Hemodynamic shear flow regulates biophysical characteristics and functions of circulating breast tumor cells reminiscent of brain metastasis

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Abstract

Tumor cells disseminate to distant organs mainly through blood circulation, where they experience considerable levels of fluid shear flow. However, its influence on circulating tumor cells remains less understood. This study elucidates the effects of hemodynamic shear flow on biophysical properties and functions of breast circulating tumor cells with metastatic preference to brain. Only a small subpopulation of tumor cells are able to survive in shear flow with enhanced anti-apoptosis ability. Compared to untreated cells, surviving tumor cells spread more on soft substrates that mimic brain tissue but less on stiff substrates. They exhibit much lower expression of F-actin and cell stiffness but generate significantly higher cellular contractility. In addition, hemodynamic shear flow upregulates the stemness genes and considerably changes the expression of the genes related to brain metastasis. The enhanced cell spreading on soft substrates, reduced stiffness, elevated cellular contractility, and upregulation of the stemness and brain metastasis genes in tumor cells after shear flow treatment may be related to breast cancer metastasis in soft brain tissues. Our findings thus provide the first piece of evidence that hemodynamic shear flow regulates biophysical properties and functions of circulating tumor cells that are associated with brain metastasis, suggesting that tumor cells surviving in blood shear flow may better recapitulate the characteristics of organ preference in metastasis.

Introduction

Metastasis is a complex process, mainly including detachment of tumor cells from the primary lesion, invasion into the stroma, intravasation into and survival in the vascular system, extravasation into distant organs, and formation of metastatic tumors[1]. Metastasis accounts for over 90% of cancer-related deaths and hence becomes the major cause of cancer mortality[1]. Tumor cells metastasize to distant organs mainly through hematogenous dissemination, where the frequency of circulating tumor cells is correlated with poor prognosis and overall survival of cancer patients[2,3]. Less than 0.01% of circulating tumor cells may eventually grow into metastatic tumors, suggesting the inefficiency of metastasis[1]. Nevertheless, metastasis is a prevalent clinical phenomenon in many types of cancer, indicating that a minor subpopulation of tumor cells can survive the metastatic process and initiate secondary tumors. Thus, it is critical to understand the roles of various factors in circulating tumor cells during metastasis.

Apart from the importance of many biochemical factors[1], circulating tumor cells experience considerable levels of shear flow in circulation[4]. It is documented that cells have the ability to sense and respond to mechanical signals through mechanotransduction[5] that can regulate mRNA transcription and cellular functions[6,7]. Accumulating evidence has demonstrated that biophysical factors play important roles in tumor metastasis[4,8]. Recent findings show that fluid shear stress significantly influences the functions of tumor cells that are adhered to solid substrates, including cell viability[9], proliferation[10], migration[11], and mobility[12]. However, the effects of hemodynamic shear flow on circulating tumor cells in suspension are less understood[13–15], especially their biophysical properties and metastatic preference.

In this work, we developed a circulatory system to mimic hemodynamic shear flow in blood circulation and explored the effects of fluid shear flow on the biophysical properties and functions of circulating tumor cells. We found that physiologic levels of shear flow significantly influenced the survival of tumor cells in circulation. The surviving tumor cells exhibited unique biophysical properties, including enhanced cell spreading on soft substrates that mimic brain tissues, lower F-actin and cell stiffness, and elevated cellular contractility. Importantly, tumor cells after shear flow treatment up-regulated the stemness genes and considerably changed the expression of the genes related to brain metastasis in breast cancer. These biophysical characteristics and functions of surviving tumor cells may be related to metastatic preference of breast cancer to brain.

