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Supplemental Information

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Supplementary Materials

Materials and Methods

Cell stiffness measurement by atomic force microscope. Cell stiffness was measured using atomic force microscope (AFM, Bruker Catalyst) with silicon nitride cantilevers (spring constant k: 0.02 to 0.08 N/m) at room temperature. The force F between tip and cell was the product of the cantilever deflection δ and k, i.e., $F = k \times \delta$. Cell Young's modulus E could be measured by fitting force-indentation curves with Sneddon's modification of the Hertzian model for a pyramidal tip, i.e., $F=2/\pi\times\tan(\alpha)\times E/(1-\nu^2)\times d^2$, where d is the indentation depth, α is the half tip angle, ν is 0.5. d was kept within 500 nm at 1 Hz to avoid potential substrate effects and cell damage.

Immunofluorescence staining. Cells after various treatment were cultured on coverslips and fixed with 4% formaldehyde (Sigma Aldrich) for 15 min at room temperature and rinsed with PBS for 3 times to remove excess reagent. 0.1% Triton X-100 (SAFC) in 1% BSA was added to coverslips and incubated for 1 h at room temperature for permeabilization. Primary

antibodies: MnSOD, Bcl2, MRP3 (Abcam) were diluted in 1% BSA and added to coverslips at 4°C overnight. Cells were then incubated with secondary antibodies: Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (Abcam) and Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 (Invitrogen) for 1 h at room temperature. The nucleus was then counterstained with DAPI. For F-actin staining, cells were permeabilized with 0.1% Triton X-100 for 5 min. 200 µl of 1x Green Fluorescent Phalloidin Conjugate working solution (Abcam) was then added into fixed cells for 60 min. Cells were rinsed with PBS for 3 times to remove excess dye and placed onto another coverslip with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). At least 100 cells/condition were imaged by the inverted fluorescent microscope (Nikon) using FITC and DAPI channel, respectively. Fluorescence intensity was quantified using ImageJ (NIH).

Fibrin gel preparation. Fibrin gels were prepared as previously described (1, 2). Briefly, fibrinogen (Sea Run Holdings Inc) in T7 buffer (pH 7.4, 50 mM Tris, 150 mM NaCl) was mixed with single cell solution into the concentration of 1mg/ml. 50 µl cell/fibrinogen solution was seeded into each well of 96-well plate and mixed with the pre-added 5 µl thrombin (20 U/ml). The plate with fibrin gels was incubated at 37 °C for 10 min. After complete gelation, 150 µl cell culture medium was gently added into each well.

Statistical analysis. Two-tailed Student's t-test or ANOVA analysis was used for the statistics among two or more conditions.

Reference

1. Liu, J., Y. Tan, H. Zhang, Y. Zhang, P. Xu, J. Chen, Y.-C. Poh, K. Tang, N. Wang, and B. Huang. 2012. Soft fibrin gels promote selection and growth of tumorigenic cells. Nat. Mater. 11: 734–41.

2. Tan, Y., A. Tajik, J. Chen, Q. Jia, F. Chowdhury, L. Wang, J. Chen, S. Zhang, Y. Hong, H. Yi, D.C. Wu, Y. Zhang, F. Wei, Y.-C. Poh, J. Seong, R. Singh, L.-J. Lin, S. Doğanay, Y. Li, H. Jia, T. Ha, Y. Wang, B. Huang, and N. Wang. 2014. Matrix softness regulates plasticity of tumour-repopulating cells via H3K9 demethylation and Sox2 expression. Nat. Commun. 5: 4619.

Supplementary Table 1: List of primers

Genes		Quantitative RT-PCR
E-cadherin	5’ primer	TGCCCAGAAAATGAAAAAGG
	3’ primer	GTGTATGTGGCAATGCGTTC
Fibronectin	5’ primer	CAGTGGGAGACCTCGAGAAG
	3’ primer	TCCCTCGGAACATCAGAAAC
Twist	5’ primer	GGAGTCCGCAGTCTTACGAG
	3’ primer	TCTGGAGGACCTGGTAGAGG
Vimentin	5’ primer	ACTCCCTCTGGTTGATAC
	3’ primer	ATCGTGATGCTGAGAAGT
Bcl2	5’ primer	GTCATGTGTGTGGAGAGCGTCAACC
	3’ primer	CCAGGGCCAAACTGAGCAGAGTC
SOD2	5’ primer	GCACATTAACGCGCAGATCA
	3’ primer	AGCCTCCAGCAACTCTCCTT
ABCG2	5’ primer	TGGCTGTCATGGCTTCAGTA
	3’ primer	GCCACGTGATTCTTCCACAA
GAPDH	5’ primer	GCGACACCCACTCCTCCACCTTT
	3’ primer	TGCTGTAGCCAAATTCGTTGTCATA
GSTP1	5’ primer	TCTACGCAGCACTGAATCCG
	3’ primer	GGAGCTGCCCATACAGACAA
MDR1	5’ primer	CCCATCATTGCAATAGCAGG
	3’ primer	GTTCAA ACTTCTGCTCCTGA
MRP1	5’ primer	AGGCCTACTACCCCAGCATT
	3’ primer	CAGTCTCTCCACTGCCACAA
MRP3	5’ primer	CTTAAGACTTCCCCTCAACATGC
	3’ primer	GGTCAAGTTCCTCTTGGCTC

