

Mechanics and actomyosin-dependent survival and chemoresistance of suspended tumor cells in shear flow

Ying Xin, Xi Chen, Xin Tang, Keming Li, Mo Yang, William Chi-Shing Tai, Yiyao Liu,
Youhua Tan

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Abstract

Tumor cells disseminate to distant organs mainly through blood circulation, in which they experience considerable levels of fluid shear stress. However, the effects of hemodynamic shear stress on biophysical properties and functions of circulating tumor cells in suspension are not fully understood. In this study, we found that the majority of suspended breast tumor cells could be eliminated by fluid shear stress, while cancer stem cells held survival advantages over conventional cancer cells. Compared to untreated cells, tumor cells surviving shear stress exhibited unique biophysical properties: cell adhesion was significantly retarded; these cells exhibited elongated morphology and enhanced spreading and expressed genes related to epithelial-mesenchymal transition or hybrid phenotype; surviving tumor cells showed reduced F-actin assembly and stiffness. Importantly, inhibiting actomyosin activity promoted the survival of suspended tumor cells in fluid shear stress, while activating actomyosin suppressed cell survival, which might be explained by the up- and down-regulation of the anti-apoptosis genes. Soft surviving tumor cells held survival advantages in shear flow and higher resistance to chemotherapy. Inhibiting actomyosin activity in untreated cells enhanced chemoresistance, while activating actomyosin in surviving tumor cells suppressed this ability. These findings might be associated with the corresponding changes in the genes related to multidrug resistance. In summary, these data demonstrate that hemodynamic shear stress significantly influences biophysical properties and functions of suspended tumor cells. Our study unveils the regulatory roles of actomyosin in the survival and drug resistance of suspended tumor cells in hemodynamic shear flow, which suggest the importance of fluid shear stress and actomyosin activity in tumor metastasis. These findings may reveal a new mechanism by which circulating tumor cells are able to survive hemodynamic shear stress and chemotherapy and may offer a new potential

strategy to target circulating tumor cells in shear flow and combat chemoresistance through actomyosin.

Introduction

Metastasis is a complex process, mainly including detachment of tumor cells from primary lesions, invasion into tumor stroma, intravasation into the vascular system, survival in circulation, extravasation into distant organs, and formation of metastatic tumors (1). Tumor cells metastasize to distant organs mainly through hematogenous dissemination, where the frequency of circulating tumor cells (CTCs) is correlated with poor prognosis and overall survival in cancer patients (2, 3). CTCs are heterogeneous with diverse subpopulations of distinct genotypes and phenotypes (4, 5). Less than 0.01% of them may eventually generate metastatic tumors in secondary sites, indicating the inefficiency of metastasis (1). Nevertheless, metastasis accounts for over 90% of cancer-related deaths (1), suggesting that a subpopulation of CTCs are able to survive the metastatic process and form metastases. To target metastasis, it is thus essential to understand the roles of various factors during dissemination in the survival and functions of CTCs.

Apart from many biochemical factors that affect CTC functions and metastasis (6), cells are able to sense and respond to forces through mechanotransduction (7) that regulate mRNA transcription and cellular functions (8, 9). Emerging evidence has demonstrated that mechanical factors play important roles in tumor metastasis (10, 11), including fluid shear stress tumor cells experience in blood circulation, which is hypothesized to affect the survival and functions of CTCs. Fluid shear stress significantly influences the functions of tumor cells that are adhered to solid substrates. High levels of shear stress promote the production of reactive oxygen species in

lung cancer cells and induce cell damage (12). Fluid shearing sensitizes cancer cells to radiation-induced apoptosis by regulating integrin and focal adhesion kinase activity (13). Shear forces modulate global gene expression and affect the proliferation of colon cancer cells (14). Interstitial shear flow arrests tumor cell cycle and proliferation through integrin and Smad (15). Shear stress gradients stimulate the expression of insulin-like growth factor-1 and proliferation of Ewing sarcoma cells (16). Shear stress in lymphatic vasculature regulates ROCK-YAP1 signaling and enhances cancer cell migration (17). Shear flow increases the secretion of matrix metalloproteinases and cancer cell invasion (18). Fluid flow facilitates epithelial-mesenchymal transition (EMT) and cell mobility (19) and confers tumor cells CSC properties (20). However, the effects of fluid shear stress on tumor cells in suspension remain less understood. The viability and proliferation of colon CTCs are related to the magnitude of shear stress and circulating time (21). Shear stress sensitizes suspended colon and prostate tumor cells to apoptosis (22). High fluid shear stress induces considerable levels of apoptosis in tumor cells (23) and facilitates migration and extravasation (24). Malignant tumor cells exhibit resistance to hemodynamic shear stress, which is regulated by transforming oncogenes (25) and nuclear lamin A/C (26). Nevertheless, the influence of hemodynamic shear stress on biophysical properties and chemoresistance of CTCs remains elusive. The roles of cell mechanics in the survival and drug resistance of CTCs in fluid shear flow are unclear.

In this study, we developed a circulatory system to generate physiologic levels of hemodynamic shear stress, which mimicked certain important aspects of the CTC microenvironment in blood circulation. The survival of tumor cells in suspension, as a model for real CTCs, under different shear stress and circulation duration was examined. The properties of tumor cells surviving hemodynamic shear stress were characterized, including adhesion, morphology, spreading,

cytoskeleton, cellular stiffness, and chemoresistance. Importantly, the roles of actomyosin activity in the survival of suspended tumor cells in fluid shear stress and in drug resistance of surviving tumor cells were elucidated.

Materials and Methods

Cell culture. Human breast cancer cell lines MDA-MB-468, MCF-7, MDA-MB-231, and MDA-MB-453 were purchased from ATCC. Cells were maintained with Dulbecco's Modified Eagle Medium (DMEM; HyClone, Logan, UT) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (HyClone) in petri dishes at 37°C and 5% CO₂. Cells were passaged every 2-3 days using 0.25% Trypsin (HyClone).

Shear stress treatment. The circulatory system consisted 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. The system could generate pulsatile flow, which mimicked hemodynamic shear stress in blood circulation. According to Poiseuille's law, wall shear stress τ (dyne/cm²) in the tubing was calculated by $\tau=4\mu Q/(\pi R^3)$, where Q is the flow rate (from 0.001 to 230 ml/min), μ is the dynamic viscosity of the fluid (0.01 dyne*s/cm² for cell culture medium), and R is the radius of the tube (0.255 mm). The whole system was sterilized by 75% ethanol before experiments and then rinsed with 4 ml phosphate buffered saline (PBS, HyClone). To reduce the adhesion of suspended tumor cells to the tube and syringe, the system was washed with 4 ml 1% bovine serum albumin (BSA, VWR Life Science). During experiments, 2 ml cell suspension solution (2×10^5 cells/ml) was added into the circulatory system and subjected to various magnitudes of shear stress for different durations (0-20 dyne/cm²; 0-24 h) in the cell culture incubator at 37°C and 5% CO₂.

Pharmacologic treatment and plasmid transfection. For pharmacologic treatment, cells were treated with 2 μ M Y-27632 (Selleckchem), 4 μ M blebbistatin (Sigma), 0.3 μ M cytochalasin D (TOCRIS), and 10 or 50 μ M 5-FU (TOCRIS) for different durations. Lipofectamine 3000 Reagent (Thermo Fisher) was used for plasmid transfection, including pSLIK-Venus, pSLIK CA-ROCK2, and pSLIK CA-MLCK (gifts from Professor Sanjay Kumar). In brief, cells were seeded in 24-well plate until 70%-90% of confluence was reached. Lipofectamine 3000 Reagent and plasmids were diluted using Opti-MEM, respectively. Diluted plasmids were then mixed with P3000 reagent. The same amount of Lipofectamine 3000 and plasmids was mixed and incubated at room temperature for 15 min. The plasmid-lipid mixture was used to transfect cells for 2 days, when 50 ng/ml doxycycline (TargetMol) was added for another 2 days.