Results and discussion

To explore the influence of hemodynamic shear flow, a circulatory microfluidic system was developed to mimic shear flow in blood circulation[13], which mainly included a peristaltic pump (P-230, Harvard Apparatus), silicone tubing, and a syringe as cell solution reservoir (Fig. 1a; see more experimental details in the supplementary information). The whole system was calibrated and kept in an incubator to maintain cells at 37 °C and 5% CO_2. The wall shear stress τ_w in tubing was calculated according to $\tau_w = 4\mu Q/(\pi R^3)$, where μ was fluid viscosity (0.01 dyne.s/cm^2 for the medium), Q was flow rate, and R was tubing radius (0.255 mm)[16]. To be physiologically relevant, wall shear stress within 0-20 dyne/cm^2 and circulating time within 0-24 h were chosen in this study[2,16]. Note that wall shear stress was different from the shear stress experienced by

suspended tumor cells, which was dependent on their radial positions within the tubing $\tau = \tau_w * r/R$ for Poiseuille flow, where r was the radial position of the cell[17]. Although the cell size was much smaller than the tubing size (~20 μm vs 500 μm), the addition of living cells into fluid might introduce another layer of complexity. These might alter the distribution of shear stress in the tubing and cell membranes and thus lead to cell rotation and tumbling. It was challenging to experimentally measure local shear stress experienced by single tumor cells. The influence of fluid shear flow on cellular functions in this study could be induced by shear stress, flow rate, or others, which needs to be further explored in the future. All the stresses represented wall shear stress.

The data show that after circulation in shear flow, the viability of breast cancer cells gradually decreased along with the circulating time (Fig. 1b). Compared to the suspension condition (0 dyne/cm^2), tumor cells under 20 dyne/cm^2 shear flow showed much lower viability. Significant difference was found in cell viability between MDA-MB-231-TGL (TGL cells) and the subpopulation specifically metastasizing to brain (MDA-MB-231.BrM2-831 or BrM2 cells) after treatment for 24 h (10% vs 21%) (Fig. 1b). The viability continuously decreased when fluid shear flow increased from 0, 8 to 20 dyne/cm^2 (Fig. S1). To explore the underlying mechanisms, we interrogated the expression of the genes related to cell survival. The data show that the gene superoxide dismutase 2 (*SOD2*) was upregulated by ~5-fold and 10-fold after 0 and 20 dyne/cm^2 shear flow treatment, respectively (Fig. 1c). Compared to the cells treated by 0 dyne/cm^2 shear flow, *SOD2* expression in tumor cells surviving 20 dyne/cm^2 shear flow was further elevated by ~2-fold, suggesting that both suspension condition and shear stress influence

SOD2 expression. The upregulation of survival genes may confer survival advantages to a subpopulation of circulating tumor cells in shear flow.

Although hemodynamic shear flow eliminates the majority of circulating tumor cells, a small subpopulation of tumor cells still can persist in circulation, which may contain the cells with the ability to generate metastases[2]. It is thus critical to characterize the properties of circulating tumor cells surviving shear flow treatment. We examined the influence of fluid shear flow on biophysical properties of circulating tumor cells, including cell spreading, cytoskeleton, stiffness, and contractility. To characterize cell spreading, tumor cells after shear flow treatment were cultured on polyacrylamide gels with different stiffnesses (0.6, 5, and 35 kPa), which mimicked the mechanics of the tissues (brain, lung, and bone) where breast cancer cells usually metastasized. We found that BrM2 cells spread less on soft (0.6 kPa) than on stiff (5 and 35 kPa) substrates regardless of shear flow treatment (Fig. 2a and 2b). Similar findings were also observed in untreated TGL cells (Fig. S2). The spreading of BrM2 cells reached equilibrium at 2 h after plating and further increase in culture time had no significant effects on cell spreading. Compared to untreated cells, the spreading of BrM2 cells after treatment by 0 and 20 dyne/cm^2 shear flow was suppressed on stiff substrates, while the spreading of tumor cells surviving 20 dyne/cm^2 shear flow was significantly enhanced on soft substrates that mimicked brain tissue except at 6 h (Fig. 2b).

Further, BrM2 cells after shear flow treatment (20 dyne/cm^2) had notably lower levels of F-actin (Fig. 3a and 3b). No obvious difference was found in cell morphology among different groups (Fig. 3a). The reduction in F-actin was also observed in TGL cells after shear flow treatment (Fig. S3). Cell stiffness was measured by atomic force microscopy