Supplementary Figures

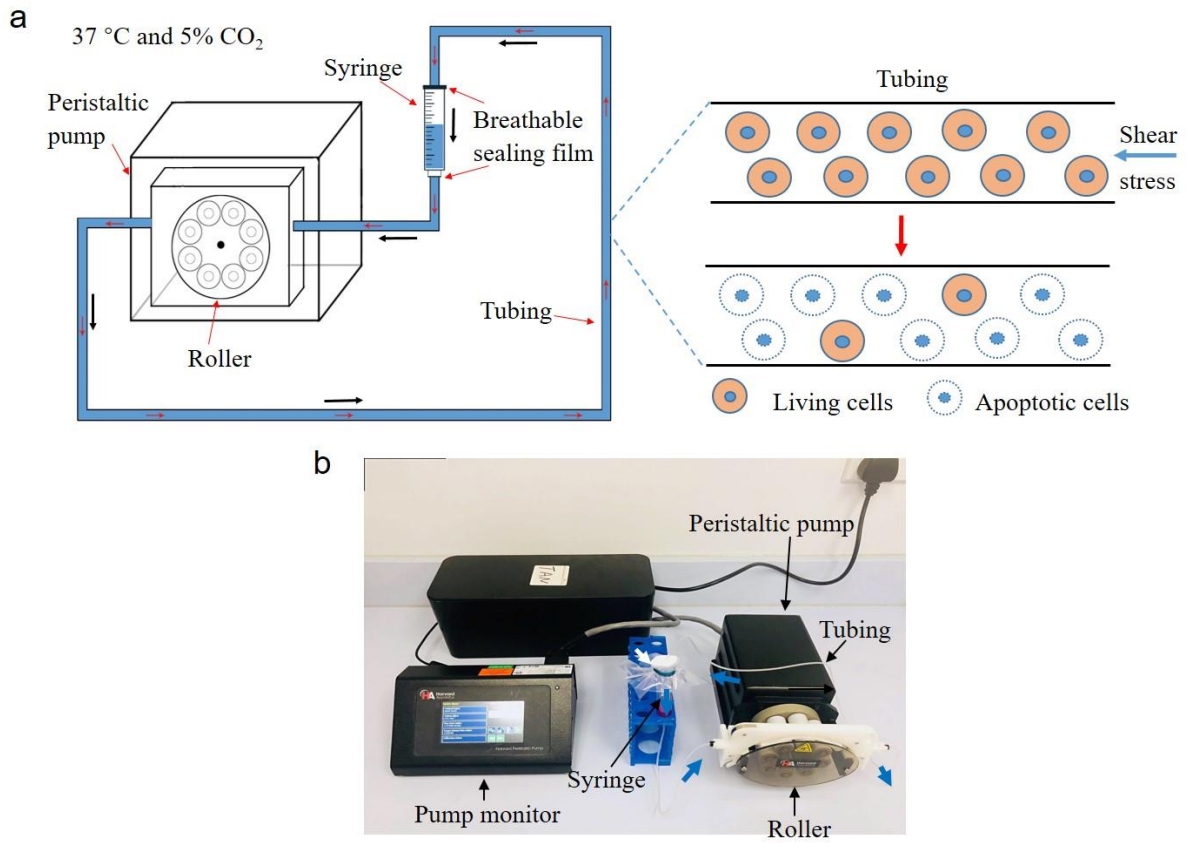

FIGURE S1 The circulatory system developed in this study. (*a*) The schematic of the circulatory microfluidic system. The system was composed of a peristaltic pump (P-230, Harvard Apparatus), a silicone micro-tubing (0.51 mm in diameter and 1.5 m in length), and a syringe as cell solution reservoir. This system could generate pulsatile flow, which mimicked the hemodynamic shear stress in blood circulation. Tumor cells in suspension were treated by various magnitudes of shear stress and circulation duration. (b) The image of the experimental system.

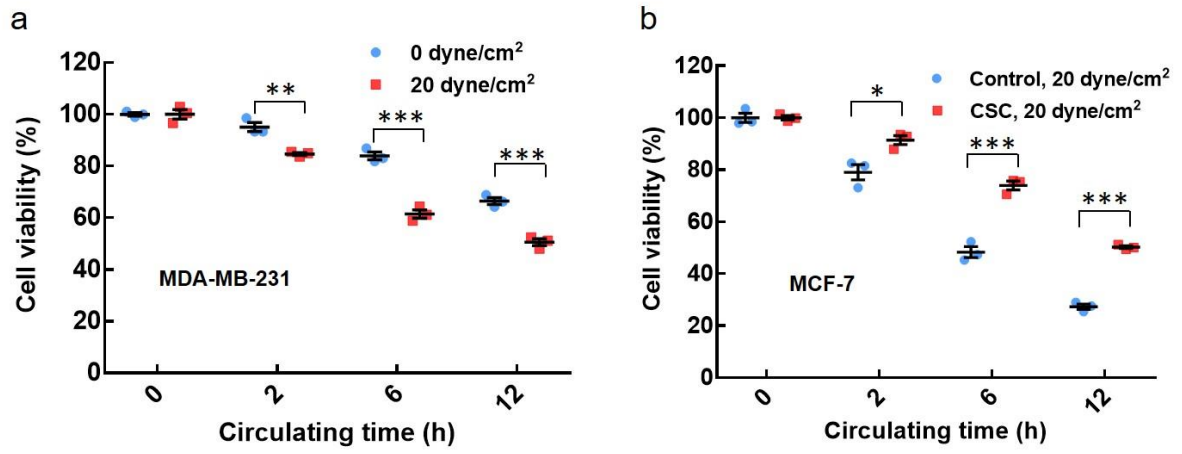

FIGURE S2 The survival of breast cancer cells and CSCs in fluid shear stress. (a) The viability of MDA-MB-231 cells decreases with shear stress and circulation duration. Suspended tumor cells were treated in the circulatory system under 0 and 20 dyne/cm^2 shear stress for 0, 2, 6, and 12 h, respectively. The viability of treated cells was measured by MTS assay and was normalized by the data at 0 h under the same treatment. n=3 independent experiments. (b) CSCs from MCF-7 exhibit higher viability than bulk tumor cells in shear stress. CSCs were acquired by culturing MCF-7 cells in soft fibrin gels for 7 days. Suspended CSCs and MCF-7 cells were then treated in 20 dyne/cm^2 shear stress for various durations, when cell viability was measured. n=3 independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

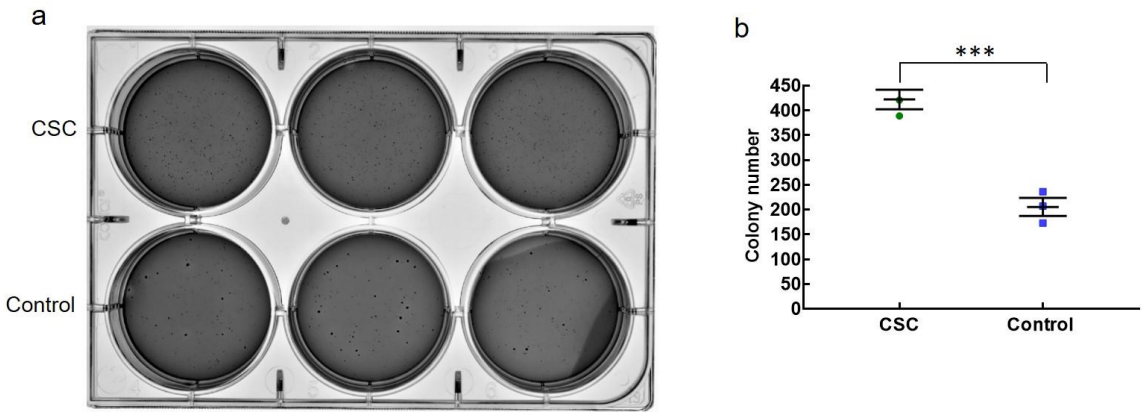

FIGURE S3 Fibrin-selected breast cancer cells are CSCs with high self-renewal. MCF7 cells were cultured in soft fibrin gels for 7 days. These fibrin-selected cells (CSCs) and control cells were cultured in soft agar for 10 days. The micrographs of the plates were shown in (a) and the colony number was quantified in (b). n=3 independent experiments. ***, $p<0.001$.