MTS assay. Cell viability was measured by MTS assay (Promega) following the manufacturer's instructions. Briefly, 100 μ l of cell suspension was collected from the circulatory system and then added into one well in a 96-well plate. After 12 h incubation, 20 μ l of sterilized CellTiter 96 Aqueous One Solution (5 mg/ml) was added to each well and the plate was incubated at 37°C for 4 h. The absorbance of the cell solution was measured at 490 nm using Benchmark Plus microplate reader (Bio-Rad).

Quantification of cell adhesion. For cell adhesion, the culture medium was changed to DMEM without FBS at 8 h before the assay. The 96-well plate was pre-treated with 40 μ g/ml collagen I solution (Corning) at 4 °C for 12 h. Cells obtained from various conditions were re-suspended in 0.1% BSA-DMEM and added into the treated 96-well plate. After plating for different durations, supernatant containing non-adherent cells was removed and fresh culture medium was added. For each condition, the same number of cells were cultured for 24 h without removing

supernatant, which were used as a reference. Cells were incubated for another 12 h, when the quantification of cell adhesion was measured by MTS assay. The percentage of adhered cells was calculated by the ratio of MTS reading over the reference under the same condition.

Quantitative RT-PCR analysis. Total mRNAs were extracted by Aurum Total RNA Mini Kit (Bio-Rad) and complementary DNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo) according to the manufacturer's instructions, respectively. Quantitative RT-PCR was performed using Forget-Me-Not EvaGreen qPCR Master Mix with Rox (Biotium) and CFX96 Real-Time System (Bio-Rad). The sequences of all the primers were obtained from the National Centre for Biotechnology Information (NCBI) database and listed in the Supplementary Table 1. For data analysis, the expressions of all genes were normalized using the $\Delta\Delta$ cycle threshold method against human glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western blotting. Cells were lysed by RIPA lysis and extraction buffer (Solarbio) together with Halt™ Phosphatase Inhibitor Cocktail (Thermo Fisher). About 50 µg of total protein was transferred from 10% SDS-PAGE gel to a PVDF western blot membrane using Trans-Blot Turbo (BIO-RAD). The membrane was incubated with TBST/5% non-fat milk (Solarbio) and stained with different primary antibodies: E-cadherin, Twist, Tubulin (Abcam) and secondary antibodies: Goat Anti-Mouse IgG (H + L)-HRP Conjugate and Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (BIO-RAD). Images were taken using Clarity™ and Clarity Max™ Western ECL Blotting Substrates and ChemiDoc™ MP Imaging System (BIO-RAD).

F/G-actin ratio measurement. F-actin/G-actin ratio was measured by G-actin/F-actin In Vivo Assay Kit (Cytoskeleton) under the manufacturer's instructions. Briefly, cells after different

treatment were lysed in Lysis and F-actin Stabilization Buffer combined with ATP solution and protease inhibitor cocktail and centrifuged at 2000 rpm for 5 min at room temperature. Then the supernatant was transferred to an ultrafast centrifuge tube and centrifuged at 100,000 g for 1 h to pellet F-actin from soluble G-actin. F-actin depolymerization buffer was added to the F-actin pellet on ice for 1 h. The expression of F-actin and G-actin protein was analyzed by Western blotting as described above.

Annexin V assay. Apoptosis assay was performed using Annexin V-FITC Apoptosis Staining/Detection Kit (Abcam) and BD Accuri C6 Flow Cytometer (BD Biosciences). 100,000 cells were collected and re-suspended in 500 µl of 1X Binding Buffer. Then 5 µl of Annexin V-FITC and 5 µl of propidium iodide were added into cell solution and incubated in dark for 5 min. 10,000 cells were counted for analysing Annexin V-FITC binding by FITC signal detector and PI staining by the PE signal detector. The results were analysed by BD Accuri C6 software.

Quantification of cell morphology and spreading. Tumour cells were exposed to 0 or 20 dyne/cm² shear stress for 12 h and then plated into petri dishes. At least 100 cells/condition were imaged by the inverted microscope (Nikon) after different durations of incubation. Cell aspect ratio and spreading area were quantified by the ImageJ software (NIH).

Statistical analysis. Two-tailed Student's t-test or ANOVA analysis was used for the statistics among two or more conditions. The post hoc Tukey or Bonferroni test was adopted in the ANOVA analysis for the comparisons with equal or unequal sample sizes, respectively.

Note that more methods can be found in the **Supporting Material**.

Results

The majority of suspended tumor cells can be eliminated by hemodynamic shear stress dependent on shear stress and circulation duration

To explore the influence of fluid shear stress on CTCs, we developed a circulatory system (Fig. S1), which included a peristaltic pump, a silicone micro-tubing, and a syringe as cell solution reservoir. The system could generate pulsatile flow at 37 °C and 5% CO₂, which mimicked shear stress in blood circulation. The wall shear stress τ in tubing was calculated according to $\tau = 4\mu Q/(\pi R^3)$ (21), where μ is fluid viscosity (0.01 dyne.s/cm² for the medium), Q is flow rate, and R is tubing radius (0.255 mm). To be physiologically relevant, fluid shear stress within 20 dyne/cm² and circulation duration within 12 h were chosen in this study (2, 21). Note that it is challenging to experimentally measure local shear stress experienced by single tumor cells.

Our data show that the viability of breast cancer cells MDA-MB-468 in suspension gradually decreased to ~21% when hemodynamic shear stress and circulating time increased from 0 to 20 dyne/cm² and from 0 to 12 h, respectively (Fig. 1a). Similar findings were also observed in several other types of breast cancer cells, including MCF-7, MDA-MB-453, and MDA-MB-231 (Fig. 1b, 1c, and Fig. S2a), suggesting that fluid shear stress eliminates the majority of CTCs dependent on the magnitudes of shear stress and circulation duration. Cancer stem cells (CSCs) have been proposed to initiate metastatic tumors (27) and thus must have the survival advantages during the whole metastatic process, including blood circulation. To this end, tumorigenic CSCs were selected from MDA-MB-468 and MCF-7 cells by 3D soft fibrin gels as we developed previously (28, 29). These fibrin-selected CSCs exhibited much higher self-renewal (Fig. S3) (30, 31) and remarkably higher viability in fluid shear flow than conventional tumor cells (Fig. 1d and Fig. S2b), suggesting that CSCs hold survival advantages in blood shear flow.

Tumor cells surviving hemodynamic shear stress exhibit unique biophysical properties

Although the majority of tumor cells in circulation can be eliminated by hemodynamic shear stress, a minor subpopulation of cells persist in the heterogeneous CTCs that may eventually generate metastatic tumors. It is thus crucial to characterize the functions of these surviving tumor cells. To this end, their biophysical properties were examined, including cell adhesion, morphology, spreading, cytoskeleton, and cellular stiffness. The data show that compared to untreated cells (“Control”), the adhesion to solid substrates was considerably suppressed at 2 h after plating for tumor cells treated by 0 dyne/cm² shear stress and at 2, 4, and 8 h for cells treated by 20 dyne/cm² shear stress (Fig. 2a and 2b). Significant differences were found in cell adhesion at 2 and 4 h between cells treated by 0 and 20 dyne/cm² shear stress (Fig. 2b). The adhesion of these tumor cells was increased to the similar level of untreated cells at 4 and 12 h after plating, respectively. These findings suggest that the adhesion of tumor cells surviving fluid shear flow may be significantly inhibited at early phase but restored to the normal level at late phase.