technique (Fig. 3c). The data show that higher forces were required in order to deform control cells and tumor cells under 0 dyne/cm^2 shear flow to the similar level of tumor cells surviving 20 dyne/cm^2 shear flow (Fig. 3c). Young's modulus was obtained by fitting the force-indentation curves with the modified Hertz model. Tumor cells after 20 dyne/cm^2 shear flow treatment exhibited much lower stiffness than control cells and tumor cells after suspension treatment (0 dyne/cm^2) (Fig. 3d). Compared to untreated cells, tumor cells surviving 0 and 20 dyne/cm^2 shear flow generated significantly higher traction as measured by traction force microscopy (Fig. 4). The contractility of tumor cells surviving 20 dyne/cm^2 shear flow was significantly higher than that of tumor cells surviving 0 dyne/cm^2 shear flow on soft (0.6 kPa) but not stiff (5 kPa) substrates (Fig. 4b). Tumor cell contractility was higher on stiff (5 kPa) than on soft (0.6 kPa) substrates, which is consistent with previous findings[18,19].

We further investigated the effects of hemodynamic shear flow on the expressions of the genes related to stemness and metastatic preference to brain. We found that the expressions of stem cell genes *Nanog, Oct4*, and *Sox2* were much lower in TGL cells than in BrM2 cells (Fig. 5a). Compared to control cells and tumor cells after suspension treatment, these stemness genes were considerably upregulated by ~5, 4, and 2-fold in BrM2 cells after shear flow treatment, respectively. These findings suggest that fluid shear flow may enrich tumor cells with high stemness. It has been demonstrated that several important genes are specifically associated with brain metastasis in breast cancer, including cyclooxygenase-2 (*COX2*) and α2,6-sialyltransferase *ST6GALNAC5*[20]. Our data show that BrM2 cells expressed higher *COX2* and *ST6GALNAC5* than TGL cells, which is consistent with previous findings (Fig. 5b and 5c)[20]. Importantly, *COX2* was

upregulated remarkably by ~18-fold and 45-fold in BrM2 cells after 0 and 20 dyne/cm^2 shear flow treatment, respectively (Fig. 5b). Notably, there was 2.5-fold further increase in *COX2* expression in tumor cells surviving 20 dyne/cm^2 compared to 0 dyne/cm^2 shear flow, suggesting that both suspension condition and shear stress affect *COX2* expression. However, the expression of *ST6GALNAC5* was significantly downregulated in surviving tumor cells (Fig. 5c), which needs to be further interrogated in the future. These findings suggest that circulating tumor cells surviving shear flow may better reflect the characteristics of organ preference in metastasis.

Our findings demonstrate that only a small subpopulation of tumor cells can survive in blood shear flow with anti-apoptosis advantages, suggesting that hemodynamic shear flow is an important biophysical factor that considerably affects the viability of heterogeneous circulating tumor cells and potentially metastasis inefficiency[21]. Previous research has shown that cancer stem cells have the ability to generate metastatic tumors[22] and that a subpopulation of circulating tumor cells with stemness can initiate metastasis[23]. We have shown that tumor cells surviving shear flow upregulate the expressions of stemness genes, suggesting that they might possess stem cell properties, which may be favourable to their survival in shear flow and the subsequent generation of metastatic tumors in distant organs. Breast cancer cells with metastatic preference to brain considerably change the expression of the genes specifically related to brain metastasis after shear flow treatment, suggesting that these subpopulation of cells may better recapitulate the characteristics of organ preference in metastasis than the whole population of heterogeneous circulating tumor cells[24]. This idea needs to be rigorously investigated in the future.

Both untreated BrM2 and TGL cells and the cells surviving shear flow exhibit higher spreading on stiff substrates than on soft substrates. These results are consistent with previous findings that prostate cancer cells from brain metastasis and many other cancer types with no metastatic preference preferentially proliferate on stiff substrates[25]. Note that several reports show that tumor cell responses are correlated with the rigidity of the metastasized organ[26,27]. The discrepancy and underlying mechanisms need to be addressed in the future. Compared to control cells, BrM2 cells with metastatic preference to brain after shear flow treatment spread more on soft substrates that mimic the stiffness of brain tissue but less on stiff substrates. The surviving cells also generate higher traction on soft substrates and exhibit lower F-actin and stiffness. These findings are consistent with the reported results that tumor cells with high malignancy or self-renewing properties tend to be soft and have metastatic advantages, including the survival in blood shear flow[19,28,29]. It is possible that fluid shearing remodels the cytoskeleton of suspended tumor cells, reduces F-actin expression and cell stiffness, and enhances their stemness. The reduced cell mechanics may be important for circulating tumor cells to survive in fluid shear flow. These unique biophysical properties may confer the surviving cells metastatic advantages in soft brain tissue, as cellular contractility, cell stiffness, and spreading are important for migration and invasion[4], which should be further studied.