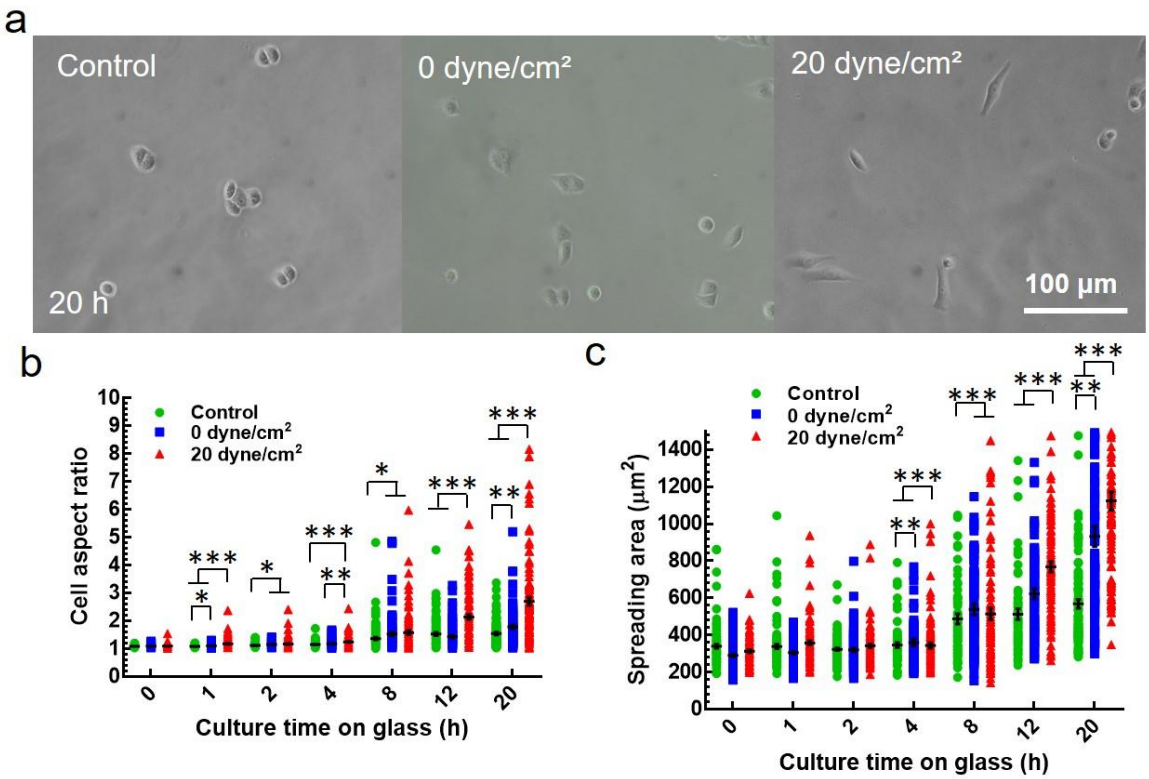

FIGURE S4 Shear stress promotes mesenchymal phenotype in MCF-7 cells. (a) Representative images of MCF-7 cells after shear stress treatment. MCF-7 cells were treated under 0 and 20 dyne/cm^2 shear stress and then cultured on collagen-coated glass. Tumor cells cultured in petri dishes were used as control. Cell images were taken at the indicated time points. Scale bar: 100 μm. (b, c) Fluid shear stress promotes elongated morphology and spreading of MCF-7 cells. The aspect ratio (b) and spreading area (c) of the treated tumor cells in (a) were quantified by ImageJ (n>95). n=2 independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

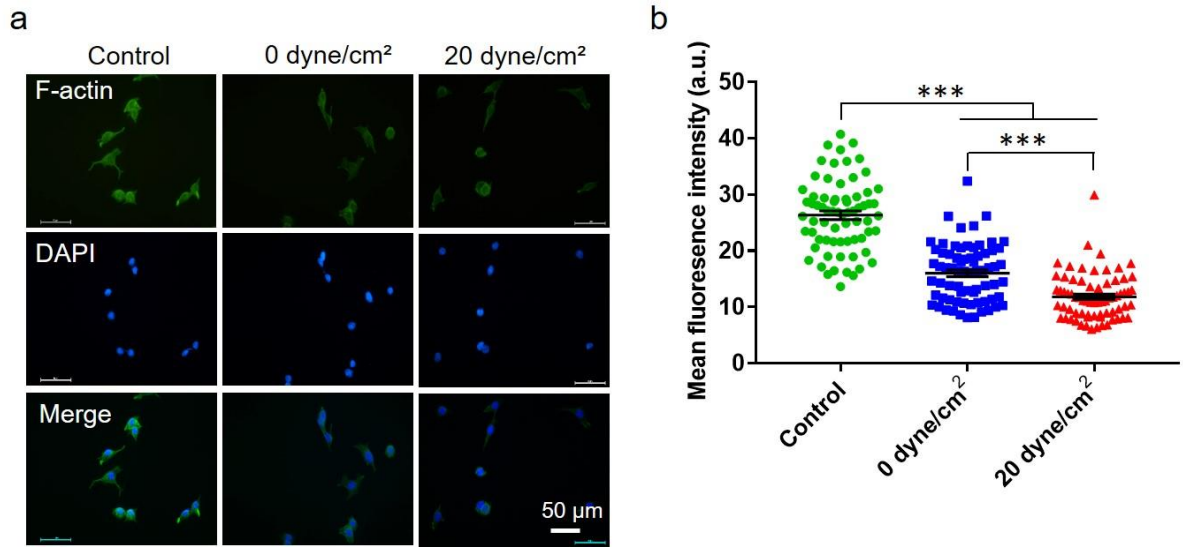

FIGURE S5 MCF-7 cells surviving shear stress exhibit low F-actin and cell stiffness. (a) Immunofluorescence imaging of F-actin in MCF-7 cells after shear stress treatment. MCF-7 cells were treated under 0 and 20 dyne/cm^2 shear stress for 12 h and then plated on glass for 10 h, when F-actin was examined by immunofluorescence staining. The nucleus was counterstained with DAPI. Scar bar: 50 μm. (b) Surviving tumor cells show much lower F-actin. F-actin assembly in (a) was quantified (n>100). a.u.: arbitrary unit. n=2 independent experiments. *, p<0.05; **, p<0.01; ***, p<0.001.

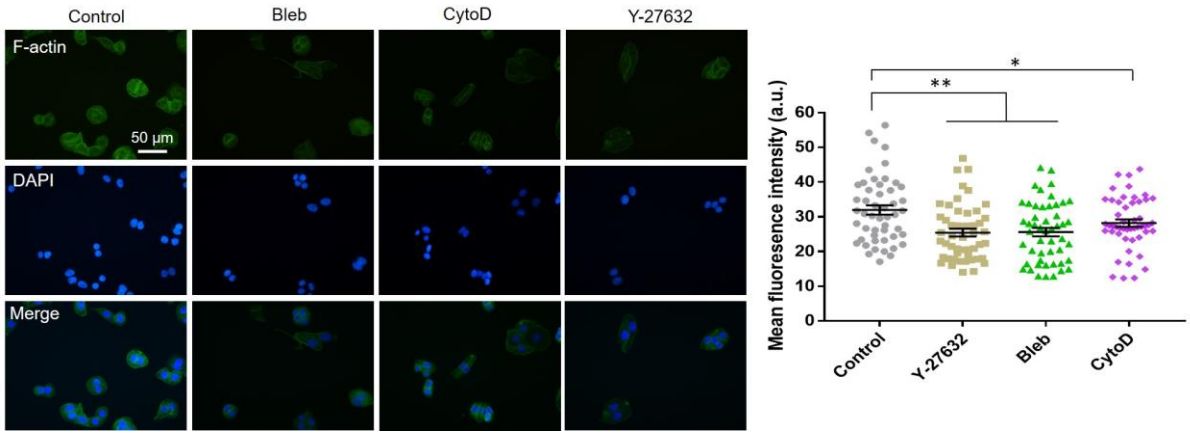

FIGURE S6 Pharmacologically inhibiting actomyosin decreases F-actin assembly. Breast cancer cells MDA-MB-468 were treated with 4 μM myosin II inhibitor blebbistatin, 2 μM ROCK inhibitor Y-27632, and 0.3 μM F-actin inhibitor cytochalasin D. The F-actin assembly in these cells were then examined by immunofluorescence staining and the nucleus was counterstained with DAPI. The fluorescence intensity was quantified by ImageJ (right panel). n> 50 cells. Scale bar: 50 μm. n=2 independent experiments. *, $p<0.05$; **, $p<0.01$.