After culture on solid substrates, cell morphology and spreading were examined. We found that although cell adhesion was suppressed at early phase for tumor cells surviving shear stress treatment (0 and 20 dyne/cm²), the morphology of the adhered cells was notably more elongated than untreated MDA-MB-468 (except 0 dyne/cm² at 8 h; Fig. 3a and 3b) and MCF-7 cells (except 0 dyne/cm² at 12 h; Fig. S4a and S4b). The spreading area of these surviving tumor cells was significantly increased especially after 4 h post plating for MDA-MB-468 and MCF-7 cells (Fig. 3c and Fig. S4c). Compared to the cells after 0 dyne/cm² shear stress treatment, tumor cells surviving 20 dyne/cm² shear stress exhibited higher aspect ratio at 2 and 20 h for MDA-MB-468

and at 1, 4, 12, and 20 h for MCF-7 cells and spread more for MDA-MB-468 (except at 20 h) and at 4, 12, and 20 h for MCF-7 cells. The findings of elongated cell morphology and enhanced spreading suggest that tumor cells surviving hemodynamic shear stress may undergo EMT. To explore this possibility, we examined the expression of EMT genes. Compared to untreated cells, surviving tumor cells expressed lower epithelial cell marker E-cadherin and higher mesenchymal cell marker Twist at the protein level (top panel in Fig. 3d). Note that tumor cells expressed higher levels of both E-cadherin and Twist after the treatment under 20 dyne/cm² shear stress than 0 dyne/cm² shear stress, representative of a hybrid epithelial/mesenchymal phenotype. Further, E-cadherin was significantly down-regulated and the mesenchymal cell markers fibronectin, twist, and vimentin were notably up-regulated in surviving tumor cells at the mRNA level (bottom panel in Fig. 3d). All these data suggest that fluid shear stress may promote EMT or hybrid epithelial/mesenchymal phenotype in surviving tumor cells.

Cell morphology and spreading are closely related to cytoskeletal structures. Compared to untreated cells, F-actin assembly and the F-actin/G-actin ratio were notably decreased in tumor cells after shear stress treatment, as measured by both immunofluorescence and western blotting (Fig. 4a, 4b, and Fig. S5). Tumor cells surviving 20 dyne/cm² shear stress exhibited significantly lower F-actin and F-actin/G-actin ratio (~0.38 vs ~0.64) than the cells after 0 dyne/cm² shear stress treatment. These findings suggest that fluid shear stress may remodel the cytoskeleton of suspended tumor cells. Since cytoskeleton largely determines cell mechanics (32), we further explored whether surviving tumor cells with remodeled cytoskeleton exhibited unique mechanical properties. The results of atomic force microscopy measurement show that the force-indentation curves of tumor cells after shear stress treatment had significantly lower values of slope (Fig. 4c). By fitting the data with the modified Hertz model (33), Young's modulus was

characterized. The results show that surviving tumor cells (20 dyne/cm²) were notably softer than tumor cells after suspension treatment (0 dyne/cm²) and untreated cells (~1600 Pa vs ~2000 and ~2300 Pa; Fig. 4d). These data suggest that suspended tumor cells surviving hemodynamic shear stress are much softer with reduced F-actin assembly.

Actomyosin activity regulates the survival of suspended tumor cells in hemodynamic shear stress

We have shown that hemodynamic shear stress considerably influences the survival of suspended tumor cells and that surviving tumor cells exhibit unique biophysical properties, including the reduced F-actin and cell stiffness. However, the roles of cell mechanics in the survival of CTCs in shear flow remain unclear. To address this problem, the cytoskeleton and mechanics of tumor cells were modulated by targeting actomyosin activity. The data show that pharmacologically inhibiting actomyosin by Rho-associated protein kinase (ROCK) inhibitor Y27632, myosin II inhibitor blebbistatin, or F-actin inhibitor cytochalasin D reduced F-actin assembly (Fig. S6) but notably enhanced the viability of suspended breast tumor cells (MDA-MB-468 and MCF-7) in fluid shear flow (Fig. 5a and Fig. S7a; except Y27632 in Fig. S7a). This effect was further confirmed by the Annexin V apoptosis assay (Fig. S8). On the other hand, activating actomyosin by expressing constitutive active (CA) mutants of myosin light-chain kinase (MLCK) or ROCK significantly increased F-actin assembly and the F-actin/G-actin ratio (Fig. S9) but suppressed tumor cell survival in hemodynamic shear stress (Fig. 5b and Fig. S7b; except CA-MLCK at 12 h in Fig. 5b and in Fig. S7b). These findings suggest that low/high actomyosin activity promotes/inhibits the survival of suspended tumor cells in blood shear flow.

Tumor cells surviving shear stress exhibited low stiffness (Fig. 4). We wondered whether these soft tumor cells possessed survival advantages in blood shear flow. The data show that surviving tumor cells held remarkably higher viability in shear flow compared to control tumor cells (Fig. 5c). CSCs are known to have lower stiffness than bulk tumor cells (30, 31). We have demonstrated that CSCs survive much better in fluid shear flow (Fig. 1d and Fig. S2b). Activating actomyosin significantly reduced the survival of CSCs in shear stress (Fig. S7c). To explore the underlying survival mechanisms, the anti-apoptosis genes were examined, including B-cell lymphoma 2 (Bcl2) and superoxide dismutase 2, mitochondrial (SOD2). We found that tumor cells after treatment by 20 dyne/cm² shear stress showed considerable increase in the expressions of these genes compared to control cells and tumor cells treated in suspension (Fig. 5d and Fig. S10). Inhibiting actomyosin significantly increased the expressions of Bcl2 and SOD2 (Fig. 5d and Fig. S11), while activating actomyosin activity suppressed their expressions (Fig. 5e and Fig. S12). These data suggest that actomyosin regulates the survival of suspended tumor cells in fluid shear flow probably via the effects on anti-apoptosis genes.

Suspended tumor cells surviving hemodynamic shear stress exhibit actomyosin-dependent chemoresistance

Research and clinical findings have demonstrated that metastatic tumors exhibit high levels of resistance to conventional chemotherapy (34–36), which is one major cause of recurrence and cancer-induced death. Tumor cells with the ability to generate metastases must survive the entire metastatic process, including blood circulation, and may be responsible for drug resistance of metastatic tumors. We thus hypothesized that tumor cells surviving hemodynamic shear stress might possess the ability to resist chemotherapy. To test this idea, breast cancer cells surviving 0

and 20 dyne/cm² shear stress were treated with different doses of conventional chemotherapy drug fluorouracil (5-FU). The data show that surviving tumor cells exhibited remarkably higher viability than untreated cells after chemotherapy (Fig. 6a and Fig. S13a), suggesting that tumor cells surviving blood shear flow or suspension hold considerable chemoresistance advantage. Note that there was no significant difference in drug resistance between 0 and 20 dyne/cm² shear stress treatment. We have shown that these surviving tumor cells exhibit low F-actin and stiffness. However, the roles of cell mechanics in chemoresistance of CTCs remain unclear. Our results show that activating CA-ROCK or CA-MLCK in soft surviving tumor cells significantly decreased cell viability after chemotherapy (Fig. 6b and Fig. S13b), suggesting that high actomyosin activity suppresses chemoresistance. Further, pharmacologic inhibition of actomyosin in untreated breast cancer cells remarkably enhanced resistance to chemotherapy (Fig. 6c and Fig. S13c). These findings demonstrate that actomyosin activity regulates chemoresistance of tumor cells surviving fluid shear flow. To explore the underlying mechanisms, the genes related to multidrug resistance (MDR) were examined. The data show that tumor cells after 5-FU treatment and the cells surviving shear stress up-regulated the expressions of MDR genes in both MDA-MB-468 (MRP3, GSTP1, ABCG2, and MRP1) and MCF-7 cells (ABCG2 and MRP3) (Fig. 6d, Fig. S13d, and Fig. S14a). Pharmacologically inhibiting actomyosin up-regulated the expressions of MDR genes (Fig. 6d, Fig. S13d, and Fig. S14b), while activating ROCK but not MLCK significantly suppressed these genes (Fig. 6e and Fig. S15). These findings suggest that suspended tumor cells surviving hemodynamic shear stress hold chemoresistance ability in an actomyosin-dependent manner.

