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

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Supplementary Information

Materials and Methods

Cell culture

Human breast cancer cell lines MDA-MB-231-TGL and MDA-MB-231.BrM2-831 were purchased from Memorial Sloan Kettering Cancer Centre. Briefly, cells were cultured in petri dishes with Dulbecco's Modified Eagle Medium (DMEM) cell culture medium supplemented with 10% fetal bovine serum (HyClone), 1% penicillin/streptomycin (HyClone) at 37°C and 5% CO_2. Cells were passaged every 2-3 days using Trypsin (HyClone).

Shear flow treatment

The circulatory system in this study 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. The system could generate pulsatile flow, which mimicked the hemodynamic shear stress in blood circulation. According to Poiseuille's law, wall shear stress τ_w in the tubing (dyne/cm^2) was calculated by $\tau_w = \frac{4\mu Q}{\pi R^3}$, where Q was the flow rate (from 0.001 to 230 ml/min), μ was the dynamic viscosity of the fluid (0.01 dyne.s/cm^2 for cell culture medium), and R was the radius of the tube (0.255 mm). Prior to the experiments, the whole system was first sterilized by 75% ethanol. To reduce the adhesion of circulating tumor cells

to the tube and syringe, the system was then washed with 4 ml phosphate buffered saline (HyClone) and 4 ml 1% bovine serum albumin (VWR Life Science), respectively. During the experiments, 2 ml cell suspension solution (2×10^5 cells/ml) was added into the circulatory system and subjected to various magnitudes of shear flow for different durations (0-20 dyne/cm^2; 0-24 h) in the cell culture incubator at 37°C and 5% CO_2.

MTS assay

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

Quantitative RT-PCR analysis

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

Quantification of cell spreading

For the cell spreading experiment, cells were plated on polyacrylamide gels with different stiffnesses (0.6, 5, and 35 kPa). At least 100 cells/condition were imaged by the inverted

microscope (Nikon) after incubation for different durations. Cell area was quantified by the ImageJ software (NIH).

Cell stiffness measurement by atomic force microscope

Cell stiffness was measured using atomic force microscope (AFM, Bruker Catalyst) with silicon nitride cantilevers of spring constant k at 0.02 N/m (MLCT, Bruker) 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 determined by fitting force-indentation curves with Sneddon's modification of the Hertzian model for a pyramidal tip[1], 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 any possible substrate effects and cell damage.

Polyacrylamide gel preparation

Polyacrylamide gels were prepared following the protocol reported previously[2]. The gel rigidity was varied by altering the concentrations of bis-acrylamide crosslinker (0.06%, 0.15%, 0.30%) and acrylamide (3%, 5%, 10%) (Bio-Rad). The corresponding gel rigidity was 0.6, 5, and 35 kPa, respectively. The gels were activated using the crosslinker Sulfo-SANPAH (sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino) hexanoate) (Pierce) and then coated with collagen (200 μg/ml) before plating cells.

Traction force measurement

Traction force was measured following the reported procedure[2]. Briefly, cells were plated on polyacrylamide gels with red fluorescent beads (0.2 μm in diameter; Molecular Probes, Invitrogen) embedded on the top surface. Before and after cell detachment, fluorescent images were taken to compute the displacement field of the beads in a homebuilt MATLAB program.

Cell tractions were then calculated from the displacement field using inverse Boussinesq mathematical model.

Immunofluorescence staining

Briefly, cells were cultured on coverslips and fixed with 4% formaldehyde (Sigma Aldrich) for 15 min at room temperature and rinsed 3 times with PBS (Hyclone). 0.1% Triton X-100 was then used to treat the fixed cells for 5 min. After cell rinse with PBS, 200 µl of 1x green fluorescent phalloidin conjugate working solution (Abcam) was incubated with cells at room temperature for 60 min. Cells were rinsed in PBS to remove excess dye and placed onto another coverslip with ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). At least 50 cells per condition were imaged using an inverted fluorescence microscope (Nikon). The intensity of fluorescence was quantified by ImageJ software (NIH).

Statistical analysis

A two-tailed Student's t-test was used for all statistics.