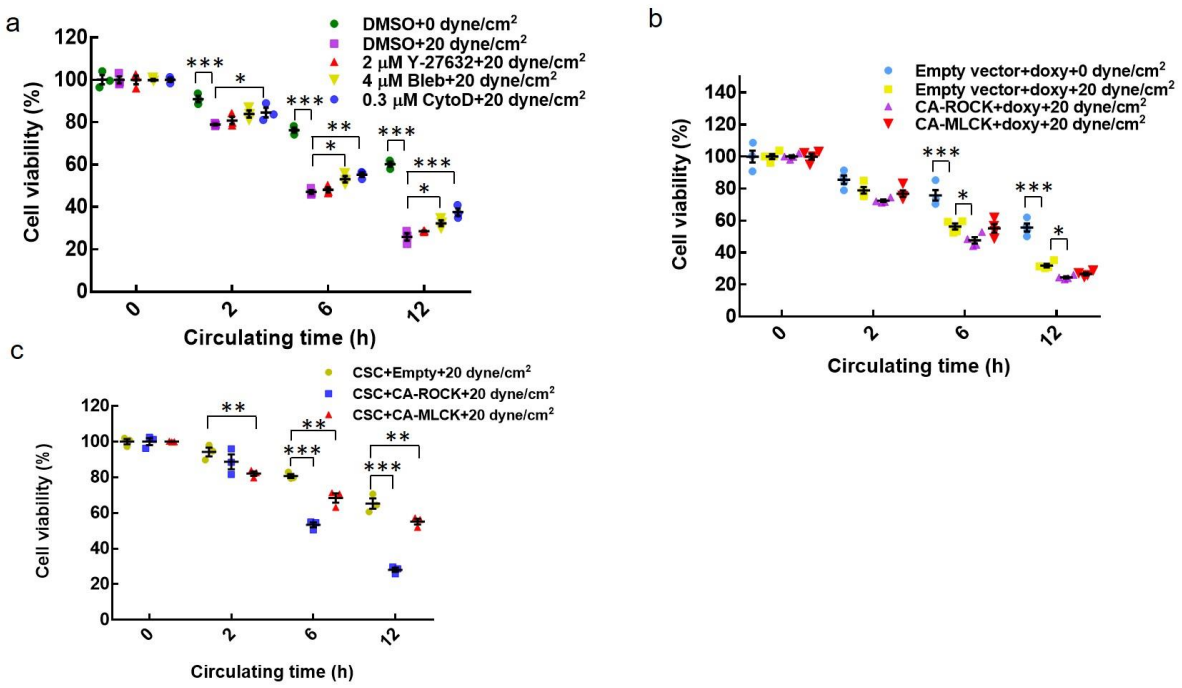

FIGURE S7 Actomyosin regulates the survival of MCF-7 cells in fluid shear stress. (a) Inhibiting actomyosin activity through myosin or actin but not ROCK enhances cell viability in shear flow. MCF-7 cells were treated with 2 μM Y-27632, 4 μM blebbistatin (Bleb), or 0.3 μM cytochalasin D (CytoD) for 48 h and then circulated under 0 or 20 dyne/cm^2 shear stress for the indicated durations, when cell viability was measured by MTS assay. Activating actomyosin in MCF-7 cells through ROCK but not MLCK (b) and MCF-7 CSCs through both MLCK and ROCK (c) suppresses the survival of suspended tumor cells in fluid shear flow. MCF-7 were transfected with CA-MLCK or ROCK or empty vector in the presence of doxycycline and then circulated under 20 dyne/cm^2 shear stress with doxycycline. CSCs were obtained by culturing these modified MCF-7 cells in soft fibrin gels for 5 days without doxycycline, where doxycycline was added at day 6 and 7 to activated ROCK or MLCK. These CSCs were then treated under 20 dyne/cm^2 shear stress with doxycycline for the indicated durations, when cell viability was measured. n=3 independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

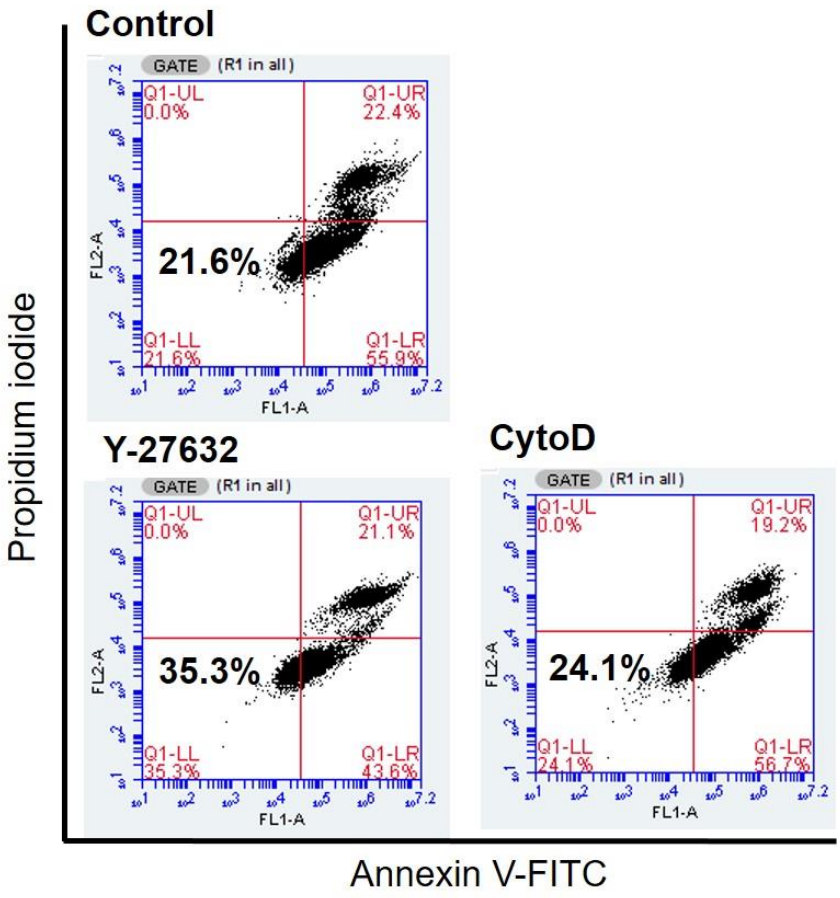

FIGURE S8 Pharmacologically inhibiting actomyosin increases the survival of tumor cells in fluid shear flow. Breast cancer cells MDA-MB-468 were treated with 2 μM ROCK inhibitor Y-27632 or 0.3 μM F-actin inhibitor cytochalasin D for 48 h and then circulated under 20 dyne/cm^2 shear stress for 12 h. Control cells treated with DMSO were used as a control. Cell survival was examined by Annexin V assay. The value in the flow cytometry figure represents the fraction of viable cells. n=2 independent experiments.