Discussion

Tumor cells disseminate to distant organs mainly through blood circulation during metastasis (1), in which they experience considerable levels of fluid shear stress. It is documented that mechanical factors play important roles in tumor progression and metastasis (10, 11). Fluid shear stress significantly affects the survival, proliferation, and other functions of adhered and suspended cells (21–25, 37, 38). Consistently, our study shows that the majority of tumor cells in suspension can be eliminated by fluid shear flow, suggesting that hemodynamic shear stress could be one major cause for the poor survival of CTCs in vasculature and metastasis inefficiency. Nevertheless, a small subpopulation of tumor cells are able to survive in fluid shear flow, which may enrich tumor cells with the ability to generate metastatic tumors. However, the biophysical properties of these cells remain poorly characterized. This study shows that tumor cells surviving suspension or shear flow appear to be mesenchymal-like or hybrid epithelial/mesenchymal, which is different from the reported finding (37), possibly due to distinct fluid shear conditions. Note that fluid shear flow induces a hybrid epithelial/mesenchymal phenotype in suspended tumor cells compared to suspension. It is still unclear how shear stress influences the EMT phenotype of suspended tumor cells. It is possible that fluid shear flow selects a subpopulation of pre-existing cells with mesenchymal or hybrid phenotype or induces the phenotypic changes, which needs to be further tested in the future. Compared to the suspension condition, cell cytoskeleton is further remodeled after shear stress treatment, especially the reduced F-actin, which may lead to the decrease in cellular stiffness and contrasts with the effect of shear flow on adhered cells (38). Note that previous studies have shown the reduction of F-actin when cells are in suspension (39). It is known that EMT or hybrid epithelial/mesenchymal phenotype promotes tumor cell malignancy (4–6, 40) and cell stiffness is inversely correlated with malignancy (41). The occurrence of EMT or hybrid phenotype and low

stiffness in CTCs after shear stress treatment may confer surviving cells enhanced metastatic potential, which may favor the subsequent generation of secondary tumors. In addition, cell adhesion is critical for tumor cell motility and metastasis (42). Although we show that the adhesion of tumor cells surviving fluid shear stress is retarded, the relationship between cell adhesion, EMT or hybrid epithelial/mesenchymal phenotype, and metastatic potential of CTCs needs to be rigorously investigated in the future. Nevertheless, our data demonstrate that hemodynamic shear stress significantly affects the biophysical properties of suspended tumor cells.

Our data, together with several others (21, 23, 25, 26, 37, 38), show that a subpopulation of suspended tumor cells can survive fluid shear flow. Except the reported oncogenes (25) and nuclear lamin A/C (26), the underlying survival mechanisms, however, remain less understood. We have found that tumor cells surviving shear stress exhibit low stiffness, which is predominantly determined by actomyosin activity (32). It is known that actomyosin is important for cell survival in various contexts, including dissociated embryonic stem cells (43), oxidative stress-induced neuronal apoptosis (44), and disruption of nuclear integrity during apoptosis (45). However, the roles of actomyosin in the survival of suspended CTCs in fluid shear stress remain unclear. Our data show that inhibiting actomyosin significantly enhances the viability of suspended tumor cells in shear flow, while activating actomyosin suppresses tumor cell survival. Soft surviving tumor cells and CSCs hold survival advantages in shear flow, while activating actomyosin in CSCs inhibits cell survival. These findings suggest that actomyosin activity regulates the survival of suspended tumor cells in fluid shear stress, which may unveil a new mechanism by which a subpopulation of CTCs are able to survive hematogenous dissemination, an essential step towards the generation of metastases. The results that low actomyosin activity

or low stiffness promotes tumor cell survival in hemodynamic shear stress are in line with many previous findings. Tumor cells with high metastatic potential exhibit low cell stiffness or actomyosin (41) and hold survival advantages during metastatic process (6). Increasing actomyosin contractility inhibits the invasion and migration of tumor cells and potentially cell survival in metastasis (46, 47). These findings suggest that low actomyosin activity is correlated with malignancy and high survival rate. Consistently, our findings have demonstrated that fibrin-selected CSCs with low stiffness exhibit high self-renewing ability (Fig. S3) (30, 31) and high viability in shear flow (Fig. 1d and Fig. S2b). Further, actomyosin regulates tumor cell survival probably through the effects on anti-apoptosis genes. In addition, EMT or hybrid epithelial/mesenchymal phenotype appears to occur in surviving tumor cells (Fig. 3 and Fig. S4), which may also promote tumor cell survival in shear flow (4, 6). Note that the mechanisms by which suspended CTCs sense hemodynamic shear stress remain to be discovered. Nevertheless, these findings may offer a new strategy to eliminate CTCs in blood shear flow by specifically delivering actomyosin activating compounds into cancer cells through sophisticated drug delivery methods (48).

Metastasis is closely linked with chemoresistance (34). However, the underlying mechanisms have not been fully understood (35, 36). In particular, the roles of hemodynamic shear stress and actomyosin-dependent cell mechanics in drug resistance of CTCs remain unclear. Our study shows that tumor cells surviving fluid shear stress exhibit low stiffness and enhanced drug resistance to chemotherapy. Note that there is no significant difference in chemoresistance between tumor cells after suspension and shear stress treatment. The underlying mechanisms need to be further studied. Nevertheless, activating actomyosin in surviving tumor cells suppresses chemoresistance, while inhibiting actomyosin in untreated cells enhances drug

resistance, suggesting actomyosin may play a regulatory role in chemoresistance of suspended tumor cells. The actomyosin-dependent chemoresistance is possibly mediated by the genes related to multidrug resistance. These results are consistent with our previous work that fibrin-selected CSCs are soft and highly chemoresistant (28) and other findings that soft cells separated from the whole tumor cell population are tumorigenic and chemoresistant (49). Note that previous research has indicated the controversial roles of Rho/ROCK signaling in drug resistance (50). Some findings show that overexpressing Rho GDP dissociation inhibitor that negatively regulates Rho activity or inhibiting ROCK signaling mediates cancer cell resistance to chemotherapeutic treatment (51, 52), while several others report that activating Rho signaling enhances drug resistance in certain types of cancer (53). The effect of ROCK on chemoresistance may be also related to its roles in apoptosis (50). Further, we also show that EMT or hybrid epithelial/mesenchymal phenotype may occur in suspended tumor cells after shear stress treatment. Since EMT or hybrid epithelial/mesenchymal phenotype has been implicated in drug resistance (54, 55), it is possible that the chemoresistance ability of surviving tumor cells may be partially mediated by EMT or hybrid phenotype. The relationship between EMT or hybrid epithelial/mesenchymal phenotype, actomyosin activity, and drug resistance of CTCs in fluid shear flow needs to be further investigated in the future. Nevertheless, these findings unveil the critical roles of actomyosin activity in chemoresistance of suspended tumor cells, which may have major implications in the treatment of metastatic tumors.

