig. 2 *B*). This is consistent with previous studies that showed that hydrostatic pressure induces sodium efflux (12). Repeating this
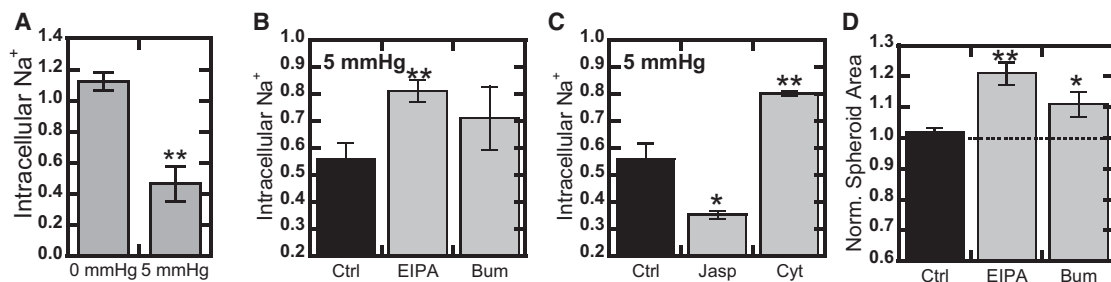

FIGURE 2 Cancer cells efflux sodium under solid stress. (*A*) A 2D monolayer of MCF7 cells was loaded with CoroNa Green sodium tracer and compressed with 5 mmHg solid stress for 2 h. CoroNa intensity was measured using a plate reader and values are reported relative to the zero time point. (*B*) Intracellular sodium under 5 mmHg stress in the presence of the NHE1 inhibitor EIPA and the NKCC1 inhibitor bumetanide (Bum). (*C*) Intracellular sodium under 5 mmHg stress in the presence of Jasplakinolide (Jasp) to enhance actin polymerization, and cytochalasin D (Cyt) to depolymerize actin. (*D*) Blockade of the sodium channels NHE1 (EIPA) and NKCC1 (Bum) in 3D tumor spheroids. Values given are mean $\pm$ SE, *$p < 0.05$, **$p < 0.01$.

experiment with modulation of actin filaments revealed that actin depolymerization blocked sodium efflux to a similar degree as NHE1 blockade. Consistent with this, actin stabilization with Jasplakinolide produced a slight but statistically insignificant ($p = 0.063$) increase in sodium efflux (Fig. 2 C). None of the inhibitors altered sodium levels in the absence of stress (Fig. S3). These results suggest that although polymerized actin may not actively support stress, it is required for cells to function properly under compression. This is consistent with recent studies that showed the importance of NHE1 for confined cell migration, where it colocalizes with polymerized actin (13).

To verify that these results would translate into our 3D spheroid model, we repeated the spheroid assay. Blockade of NKCC1 with bumetanide caused modest swelling, but consistent with the sodium efflux findings, NHE1 blockade with EIPA produced a larger increase in spheroid cross-sectional area (Figs. 2 D and S1), which was identical to that seen with actin depolymerization (Figs. 1 A and S1). This swelling was not observed in unstressed spheroids (Fig. S2).

Sodium efflux, tonicity, and actin polymerization contribute to cell viability under stress

To test whether the observed stress-induced sodium efflux was actually protective, we finally analyzed the viability of cells under compression. In our 2D system, we found that compression with 5 mmHg induced significant cell death (Fig. 3 A). Although cells in hypertonic media showed increased death without compression, 5 mmHg of compression did not significantly increase the amount of death in these cells, suggesting that it may have served to protect the surviving cells (Fig. 3 A). Increasing actin polymerization with Jasplakinolide was more protective under stress, significantly decreasing cell death relative to the compressed control. Conversely, decreased media tonicity, blockade of sodium efflux, and actin depolymerization all significantly increased the amount of cell death under compression (Fig. 3 A). Although Jasplakinolide pretreatment improved 2D sodium efflux and viability under stress, it was not sufficient to decrease the 3D spheroid size. This may be because spheroid growth selected for tumor cells that could optimally efflux sodium under stress and did not need additional polymerized actin to increase this efflux rate.