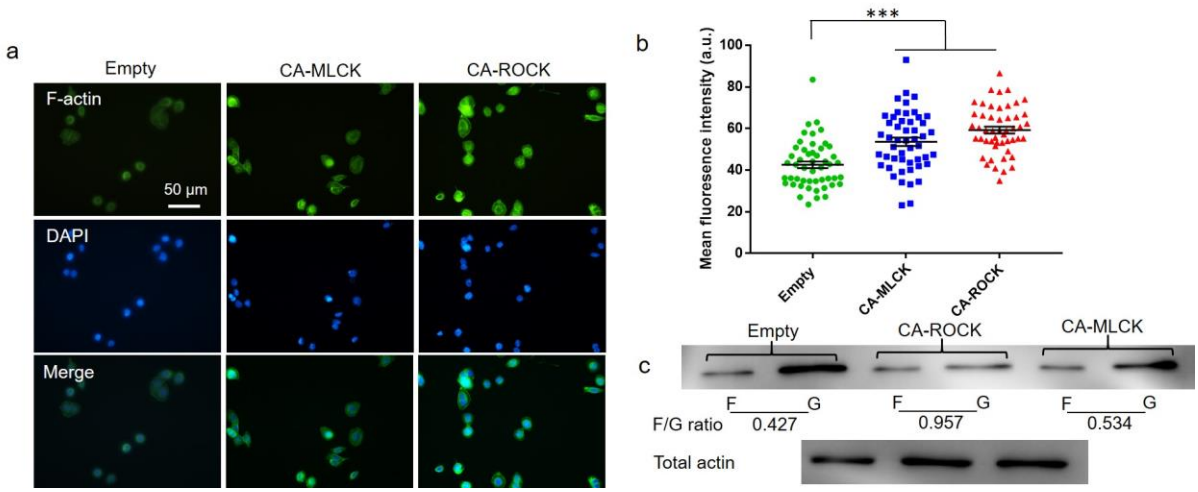

FIGURE S9 Overexpressing MLCK/ROCK enhances F-actin assembly. (a) Immunofluorescence imaging of F-actin in breast cancer cells with overexpression of MLCK or ROCK. MDA-MB-468 cells were transfected with constitutive active (CA) mutants of MLCK or ROCK in the presence of doxycycline, which could activate the plasmids. The F-actin assembly in these cells was then examined by immunofluorescence staining and the nucleus was counterstained with DAPI. Scale bar: 50 μm. (b) Quantification of F-actin assembly. The fluorescence intensity in (a) was quantified by ImageJ (right panel). n>80 cells. ***, $p<0.001$. (c) Immunoblotting of G-actin and F-actin in tumor cells with overexpression of MLCK or ROCK. MDA-MB-468 cells with overexpression of CA-MLCK or CA-ROCK were analyzed for the expression of G-actin and F-actin by western blotting. The values represent the F-actin/G-actin ratio. The level of total actin was used as loading control. n=2 independent experiments.

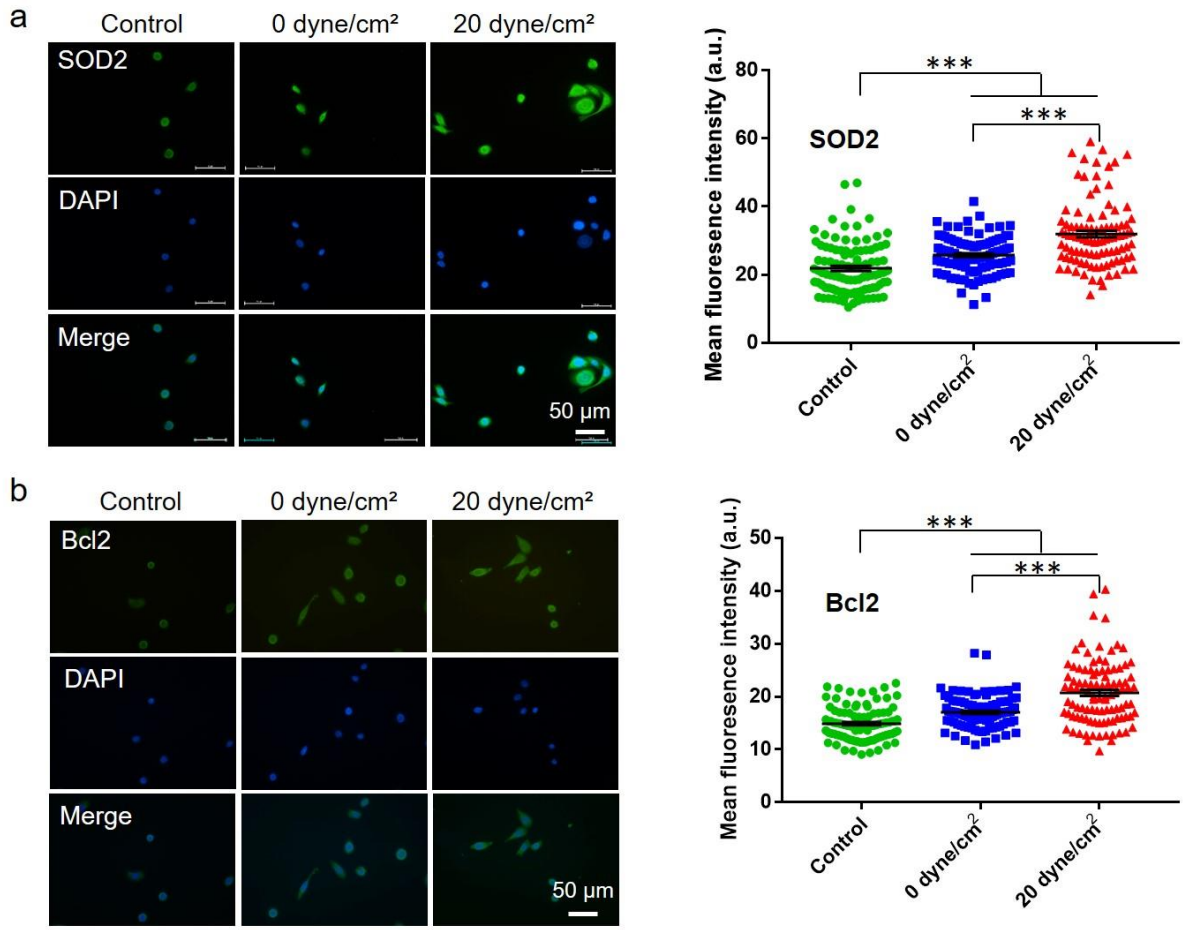

FIGURE S10 Hemodynamic shear stress up-regulates the expression of SOD2 and Bcl2. MDA-MB-468 cells were treated under 0 and 20 dyne/cm^2 shear stress for 12 h and then plated on glass for the analysis of SOD2 (a) and Bcl2 (b) by immunofluorescence staining. The nucleus was counterstained with DAPI. Tumor cells cultured on glass were used as control. The fluorescence intensity was quantified by ImageJ (right panel). At least 100 cells were used for each condition. n=2 independent experiments. Scale bar: 50 μm. ***, $p<0.001$.

