Osmotic Regulation is Required for Cancer Cell Survival under Solid Stress

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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.

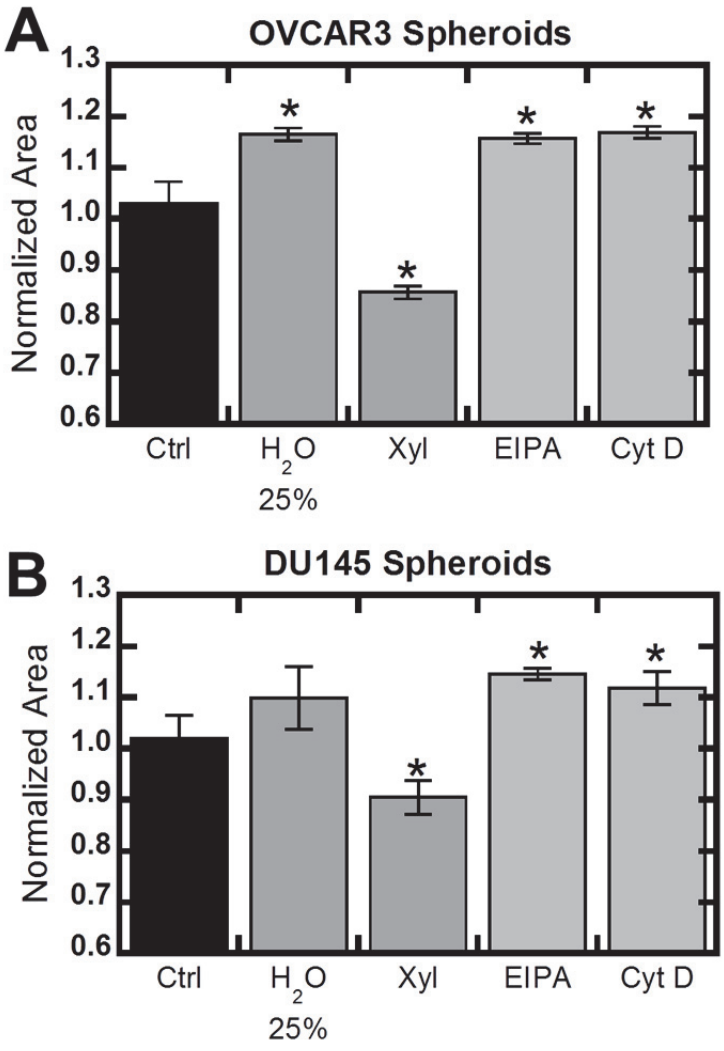

Figure S1. Changes in spheroid cross-sectional area following treatment are conserved across multiple types of cancer. Stressed spheroids were exposed to hypotonic media (25% water), hypertonic media (250 mM xylose), EIPA to block sodium channels or cytochalasin D (Cyt D) to inhibit actin polymerization and normalized to their area before treatment. (A) Ovarian cancer OVCAR-3 spheroids. (B) Prostate cancer DU145 spheroids. *P<0.05 relative to control.

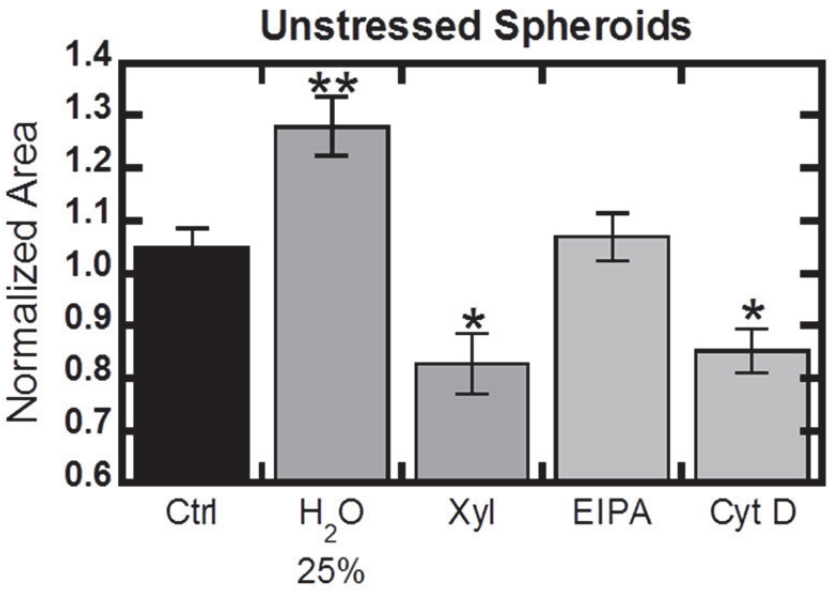

Figure S2. Changes in cross-sectional area following treatment of unstressed MCF7 spheroids. Spheroids were exposed to hypotonic media (25% water), hypertonic media (250 mM xylose), EIPA to block sodium channels or cytochalasin D (Cyt D) to inhibit actin polymerization and normalized to their area before 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.

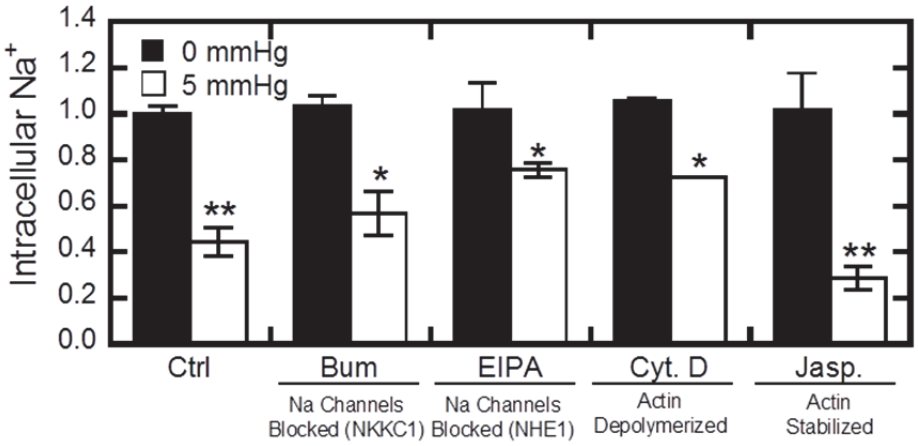

Figure S3. Intracellular sodium levels in stressed and unstressed monolayers. Cells were loaded with CoroNa Green sodium tracer and then treated with desired inhibitors or solvent. Initial readings were taken, and then cells were topped with either a glass coverslip (0 mmHg) or a weighted piston of equal diameter (5 mmHg) for 2 hours before taking a final reading. All values are normalized to the control reading before compression.