To verify that these mechanisms were conserved in our 3D model, we repeated the analysis of viability after addition of EIPA and cytochalasin D. These results are presented as the resulting cell death as a function of radial stress (Fig. 3 B). If these molecules induce compressive stress-dependent toxicity, we should see increasing cell death as a function of radial stress in the spheroids. In the control condition, there is a weak positive correlation between radial stress and cell death, which is subsequently increased by NHE1 inhibition and even further increased by actin
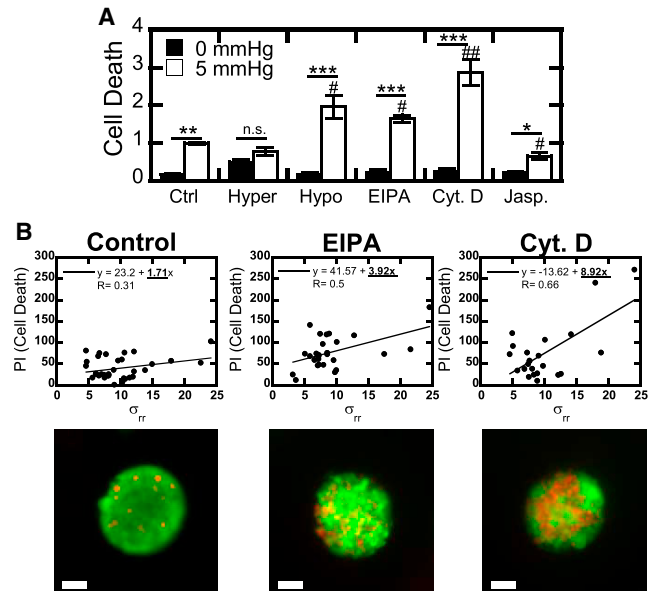

FIGURE 3 Sodium efflux and actin polymerization are required for viability under solid stress. (*A*) Quantification of cell death based on the ratio of propidium iodide to calcein AM after 4 h of compression of a 2D monolayer. Hyper: 250 mM xylose; hypo: 25% H_2O. (*B*) Cell death in 3D tumor spheroids versus radial stress. Stress-dependent toxicity is indicated by the slope value. Representative images of spheroids stained for live cells (calcein AM, *green*) and dead cells (propidium iodide, *red*) are given below. Scale bar is 50 μm. Values given are mean $\pm$ SE; # indicates significant difference compared with stressed control; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. To see this figure in color, go online.

depolymerization, demonstrating that both are required for tumor cells to survive under stress.

CONCLUSIONS

Cells use ion pumps to maintain a constant volume by modulating their tonicity and subsequently their osmotic pressure (14). During mitosis, cells also use osmotic pressure to generate a rounding force, which is counteracted by actomyosin tension (15). Here, we document that under compressive stress, multicell spheroids as well as cell monolayers will decrease their intracellular tonicity by effluxing sodium, and that although cytoskeletal filaments do not actively support compressive stress, actin polymerization is required for osmotic regulation. This finding is consistent with the work of Hui et al. (12), who found that increased hydrostatic pressure induced active sodium efflux. Recent studies using *C. elegans* suggested that these organisms may have an absolute internal pressure set point (16). This could be consistent with the model of cell volume and pressure regulation presented by Jiang and Sun (10), which relates changes in cell volume to water flux controlled by differences in hydrostatic and osmotic pressures across the cell membrane. The lower steady-state intracellular tonicity

under stress implies a negative pressure change across the cell membrane from osmotic pressure. This negative pressure change may help offset the compressive stress. Taken together, our results demonstrate that to survive under compressive stress, cells must be able to modulate their tonicity by effluxing sodium, and that polymerized actin is necessary for this process.