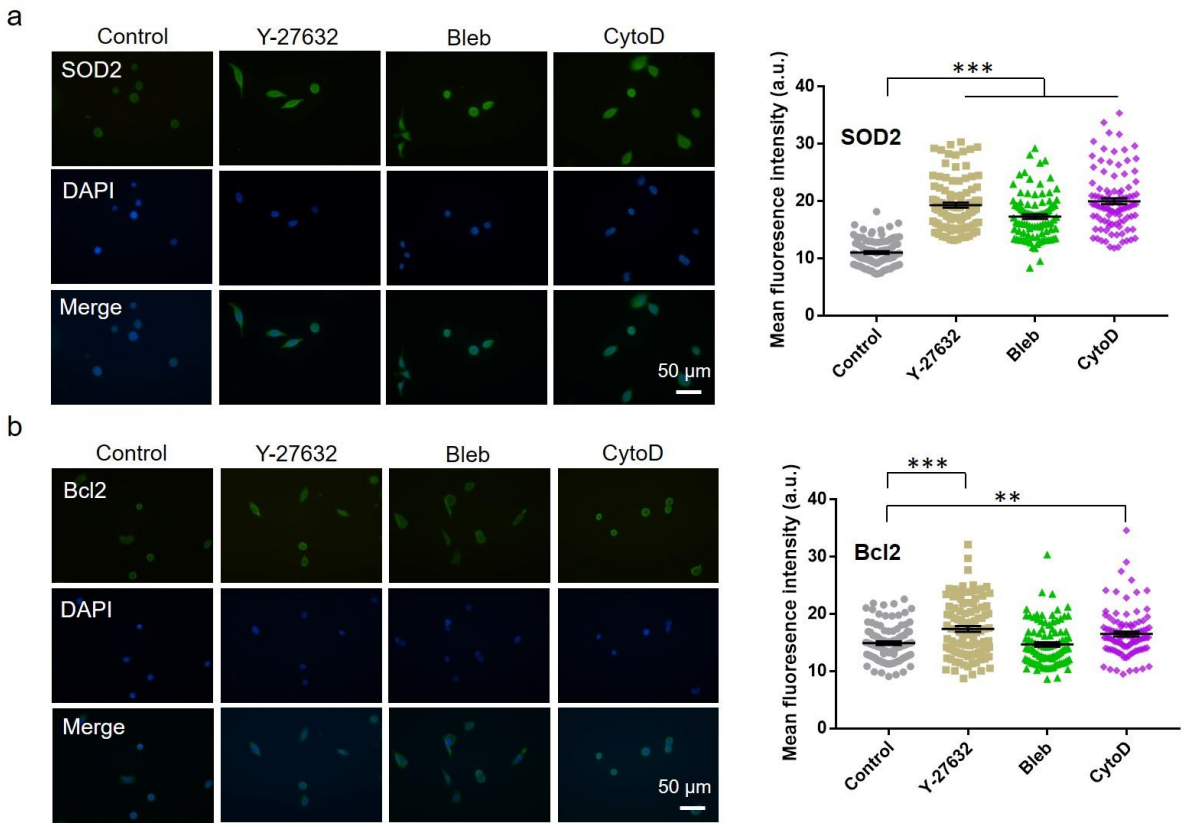

FIGURE S11 Inhibiting actomyosin up-regulates the expression of SOD2 and Bcl2. MDA-MB-468 cells were treated with 2 μM Y-27632, 4 μM blebbistatin, or 0.3 μM cytochalasin D for 48h and then analyzed for the expression of SOD2 (a) and Bcl2 (b) by immunofluorescence staining. Tumor cells treated with DMSO were used as control. The nucleus was counterstained with DAPI. The fluorescence intensity was quantified by ImageJ (right panel). At least 100 cells were used for each condition. n=2 independent experiments. Scale bar: 50 μm. **, $p<0.01$; ***, $p<0.001$.