It is known that CTCs in cancer patients are very rare (1-10 CTCs per ml blood) (2, 3). Due to this technical and other challenges, cancer cell lines in suspension have been utilized in this study as an alternative model. Note that they may recapitulate certain aspects but not the bona fide biology of CTCs and differ from primary tumor cells from patients. Further, CTCs reside in

a very complexed microenvironment, in which blood cells, immune cells, coagulation factors, and hemodynamic shear flow are present (2, 3). This study has investigated the effects of hemodynamic shear stress but not other factors in blood circulation. The influence of the interactions among various factors on the properties of CTCs has not been examined. Therefore, to extend our current findings to the clinical setting, it is necessary to rigorously test the ideas using patient-derived CTCs or CTC lines in a more sophisticated system better representative of the blood microenvironment.

Conclusion

In summary, this work reports that the majority of suspended tumor cells in suspension can be eliminated by hemodynamic shear stress. The surviving cells exhibit unique biophysical properties, including retarded cell adhesion, EMT-like or hybrid epithelial/mesenchymal phenotype, and reduced cellular stiffness. Importantly, low actomyosin activity promotes the survival of suspended tumor cells in fluid shear flow and chemoresistance, while high actomyosin inhibits tumor cell survival and drug resistance of tumor cells surviving shear stress treatment. These findings unveil the regulatory role of actomyosin activity in the survival of suspended tumor cells in hemodynamic shear stress and chemoresistance, which may have important implications in targeting CTCs and drug resistance in metastasis.

Supporting Material

Supporting Materials and Methods and 15 supplementary figures are available.

Author Contributions

Y.T. conceived the project. Y.T., Y.X., X.C. X.T., and K.L. designed and conducted the experiments, analyzed data, and wrote the manuscript. M.Y, W.C.S.T., and Y.L. provided plasmids, analyzed data, and commented the manuscript.

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The authors declare no conflicts of interest.

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Figures

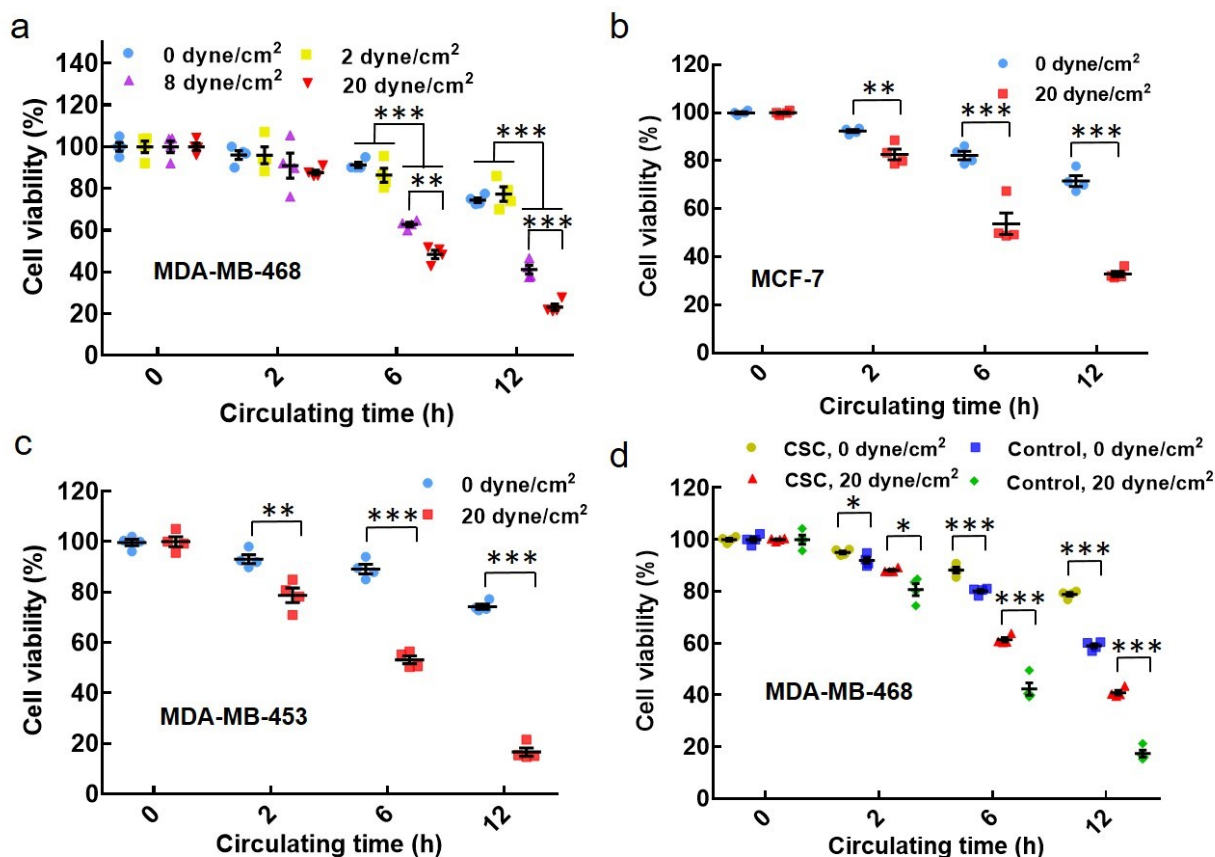


FIGURE 1 The majority of suspended tumor cells can be eliminated by hemodynamic shear flow dependent on the magnitudes of shear stress and circulation duration. (a-c) The viability of breast cancer cells in suspension decreases along with fluid shear stress and circulation duration. Single cell suspensions of MDA-MB-468 (a), MCF-7 (b), and MDA-MB-453 (c) were obtained by trypsinization from petri dish and circulated under various magnitudes of shear stress and circulation duration. The viability of treated cells was measured by MTS assay and normalized by the data at 0 h under the same treatment. n=4 independent experiments. (d) Fibrin-selected cancer stem cells exhibit considerable survival advantage in hemodynamic shear flow over non-

selected cells. Breast cancer cells MDA-MB-468 were cultured in soft (90-Pa) fibrin gels for 7 days. Suspended fibrin-selected cells (cancer stem cells or CSC) and tumor cells cultured in petri dish (Control) were treated under 0 and 20 dyne/cm² shear stress, respectively. n=4 independent experiments. The statistics were conducted using ANOVA with the post hoc Tukey test in (a) and two-tailed Student t-test in (b-d). *, p<0.05; **, p<0.01; ***, p<0.001.