SUPPORTING MATERIAL

Supporting Materials and Methods and three figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(15)00782-1.

AUTHOR CONTRIBUTIONS

D.J.M., K.M.M., C.P.B, N.R., and Q.M.N.K. performed experiments. D.J.M. designed research and analyzed data. D.J.M., K.M.M., and M.R.D. wrote the manuscript.

ACKNOWLEDGMENTS

REFERENCES

1. Helmlinger, G., P. A. Netti, …, R. K. Jain. 1997. Solid stress inhibits the growth of multicellular tumor spheroids. *Nat. Biotechnol.* 15:778–783.

2. Stylianopoulos, T., J. D. Martin, …, R. K. Jain. 2013. Coevolution of solid stress and interstitial fluid pressure in tumors during progression: implications for vascular collapse. *Cancer Res.* 73:3833–3841.

3. Fletcher, D. A., and R. D. Mullins. 2010. Cell mechanics and the cytoskeleton. *Nature.* 463:485–492.

4. Chaudhuri, O., S. H. Parekh, and D. A. Fletcher. 2007. Reversible stress softening of actin networks. *Nature.* 445:295–298.

5. Brangwynne, C. P., F. C. MacKintosh, …, D. A. Weitz. 2006. Microtubules can bear enhanced compressive loads in living cells because of lateral reinforcement. *J. Cell Biol.* 173:733–741.

6. Mendez, M. G., D. Restle, and P. A. Janmey. 2014. Vimentin enhances cell elastic behavior and protects against compressive stress. *Biophys. J.* 107:314–323.

7. McGrail, D. J., R. Mezencev, …, M. R. Dawson. 2015. SNAIL-induced epithelial-to-mesenchymal transition produces concerted biophysical changes from altered cytoskeletal gene expression. *FASEB J.* 29:1280–1289.

8. Roose, T., P. A. Netti, …, R. K. Jain. 2003. Solid stress generated by spheroid growth estimated using a linear poroelasticity model. *Microvasc. Res.* 66:204–212.

9. Wang, N., J. P. Butler, and D. E. Ingber. 1993. Mechanotransduction across the cell surface and through the cytoskeleton. *Science.* 260:1124–1127.

10. Jiang, H., and S. X. Sun. 2013. Cellular pressure and volume regulation and implications for cell mechanics. *Biophys. J.* 105:609–619.

11. Tse, J. M., G. Cheng, …, L. L. Munn. 2012. Mechanical compression drives cancer cells toward invasive phenotype. *Proc. Natl. Acad. Sci. USA.* 109:911–916.

12. Hui, T. H., Z. L. Zhou, …, H. Gao. 2014. Volumetric deformation of live cells induced by pressure-activated cross-membrane ion transport. *Phys. Rev. Lett.* 113:118101.

13. Stroka, K. M., H. Jiang, …, K. Konstantopoulos. 2014. Water permeation drives tumor cell migration in confined microenvironments. *Cell.* 157:611–623.

14. Hoffmann, E. K., I. H. Lambert, and S. F. Pedersen. 2009. Physiology of cell volume regulation in vertebrates. *Physiol. Rev.* 89:193–277.

15. Stewart, M. P., J. Helenius, …, A. A. Hyman. 2011. Hydrostatic pressure and the actomyosin cortex drive mitotic cell rounding. *Nature.* 469:226–230.

16. Gilpin, W., S. Uppaluri, and C. P. Brangwynne. 2015. Worms under pressure: bulk mechanical properties of C. elegans are independent of the cuticle. *Biophys. J.* 108:1887–1898.

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Supporting Material

Material and Methods
Supplementary Figures 1-3

MATERIALS AND METHODS

Cell Culture and Spheroid Generation

Human breast carcinoma MCF7 (ATCC, Manassas, VA) were cultured in RPMI 1640 (Mediatech, Herndon, VA) containing 10% FBS (Atlanta Biologicals, Norcross, GA). Spheroids were generated by creating a single-cell suspension in 0.75% low-gelling agarose (Type VIIA, Sigma-Aldrich, St. Louis, MO) and allowing them to grow for 3 weeks (1).