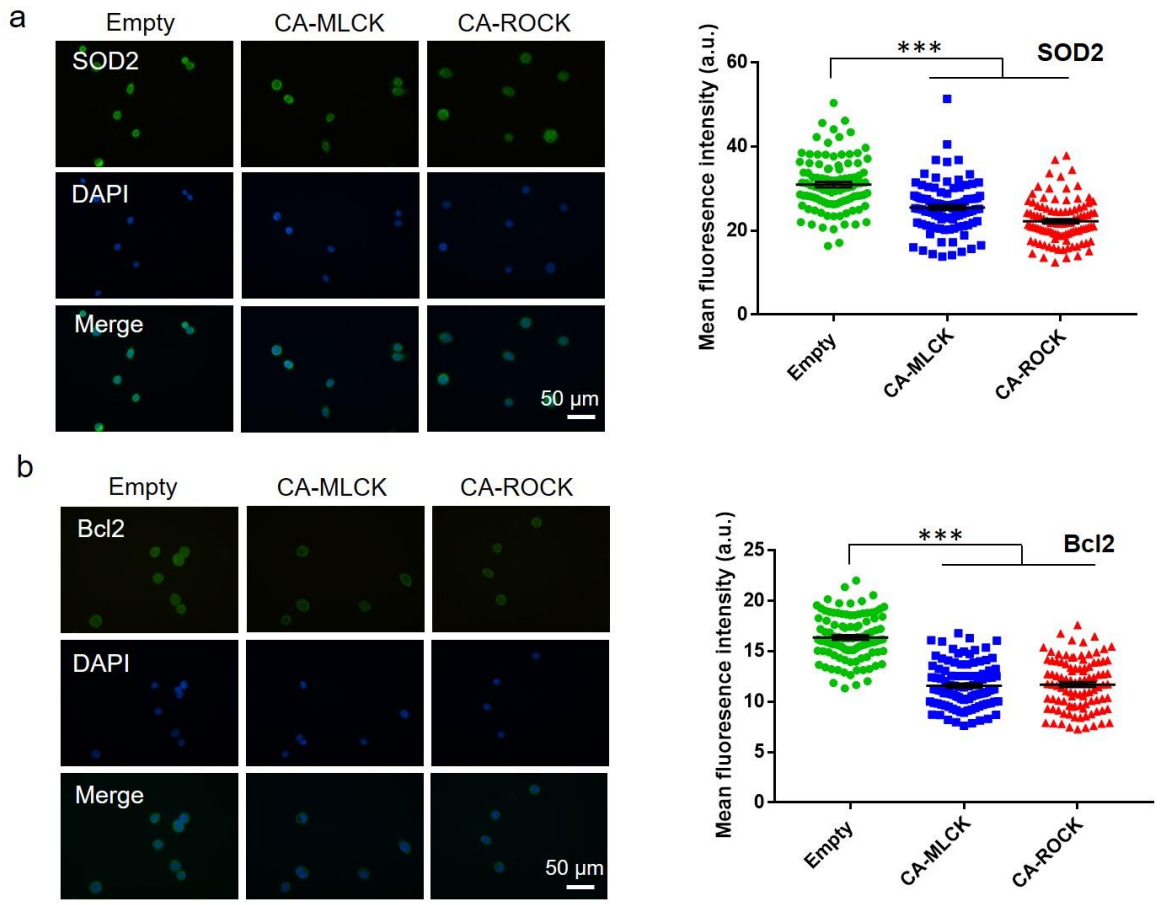

FIGURE S12 Activating actomyosin down-regulates the expression of SOD2 and Bcl2. MDA-MB-468 cells were transfected with CA-MLCK or ROCK in the presence of doxycycline for 48h and then analyzed for the expression of SOD2 (a) and Bcl2 (b) by immunofluorescence staining. Tumor cells transfected with empty vectors were used as control. The nucleus was counterstained with DAPI. The fluorescence intensity was quantified by ImageJ (right panel). At least 100 cells were used for each condition. n=2 independent experiments. Scale bar: 50 μm. ***, $p<0.001$.

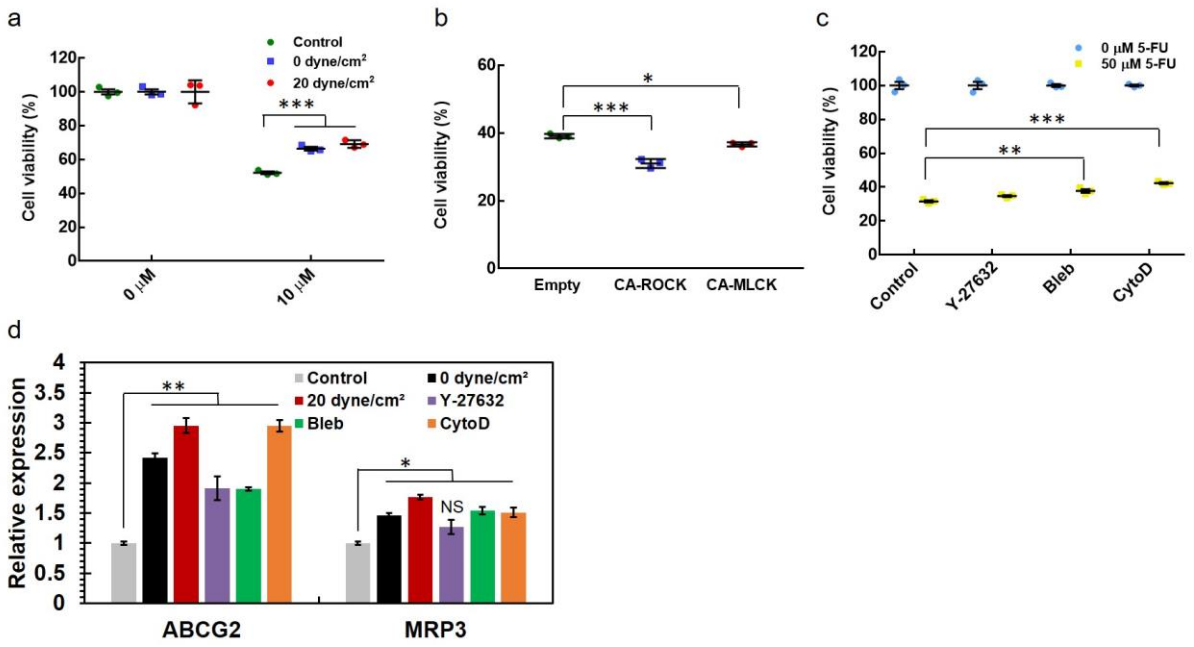

FIGURE S13 Tumor cells surviving fluid shear stress exhibit chemoresistance dependent on actomyosin. (a) Tumor cells surviving suspension or shear stress show enhanced drug resistance to chemotherapy. MCF-7 cells were circulated under 0 and 20 dyne/cm^2 shear stress for 12 h and then plated on glass with 0 or 10 μM 5-FU for 24 h. Cell viability was measured by MTS assay. The viability was normalized against to the value at 0 μM under the same condition. Tumor cells cultured in petri dishes were used as a control. n=3 independent experiments. (b) Activating actomyosin inhibits chemoresistance of surviving tumor cells. MCF-7 cells were transfected with CA-MLCK, CA-ROCK, or empty vector without doxycycline and then circulated under 20 dyne/cm^2 shear stress for 12 h. The surviving cells were treated with 50 μM 5-FU and doxycycline for 24 h, when cell viability was measured. n=3 independent experiments. (c) Inhibiting actomyosin in control cells enhances chemoresistance. MCF-7 cells were treated with 2 μM Y-27632, 4 μM blebbistatin, or 0.3 μM cytochalasin D for 24 h and then cultured with 0 or 50 μM 5-FU for another 24 h, when cell viability was measured. n=3 independent experiments. (d) Shear stress or inhibition of actomyosin in breast cancer cells up-regulates the expressions of genes related to multidrug

resistance. MCF-7 cells surviving 0 and 20 dyne/cm^2 shear stress in (a) or pre-treated by Y-27632, blebbistatin, or cytochalasin D in (c) were used for the analysis of genes related to multidrug resistance by quantitative RT-PCR, including ABCG2 and MRP3. NS: no significant difference vs 'Control'. n=3 independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