Conclusions

In summary, this study demonstrates that hemodynamic shear flow experienced by circulating tumor cells during hematogenous dissemination significantly influences cell viability, biophysical properties, and cellular functions, which may be related to the generation of metastatic tumors. These findings highlight the significant roles of

biophysical factors in tumor metastasis and provide a solid foundation for the future study of the subpopulations within circulating tumor cells, which may be the key players in driving the metastatic preference to different organs.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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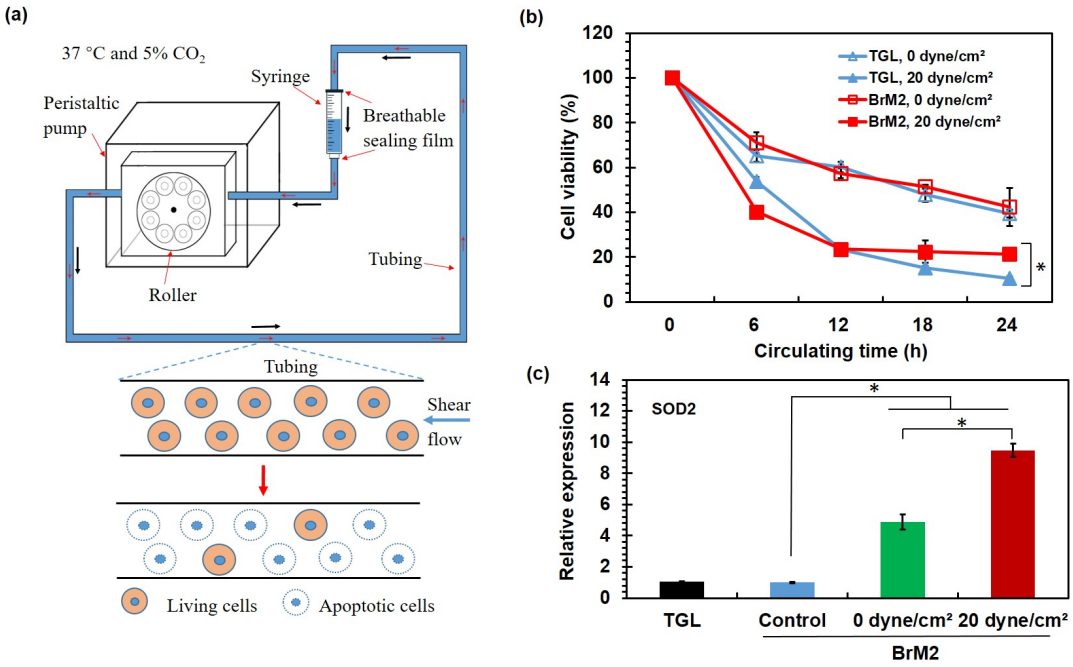

Fig. 1 Hemodynamic shear flow influences the survival of circulating tumor cells. (a) The schematic of the microfluidic circulation system. Tumor cells in suspension were treated by various magnitudes of shear flow and circulating time. (b) The viability of circulating tumor cells in shear flow decreases along with circulating time. MDA-MB-231-TGL (TGL) and the subpopulation MDA-MB-231.BrM2-831 (BrM2) cells with metastatic preference to brain were trypsinized from petri dishes and circulated in the system at 0 and 20 dyne/cm^2 shear flow. Cell viability was examined at the indicated time points by MTS assay (n=3). (c) Tumor cells after shear flow treatment upregulate the survival gene SOD2. BrM2 cells were treated under 0 and 20 dyne/cm^2 shear flow for 12 h, when the mRNAs were extracted for gene analysis (n=3). BrM2 (Control) and TGL cells cultured in petri dishes were used as controls. *, $p<0.05$.