Chemical Perturbations

For hypotonic media, DI water was added to 25-50% v/v, for hypertonic media Xylose (Spectrum), Sucrose (BDH), NaCl (Amresco) or PEG400 (TCI) were added to final concentration of 250 mM. Chemical inhibitors were used at the following concentrations:

Chemical	Manufacturer	Concentration	Effect
Cytochalasin D	Enzo	1 µM	Depolymerize actin
Jasplakinolide	Enzo	100 nM	Increase actin polymerization
Nocodazole	Sigma	20 µM	Depolymerize microtubules
Taxol	Enzo	100 nM	Stabilize microtubules
Acrylamide	Acros	4 mM	Depolymerize intermediate filaments
Triton-X100	BioRad	0.0025%	Permeabilize cells
EIPA	Enzo	50 µM	Blocks sodium channel NHE1
Bumetanide	Enzo	20 µM	Blocks sodium channel NKCC1

3D Spheroid Compression Model

After 3 weeks of growth to accumulate solid stress, spheroids were analyzed by live-cell microscopy on a Nikon Eclipse Ti inverted epifluorescent microscope, maintained at 37° C and 5% carbon dioxide throughout the experiment using an In Vivo Scientific environmental cell chamber and Bioscience Tools CO_2 controller. After capturing initial images of spheroids, spheroids were treated with inhibitors described above and returned to the microscope for continued imaging. Spheroid areas were manually traced, the value given represents the spheroid area 6 hours post treatment normalized to the initial area. Solid stress was determined as previously described (1). In brief, the deformation in the gel is determined from the final spheroid size relative to the initial radius of the single cells they originated

from (taken to be 10 μm), and this deformation can be used to determine stresses based on the known mechanical properties of agarose.

Stress-free 3D Spheroid Model

For an uncompressed spheroid control, spheroids were generated by anchorage independence on top of a layer 2.0% agarose. Spheroids were allowed to grow for one week until they reached roughly the diameter of week 3 compressed spheroids, and then embedded in 0.4% agarose. Spheroids were allowed to equilibrate overnight before analysis as described for the 3D spheroid compression model.

2D Compression Model

Cells were grown as a monolayer and then compressed with weighted pistons as previously described (2). After reaching 80% of confluence, monolayers were washed with PBS and a 2% agarose solution and flattened using a custom well plate insert. After polymerization, this agarose cushion was topped with media and allowed to equilibrate in standard culture conditions. To apply stress, custom pistons that applied 5 mmHg of solid stress (piston weight divided the piston surface area) were then added and incubated for desired period of time. Unstressed controls were topped with coverslips of equal size to the pistons to account for differences in oxygen diffusion.

Live-Dead Quantification

In order to quantify cell viability, cells were labeled with 10 μg/mL propidium iodide and 2 μM Calcein AM (Enzo) to identify dead and live cells, respectively. Images were then collected on a Nikon Eclipse Ti inverted epifluorescent microscope as described above. Image analysis was then performed in MATLAB, taking cell death as total propidium iodide signal normalized to total Calcein signal. For 2D studies, cells were pre-treated with inhibitors for 2 hours before applying stress for 4 hours. For 3D studies, spheroids were incubated with inhibitors for 6 hours before performing analysis.

Intracellular Sodium

Intracellular sodium was measured using CoroNa Green (Invitrogen, Carlsbad, CA) per manufacturer's instructions. Individual aliquots were solubilized to 1 mM in DMSO. Cell monolayers were washed twice with HBSS before incubation in 10 μM CoroNa green in HBSS for 45 minutes. Cells were washed two more times before returning to growth media, and then treated as described in the 2D compression model. For quantification, wells were read on a DTX-800 Multimode Detector microwell plate reader (Beckman Coulter) at 485nm excitation, 535nm emission. An initial reading was taken before applying

5 mmHg stress (or coverslip control) and then a final reading taken two hours later after removing weights. Values are reported as final CoroNa signal normalized to initial signal after blank subtraction.