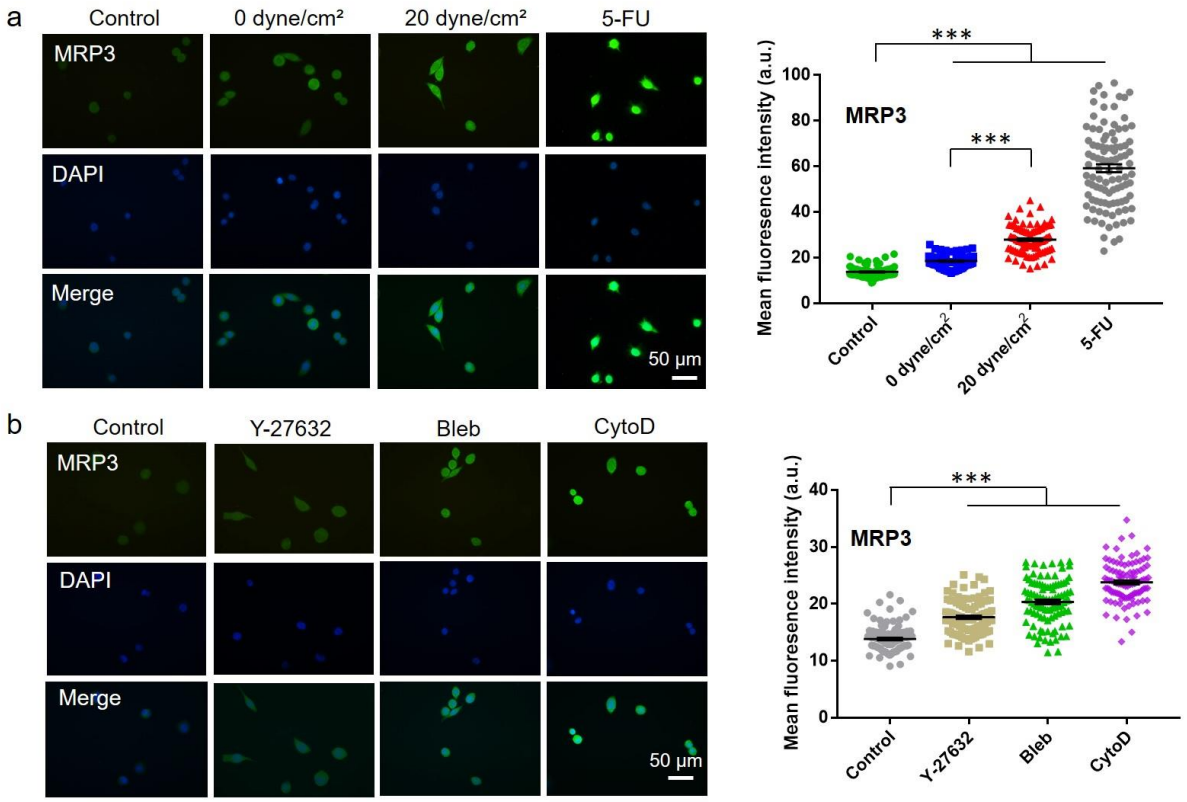

FIGURE S14 Hemodynamic shear stress or inhibiting actomyosin up-regulates MRP3 expression. MDA-MB-468 cells were circulated under 0 and 20 dyne/cm^2 shear stress for 12 h (a) or treated with 50 μM 5-FU, 2 μM Y-27632, 4 μM blebbistatin, or 0.3 μM cytochalasin D (b) for 24 h. The expression of MRP3 was analyzed by immunofluorescence staining. Tumor cells treated with DMSO were used as control. The nucleus was counterstained with DAPI. The fluorescence intensity was quantified by ImageJ (right panel). At least 100 cells were used for each condition. n=2 independent experiments. Scale bar: 50 μm. ***, $p<0.001$.

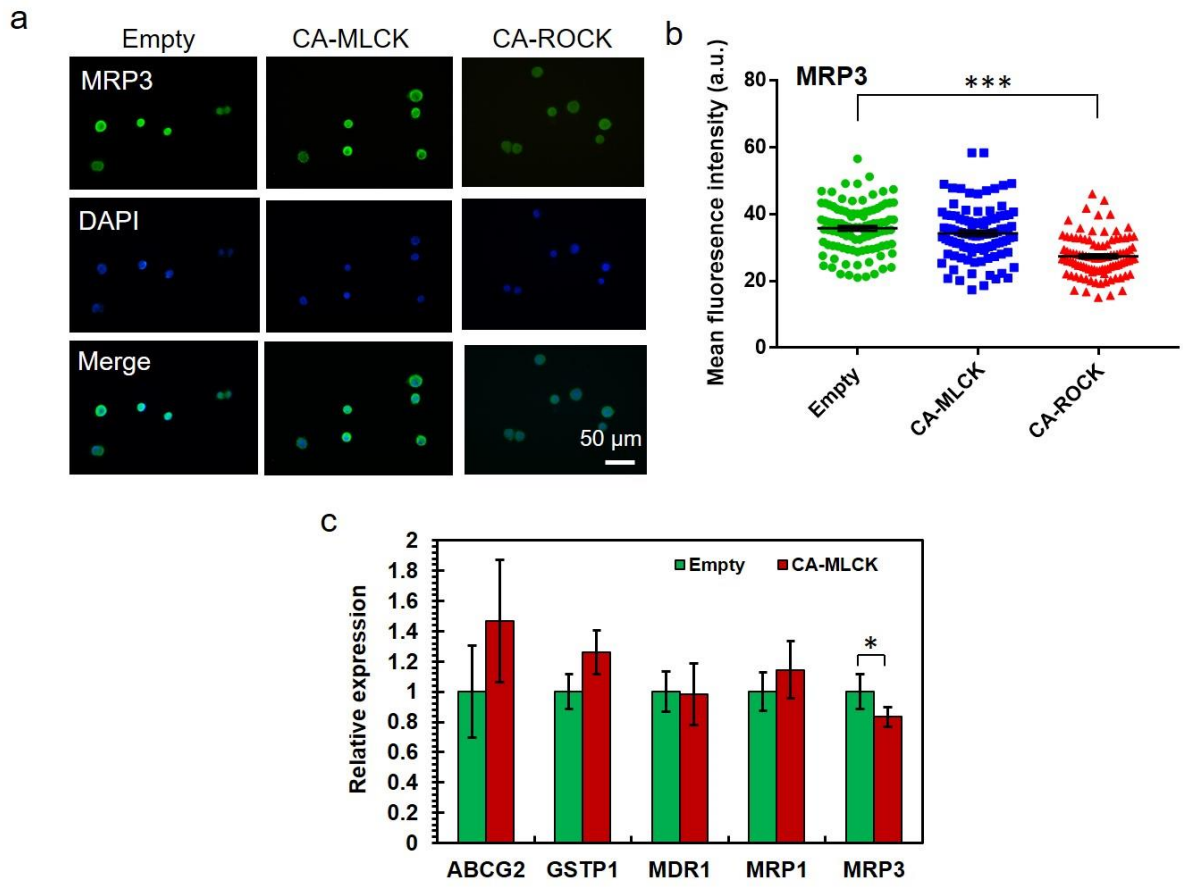

FIGURE S15 Activating actomyosin through ROCK but not MLCK down-regulates the genes related to multidrug resistance. (a) Immunofluorescence imaging of MRP3 in tumor cells with overexpression of MLCK or ROCK. MDA-MB-468 cells were transfected with CA-MLCK or CA-ROCK in the presence of doxycycline for 48 h and then analyzed for the expression of MRP3 by immunofluorescence staining. Tumor cells transfected with empty vectors were used as control. The nucleus was counterstained with DAPI. Scale bar: 50 μm. (b) Quantification of fluorescence intensity in (a). The fluorescence intensity was quantified by ImageJ. At least 100 cells were used for each condition. n=2 independent experiments. (c) Activating MLCK in surviving tumor cells has no significant effects on the genes related to multidrug resistance. MDA-MB-468 cells were transfected with CA-MLCK plasmids in the absence of doxycycline and then circulated under 20 dyne/cm^2 shear stress for 12 h. The surviving cells were treated

with doxycycline for 24 h. The expressions of the genes related to multidrug resistance were examined by quantitative RT-PCR. n=3 independent experiments. *$p<0.05$; ***, $p<0.001$.