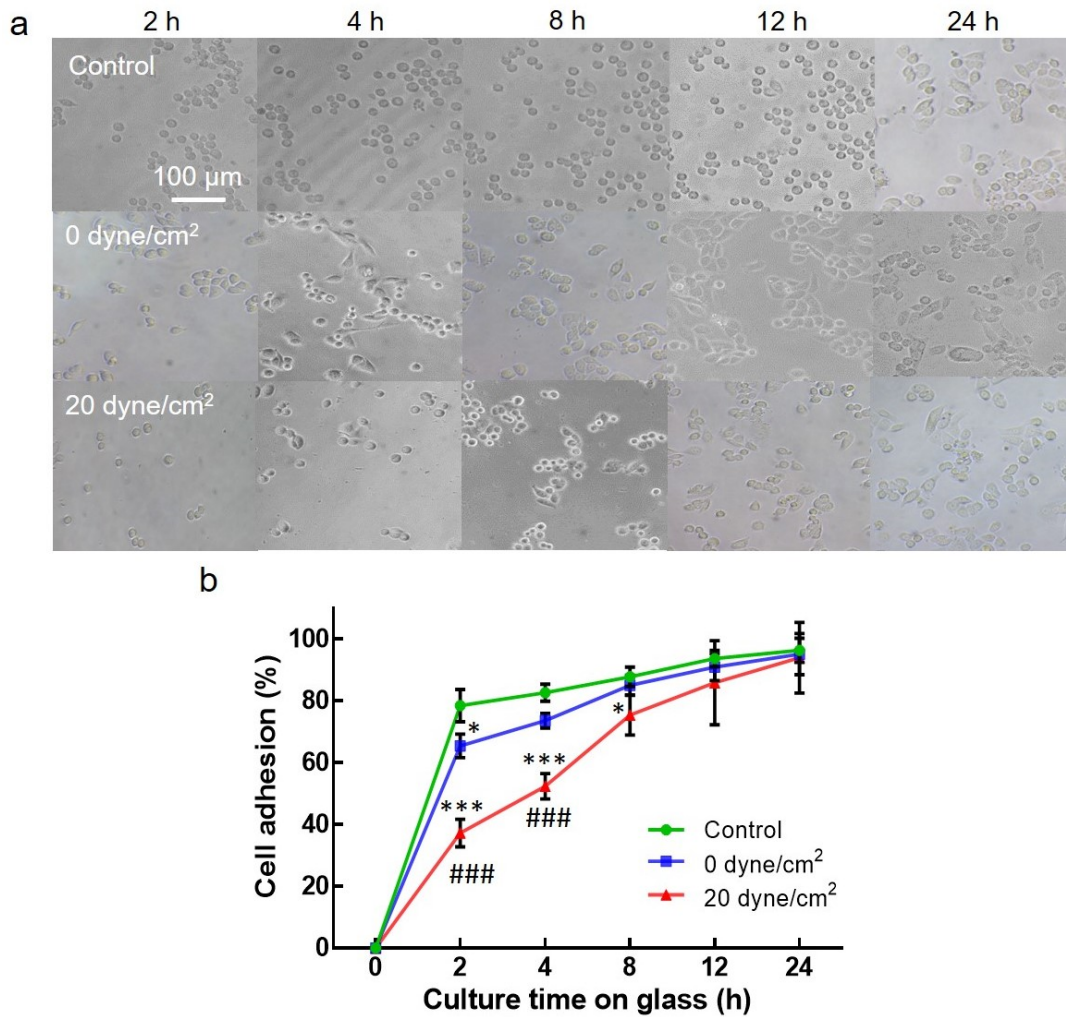


FIGURE 2 Hemodynamic shear stress retards the adhesion of suspended tumor cells. (a) Representative images of tumor cell adhesion after shear stress treatment. Suspended MDA-MB-468 cells were circulated under 0 and 20 dyne/cm² shear stress for 12 h, respectively. Tumor

cells cultured in petri dish were used as a control. The treated and control cells were then cultured on collagen-coated glass for 2, 4, 8, 12, and 24 h, respectively. Non-adhered cells were removed by gentle washing before imaging. Scale bar: 100 μm . (b) Quantification of tumor cell adhesion. The percentage of the adhered cells was calculated by the ratio of the MTS reading at the indicated time point over the reading of cells at 24 h after plating under the same condition ($n=3$ wells for each condition). The statistics were conducted using ANOVA with the post hoc Tukey test. *, $p<0.05$ and ***, $p<0.001$, showing significant differences between “Control” and “0 dyne/cm²” or “20 dyne/cm²”, and ###, $p<0.001$, showing significant differences between “0 dyne/cm²” and “20 dyne/cm²”. $n=3$ independent experiments.

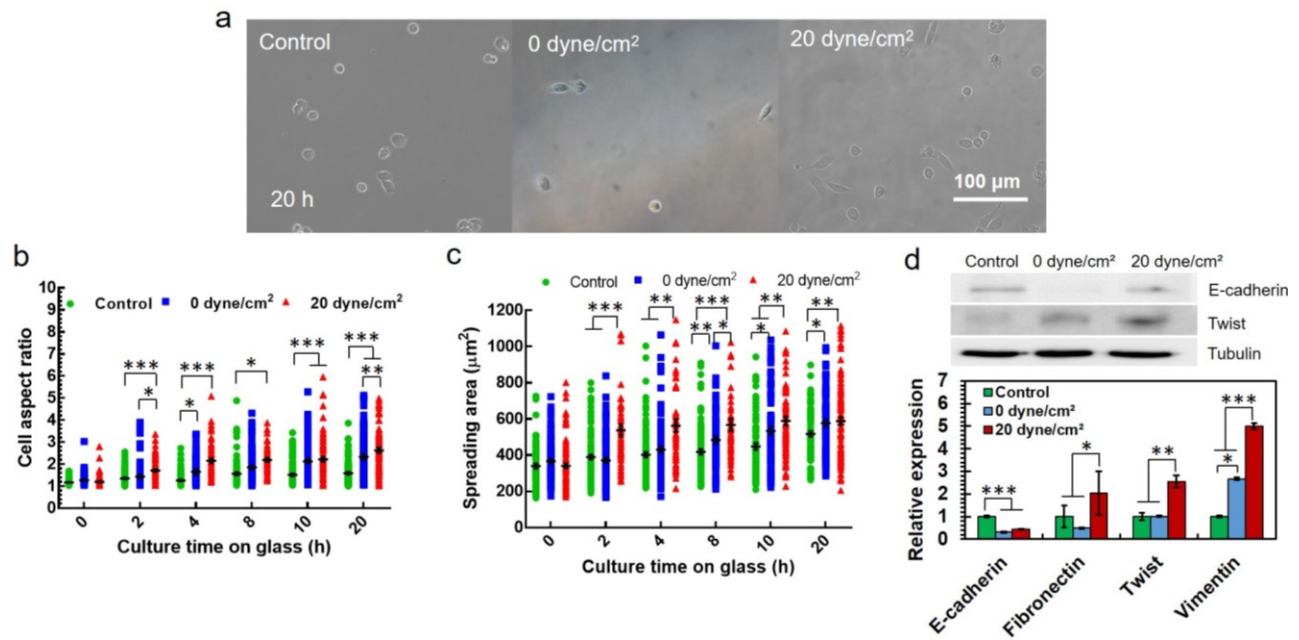


FIGURE 3 Hemodynamic shear stress influences the epithelial/mesenchymal phenotype of suspended tumor cells. (a) Representative images of tumor cell morphology after shear stress treatment. Suspended MDA-MB-468 cells treated under different shear stresses were cultured on collagen-coated glass. Cell images were taken at the indicated time points after removing non-

adhered cells. Scale bar: 100 μm . (b, c) Fluid shear stress promotes elongated morphology and spreading of circulating tumor cells. The aspect ratio (b) and spreading area (c) of the treated tumor cells in (a) were quantified ($n>92$). $n=3$ independent experiments. (d) Fluid shear stress influences the expressions of mesenchymal/epithelial genes in CTCs. The expressions of epithelial marker (E-cadherin) and mesenchymal markers (Twist, fibronectin, and vimentin) in surviving tumor cells were measured by quantitative RT-PCR. E-cadherin and Twist were analyzed by western blotting. $n=2$ and 3 independent experiments for the top and bottom panel, respectively. The statistics were conducted using ANOVA with the post hoc Bonferroni test in (b, c) and Tukey test in (d). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