Supplementary Table 1: List of primers

Genes		Quantitative RT-PCR
SOD2	5' primer	GCACATTAACGCGCAGATCA
	3' primer	AGCCTCCAGCAACTCTCCTT
Nanog	5' primer	CCCAAAGGCAAACAACCCACTTCT
	3' primer	AGCTGGGTGGAAGAGAACACAGTT
OCT4	5' primer	AGCAAAACCCGGAGGAGT
	3' primer	CCACATCGGCCTGTGTATATC
Sox2	5' primer	TACAGCATGTCCTACTCGCAG
	3' primer	GAGGAAGAGGTAACCACAGGG
COX2	5' primer	TCCACCAACTTACAATGCTGAC
	3' primer	CACAGGAGGAAGGGCTCTAGTA
ST6GALNAC5	5' primer	GGATCCCAATCACCCTTCAG
	3' primer	TAGCAAGTGATTCTGGTTCCA
GAPDH	5' primer	GCGACACCCACTCCTCCACCTTT
	3' primer	TGCTGTAGCCAAATTCGTTGTCATA

Reference

1 C. Rotsch, K. Jacobson and M. Radmacher, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 921–6.

2 Y. Tan, 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, *Nat. Commun.*, 2014, **5**, 4619.

Supplementary figures

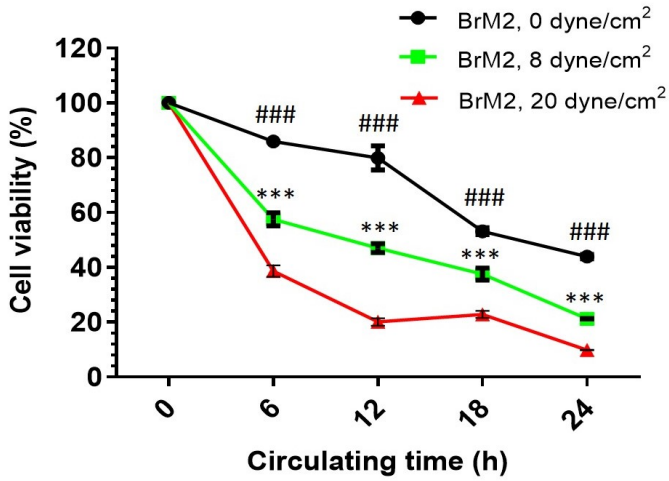

Fig. S1 The survival of circulating tumor cells depends on the magnitude of shear flow. BrM2 cells were treated under various levels of fluid shear flow (0, 8, and 20 dyne/cm^2) for the indicated circulating time, when cell viability was measured by MTS assay (n=3). ***, $p<0.001$ represents the significant difference between "8 dyne/cm^2" and "20 dyne/cm^2"; ### $p<0.001$ represents the significant difference between "0 dyne/cm^2" and "8 dyne/cm^2".