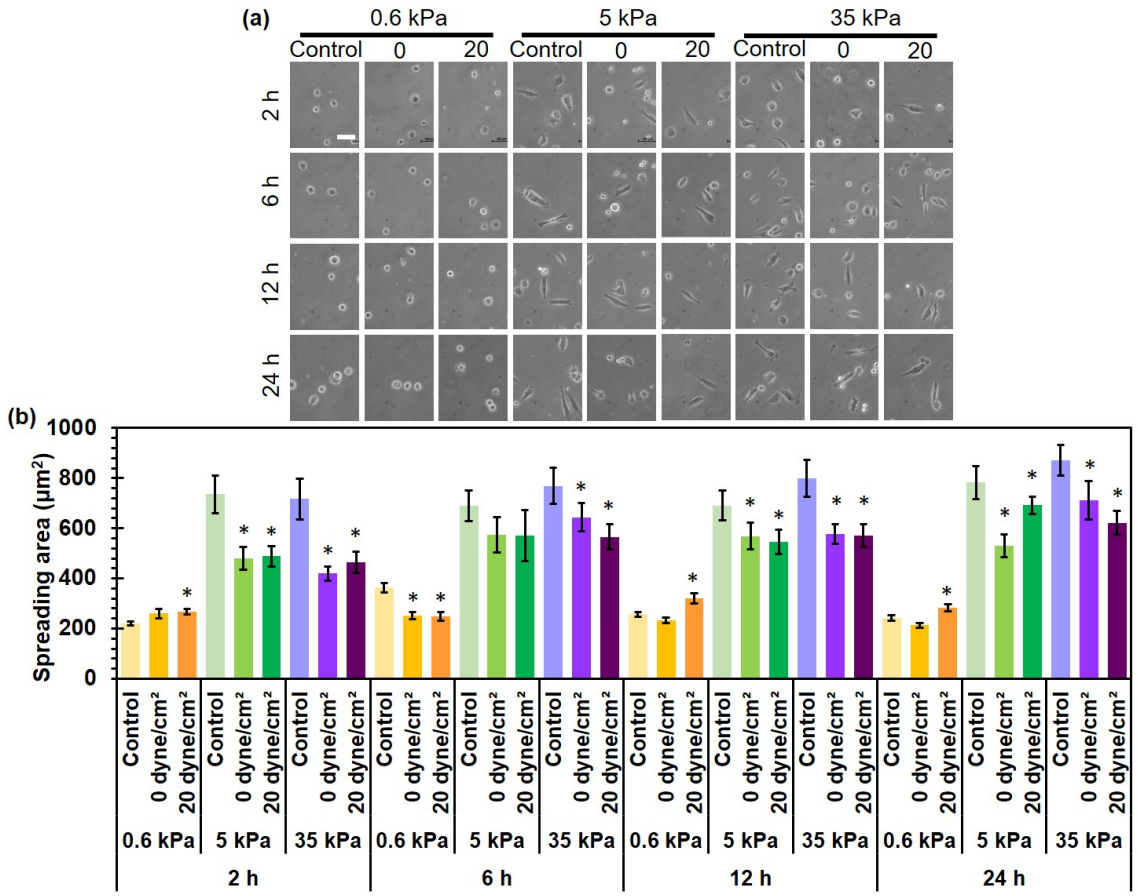

Fig. 2 Hemodynamic shear flow enhances/suppresses the spreading of circulating tumor cells with metastatic preference to brain on soft/stiff substrates. (a) Representative images of tumor cells under different conditions. Scale bar: 100 μm. (b) Quantification of the spreading area of circulating tumor cells after shear flow treatment. BrM2 cells were treated under 0 and 20 dyne/cm^2 shear flow for 12 h and then cultured on polyacrylamide gels with the stiffness of 0.6, 5, and 35 kPa, respectively. Cell images were taken at the indicated time points for analysis (n>100 cells). BrM2 cells cultured in petri dishes were used as control. Two independent experiments were conducted. *, $p<0.05$ (vs Control).