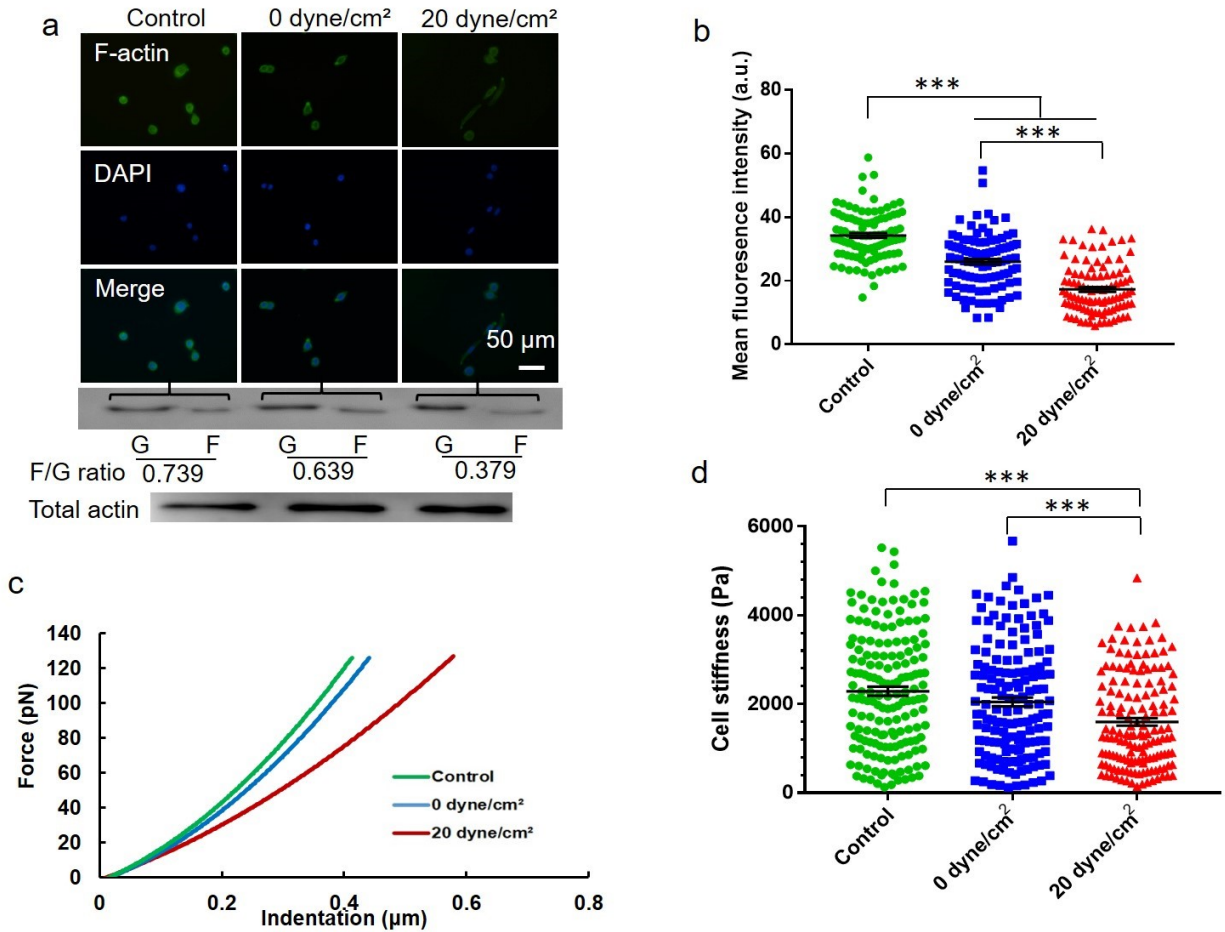


FIGURE 4 Suspended tumor cells surviving fluid shear stress exhibit reduced F-actin assembly and cell stiffness. (a) Immunofluorescence imaging and immunoblotting of F-actin in breast cancer cells after shear stress treatment. MDA-MB-468 cells were treated under 0 and 20 dyne/cm² shear stress for 12 h and then plated on glass for 10 h, when F-actin was stained and the immunoblotting of G-actin and F-actin was conducted. The level of total actin was used as a control. G: G-actin; F: F-actin. The value represents the F-actin/G-actin ratio. n=2 independent experiments. The nucleus was counterstained with DAPI. Scar bar: 50 μ m. (b) Surviving tumor cells show much lower F-actin assembly. F-actin in the top panel of (a) was quantified (n>100). a.u.: arbitrary unit. n=3 independent experiments. (c) Typical force-indentation curves of breast cancer cells after shear stress treatment measured by atomic force microscopy. (d) Breast cancer cells surviving hemodynamic shear stress exhibit much lower stiffness. MDA-MB-468 cells after shear stress treatment were plated on glass for 10 h, when cell stiffness was measured. At least 150 cells were measured for each condition. n=3 independent experiments. The statistics were conducted using ANOVA with the post hoc Bonferroni test in (b, d). ***, p<0.001.