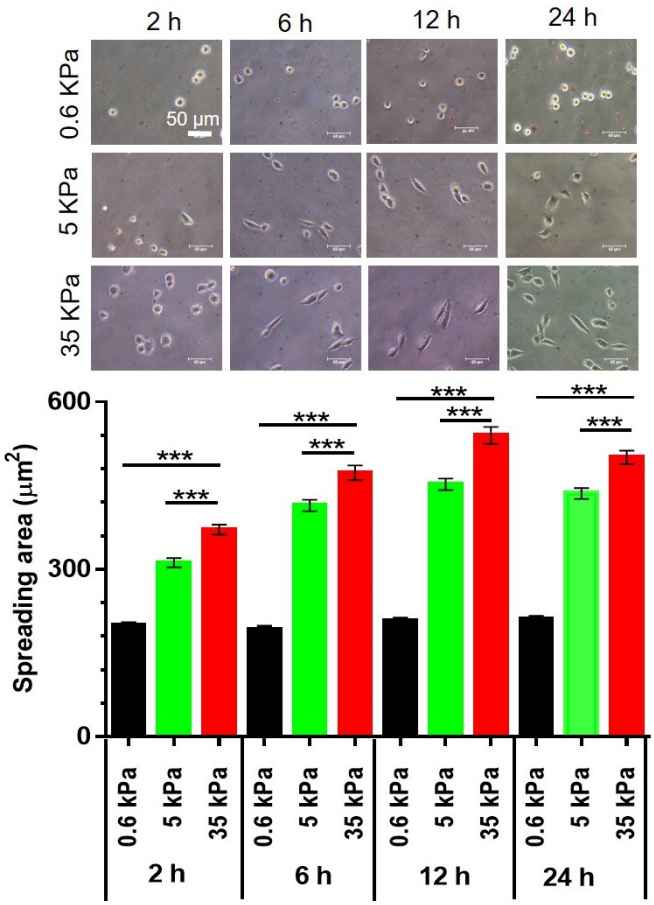

Fig. S2 TGL cells spread more on stiff substrates than soft substrates. TGL cells were cultured on 0.6, 5, and 35 kPa polyacrylamide gels for 2, 6, 12, and 24 h, respectively, when cell images were taken (top panel). Cell area was quantified by ImageJ (n>100). Scale bar: 50 μm. ***, $p<0.001$.

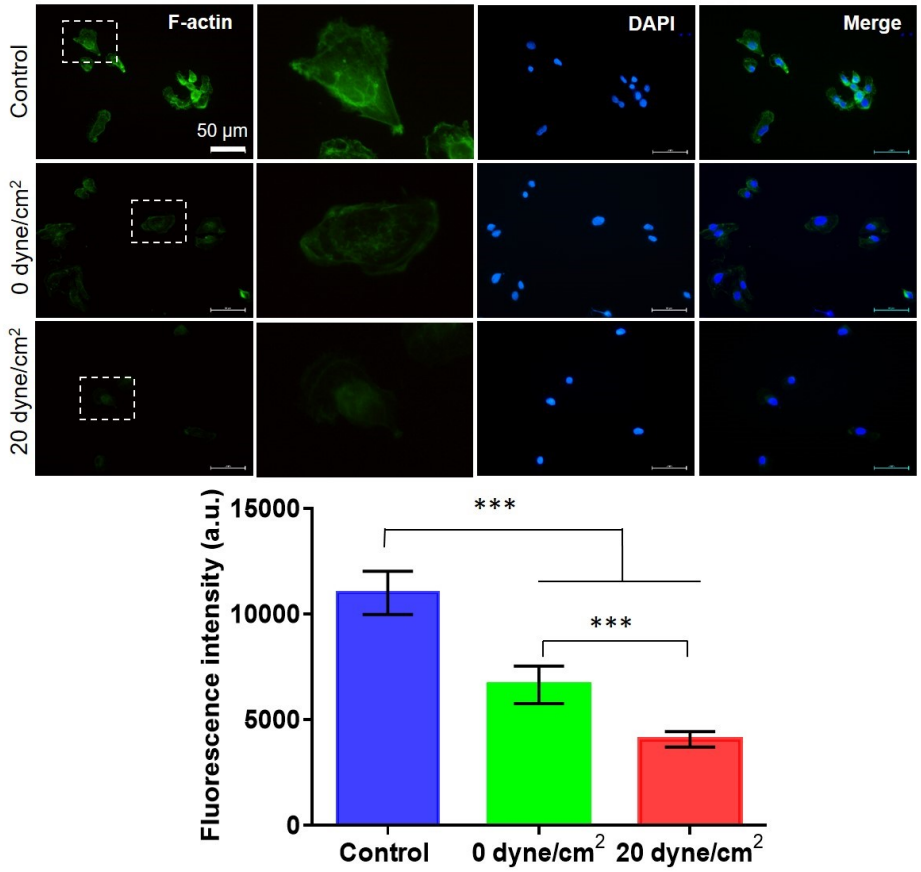

Fig. S3 Hemodynamic shear flow decreases F-actin expression in TGL cells. TGL cells were treated under 0 and 20 dyne/cm^2 shear flow for 12 h and then cultured on glass for 8 h, when F-actin was measured by immunofluorescence (n>100). Fluorescence intensity was quantified by ImageJ. Tumor cells cultured in petri dishes were used as a control. ***, $p<0.001$.