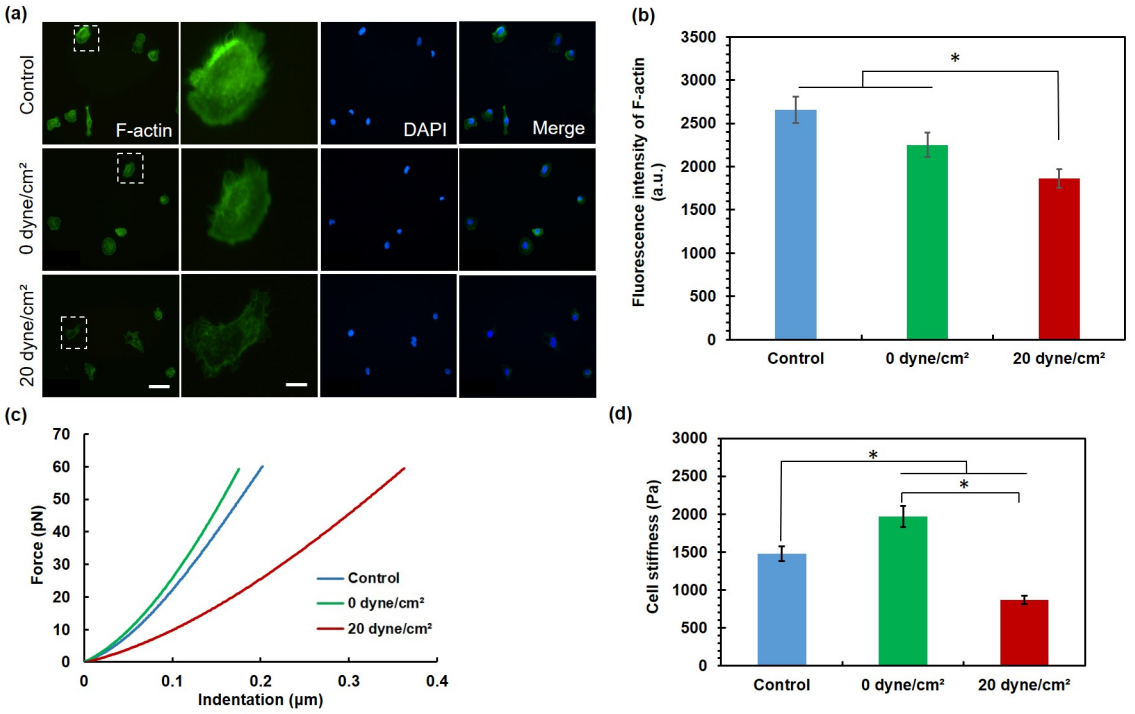

Fig. 3 Hemodynamic shear flow decreases F-actin expression and stiffness of circulating tumor cells. (a) The expression of F-actin after shear flow treatment. The images in the dotted line of the first column were magnified in the second column. The scale bar in the first and second column is 50 μm and 10 μm, respectively. (b) Quantification of F-actin expression. a.u.: arbitrary unit. (c) Typical force-indentation curves for tumor cells treated under different conditions. (d) Quantification of cell stiffness after shear flow treatment. BrM2 cells were treated under 0 and 20 dyne/cm^2 shear flow for 12 h and then cultured on glass for 8 h, when F-actin and cell stiffness were measured by immunofluorescence (n>50/condition) and atomic force microscopy (n>80 cells/condition), respectively. BrM2 cells cultured in petri dishes were used as control. The fluorescence intensity of F-actin was measured by ImageJ in (b). *, $p<0.05$.