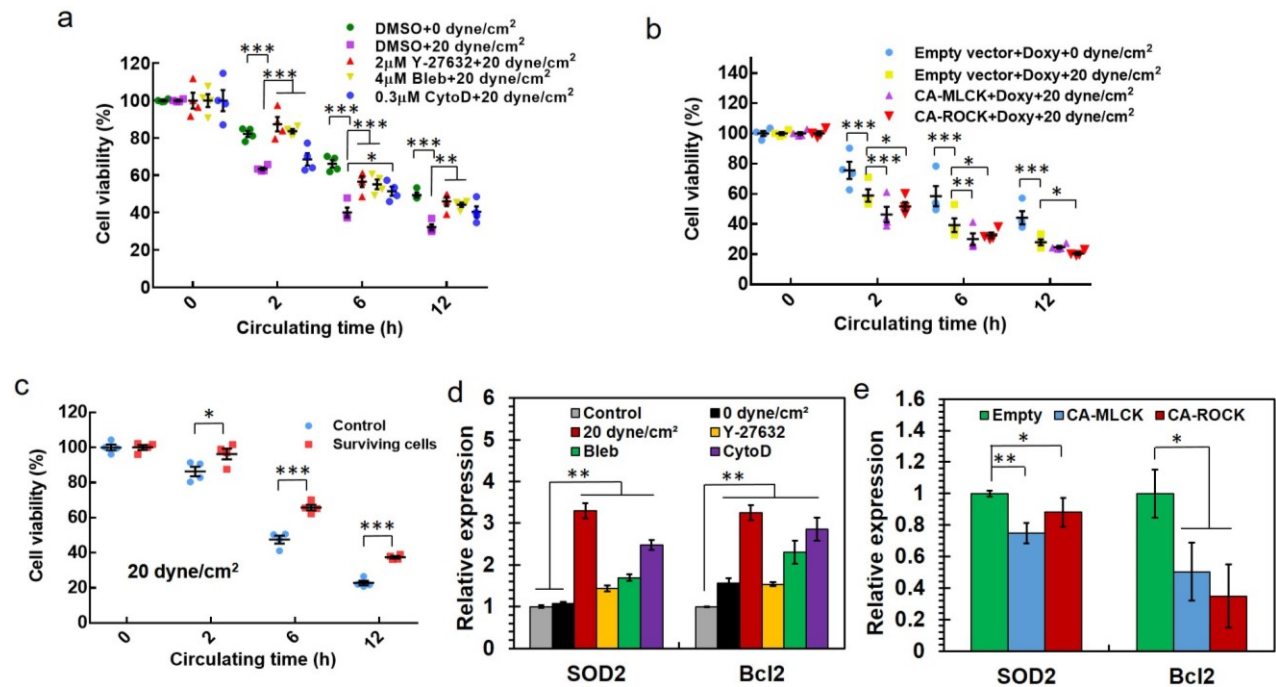


FIGURE 5 Actomyosin activity regulates the survival of suspended tumor cells in hemodynamic shear stress. (a) Pharmacologically inhibiting actomyosin enhances the viability of suspended breast tumor cells in fluid shear flow. MDA-MB-468 cells were treated with 2 μ M Y-27632, 4 μ M blebbistatin (Bleb), or 0.3 μ M cytochalasin D (CytoD) for 48 h and then circulated under 20 dyne/cm² shear stress with the inhibitor for the indicated durations. Cell viability was measured by MTS assay (3 wells per condition). n=4 independent experiments. (b) Activating actomyosin suppresses the survival of suspended breast tumor cells in fluid shear flow. Tumor cells were transfected with constitutive active (CA) MLCK or ROCK in the presence of doxycycline and then circulated under 20 dyne/cm² shear stress with doxycycline. Cell viability was measured by MTS assay. n=4 independent experiments. (c) Tumor cells surviving shear flow hold survival advantages in hemodynamic shear stress. MDA-MB-468 cells were circulated under 20 dyne/cm² shear stress for 12 h. The surviving cells were cultured in petri dish for 10 h and the retrieved suspended cells were then re-treated under 20 dyne/cm² shear stress. Tumor cells that

were cultured in petri dish were used as a control. n=4 independent experiments. (d) Shear stress or inhibition of actomyosin up-regulates the anti-apoptosis genes. MDA-MB-468 cells were treated with various inhibitors for 48 h as in (a) or circulated in 0 and 20 dyne/cm² shear stress for 12 h, respectively. The treated cells were collected for the analysis of anti-apoptosis genes Bcl2 and SOD2. n=3 independent experiments. (e) Actomyosin activation in tumor cells suppresses the expressions of anti-apoptosis genes. MDA-MB-468 cells were transfected with CA-MLCK or CA-ROCK with doxycycline for 48 h, when the gene expressions were examined. Bleb: blebbistatin; CytoD: cytochalasin D; Doxy: doxycycline. n=3 independent experiments. The statistics were conducted using ANOVA with the post hoc Tukey test in (a, b, d, e) and two-tailed Student t-test in (c). *, p<0.05; **, p<0.01; ***, p<0.001.

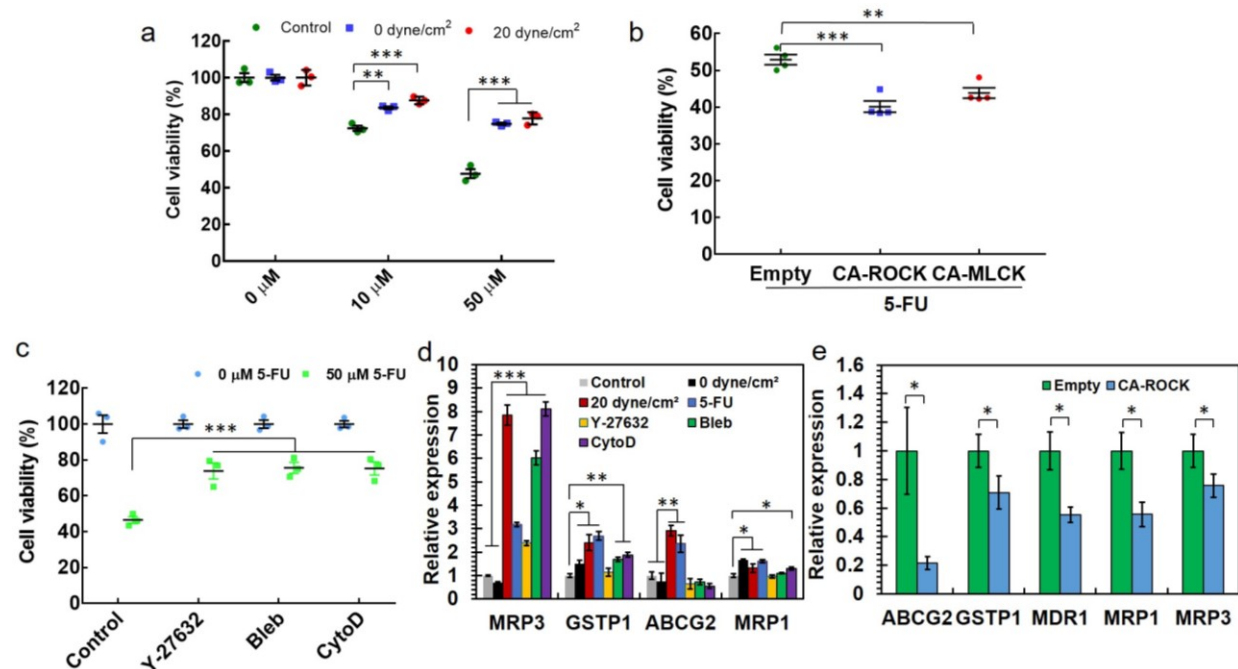


FIGURE 6 Actomyosin activity regulates chemoresistance of suspended tumor cells surviving hemodynamic shear stress. (a) Breast cancer cells surviving hemodynamic shear stress or suspension exhibit resistance to chemotherapy drug treatment. MDA-MB-468 cells were

circulated under 0 and 20 dyne/cm² shear stress for 12 h, respectively. The surviving cells were cultured on glass in the presence of 0, 10, and 50 μ M fluorouracil (5-FU) for 24 h, respectively, when cell viability was examined by MTS assay. The viability was normalized against to the value at 0 μ M under the same condition. Tumor cells cultured in petri dishes were used as a control. n=3 independent experiments. (b) Activating actomyosin in surviving tumor cells suppresses chemoresistance. Cancer cells were transfected with CA-MLCK, CA-ROCK, or empty vector in the absence of doxycycline and then circulated under 20 dyne/cm² shear stress for 12 h. The surviving cells were treated with 50 μ M 5-FU and 50 ng/ml doxycycline for 24 h, when cell viability was measured. n=4 independent experiments. (c) Inhibiting actomyosin in untreated tumor cells enhances chemoresistance. Tumor cells were pre-treated with 2 μ M Y-27632, 4 μ M blebbistatin, or 0.3 μ M cytochalasin D for 24 h and then cultured with 0 or 50 μ M 5-FU for another 24 h. 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. MDA-MB-468 cells were circulated under 0 and 20 dyne/cm² shear stress for 12 h or treated with 50 μ M 5-FU, 2 μ M Y-27632, 4 μ M blebbistatin, or 0.3 μ M cytochalasin D for 24 h. The expressions of multidrug resistance genes were examined by quantitative RT-PCR. n=3 independent experiments. (e) Activating actomyosin through ROCK in tumor cells surviving shear stress suppresses the genes related to multidrug resistance. Tumor cells were transfected with CA-ROCK plasmids in the absence of doxycycline and then circulated under 20 dyne/cm² 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. The statistics were conducted using ANOVA with the post hoc Tukey test in (a-d) and two-tailed Student t-test in (e). *, p<0.05; **, p<0.01; ***, p<0.001.