Statistics

All studies were performed in triplicate or more. The data are reported as mean ± standard error of the mean. Statistical analysis was carried out using a student's t-test for comparison considering $p < 0.05$ to be significant (***$p<0.001$,**$p<0.01$,*$p<0.05$).

REFERENCES

1. Roose, T., P. a. Netti, L.L. Munn, Y. Boucher, and R.K. Jain. 2003. Solid stress generated by spheroid growth estimated using a linear poroelasticity model. Microvasc. Res. 66: 204–212.

2. Tse, J.M., G. Cheng, J. a Tyrrell, S. a Wilcox-Adelman, Y. Boucher, et al. 2012. Mechanical compression drives cancer cells toward invasive phenotype. Proc. Natl. Acad. Sci. U. S. A. 109: 911–6.
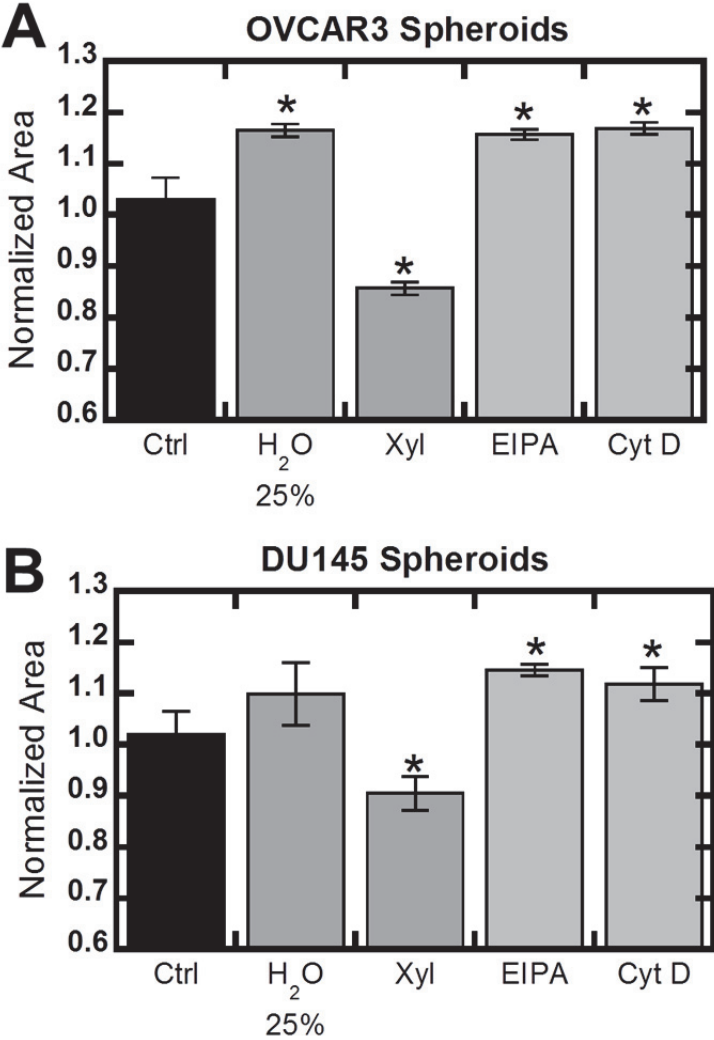
Figure S1. Changes in spheroid cross-sectional area following treatment are conserved across multiple types of cancer. Stressed spheroids were exposed to hypotonic media (25% water), hypertonic media (250 mM xylose), EIPA to block sodium channels or cytochalasin D (Cyt D) to inhibit actin polymerization and normalized to their area before treatment. (A) Ovarian cancer OVCAR-3 spheroids. (B) Prostate cancer DU145 spheroids. *$P<0.05$ relative to control.