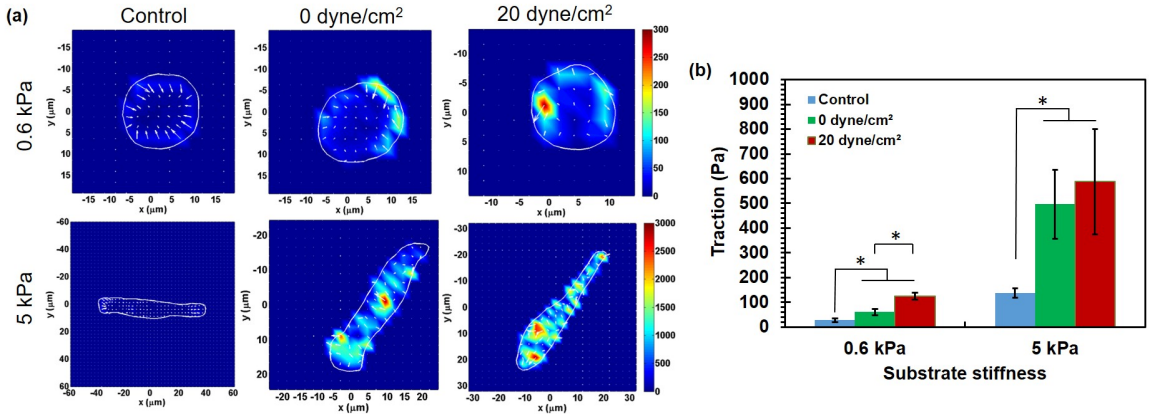

Fig. 4 Hemodynamic shear flow enhances the contractility of circulating tumor cells on soft substrates. (a) Representative traction maps. (b) Quantification of cellular traction after shear flow treatment. BrM2 cells were treated under 0 and 20 dyne/cm^2 shear flow for 12 h and then cultured on 0.6 and 5 kPa polyacrylamide gels for 6 h, when the traction was measured by traction force microscopy (n>10 cells/condition). BrM2 cells cultured in petri dishes were used as control. *, $p<0.05$.

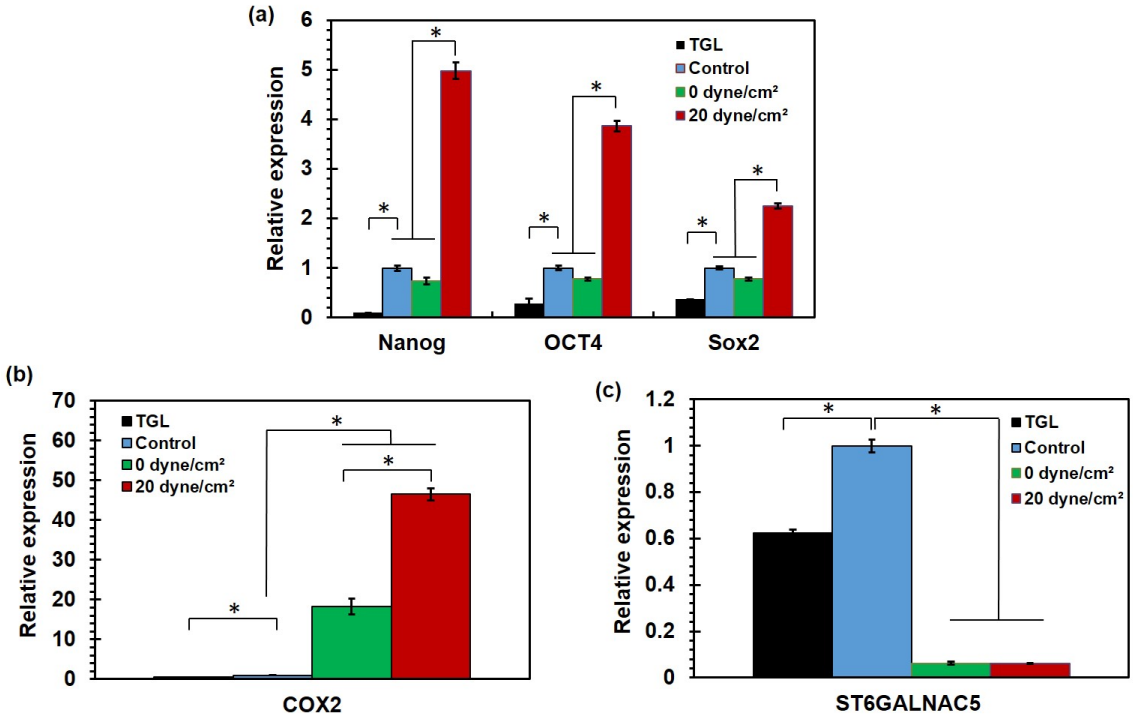

Fig. 5 Hemodynamic shear flow influences the expressions of the genes related to stemness (a) and brain metastasis (b, c) in circulating tumor cells. BrM2 cells were treated under 0 and 20 dyne/cm^2 shear flow for 12 h, when the mRNAs were extracted for analysis of gene expression (n=3). BrM2 (Control) and TGL cells cultured in petri dishes were used as controls. *, $p<0.05$.