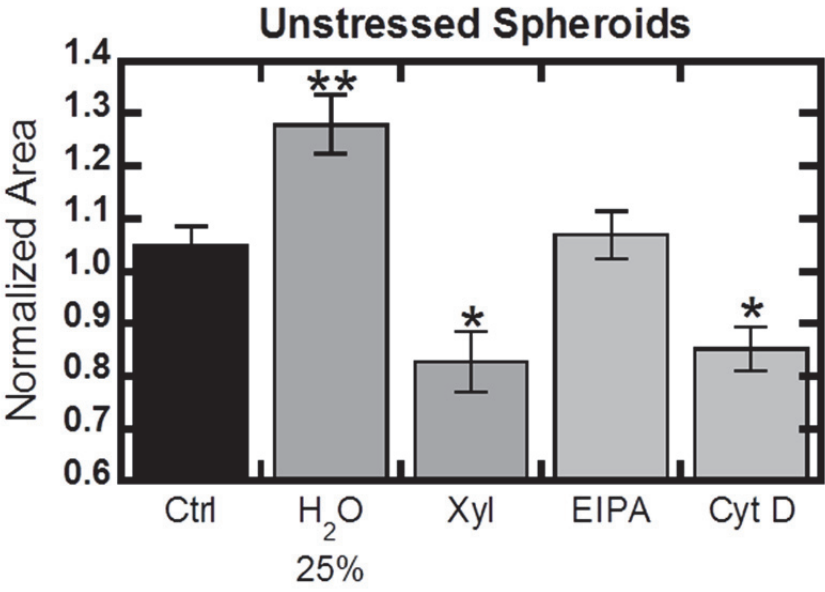
Figure S2. Changes in cross-sectional area following treatment of unstressed MCF7 spheroids. Spheroids were exposed to hypotonic media (25% water), hypertonic media (250 mM xylose), EIPA to block sodium channels or cytochalasin D (Cyt D) to inhibit actin polymerization and normalized to their area before treatment. While hypotonic and hypertonic showed larger changes than stressed spheroids, sodium channel blockade with EIPA did not induce any significant change in spheroid area and actin depolymerization induced spheroid collapse instead of swelling. *$P<0.05$, **$P<0.01$ relative to control.
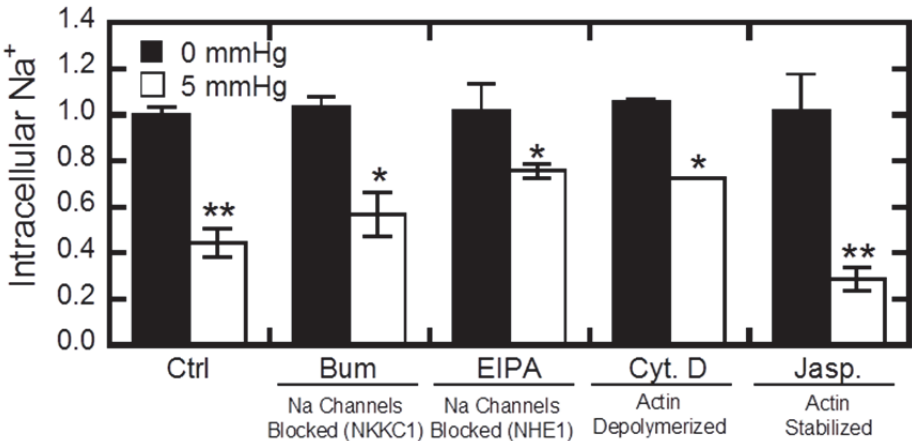
Figure S3. Intracellular sodium levels in stressed and unstressed monolayers. Cells were loaded with CoroNa Green sodium tracer and then treated with desired inhibitors or solvent. Initial readings were taken, and then cells were topped with either a glass coverslip (0 mmHg) or a weighted piston of equal diameter (5 mmHg) for 2 hours before taking a final reading. All values are normalized to the control reading before compression.