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MnSOD mediates shear stress-promoted tumor cell migration and adhesion

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Abbreviations: CTCs: circulating tumor cells; ECM: extracellular matrix; ERK: extracellular signal-regulated kinases; FAK: focal adhesion kinase; FBS: fetal bovine serum; HUVEC: human umbilical vein endothelial cells; LMW-PTP: low molecular weight protein tyrosine phosphatase; MnSOD: manganese superoxide dismutase; NAC: N-acetyl-cysteine; PG: propyl gallate; PTP: protein tyrosine phosphatase; ROS: reactive oxygen species; SS: shear stress.

Abstract

Circulation of cancer cells in the bloodstream is a vital step for distant metastasis, during which cancer cells are exposed to hemodynamic shear stress (SS). The actions of SS on tumor cells are complicated and not fully understood. We previously reported that fluidic SS was able to promote migration of breast cancer cells by elevating the cellular ROS level. In this study, we further investigated the mechanisms regulating SS-promoted cell migration and identified the role of MnSOD in the related pathway. We found that SS could enhance tumor cell adhesion to extracellular matrix and endothelial monolayer, and MnSOD also regulated this process. Briefly, SS stimulates the generation of mitochondrial superoxide in tumor cells. MnSOD then converts superoxide into hydrogen peroxide, which activates ERK1/2 to promote tumor cell migration and activates FAK to promote tumor cell adhesion. Combining our previous and present studies, we present experimental evidence on the pro-metastatic effects of hemodynamic SS and reveal the underlying mechanism. Our findings provide new insights into the nature of cancer metastasis and the understanding of tumor cell responses to external stresses and have valuable implications for cancer therapy development.

Key words: Shear stress; Tumor cell adhesion; Tumor cell migration; Reactive oxygen species; MnSOD; FAK

1. Introduction

Cancer metastasis is defined as the spread of cancer cells from the site of origin to other parts of the body and is accountable for ninety percent of deaths from solid tumors [1, 2]. Circulation of tumor cells in the bloodstream is a vital step for successful distant metastasis, during which tumor cells are exposed to hemodynamic shear stress (SS) [3-5]. Recent studies investigating the effects of SS on tumor cells mainly focused on how the cells are mechanically damaged [5, 6]. However, whether SS could affect the biochemical properties of tumor cells and how the cells respond to SS remains unclear.

We previously reported that the levels of reactive oxygen species (ROS) were increased in breast cancer cells when they were circulated under arterial levels of pulsatile SS, such as 15-30 dyne/cm² [7]. The excessive cellular ROS could activate ERK1/2 and promote tumor cell migration and extravasation [8]. In this study, we further explored the mechanisms that regulate SS-enhanced cell migration and identified an antioxidant enzyme, manganese superoxide dismutase (MnSOD), as an indispensable signaling molecule in the related pathway. In this pathway, pulsatile SS stimulates the generation of mitochondrial superoxide in tumor cells [7]. As superoxide cannot penetrate through the mitochondrial membrane, it is accumulated in the mitochondria and induces cell apoptosis [7, 9, 10]. MnSOD can convert superoxide to hydrogen peroxide, which is less toxic and can be released into cytosol to protect cells from apoptosis [7, 11]. We found that by increasing cytosol level of hydrogen peroxide, MnSOD could activate ERK1/2 and stimulate the migration of tumor cells.

We also investigated the effects of SS on adhesion of tumor cells. The adhesion capacity of cells to extracellular matrix (ECM), which indicates how efficiently cells can form stable interactions with ECM, is very important in determining cell survival and growth [12, 13].

Appropriate and effective adhesions of the extravasated tumor cells to ECM of the secondary organs have a major influence on the invasion and colonization of the tumor cells in a new microenvironment [14, 15]. The adherence of tumor cells to endothelium is also important, which is an indispensable step before extravasation [16, 17]. We found that pulsatile SS can promote tumor cell adhesion to both ECM and endothelial monolayer, which is also regulated by MnSOD. MnSOD converts SS-generated superoxide to cytosolic hydrogen peroxide to promote activation of FAK and thus enhances tumor cell adhesion.

Taking our previous reports [7, 8] and the present findings together, we revealed the pro-metastasis effects of SS. We found that SS can promote migration, extravasation and adhesion of tumor cells. The underlying mechanism was explored, and we identified the roles of cellular ROS and antioxidant enzyme MnSOD in regulating the responses of tumor cells to SS.

2. Materials and Methods

2.1 Cell culture

The breast cancer cell line MDA-MB-231 was provided by Professor Xiaofeng Le when he was working in the Department of Experimental Therapeutics at The M.D. Anderson Cancer Center, University of Texas. MCF7, MDA-MB-157, BT-549 and human umbilical vein endothelial cells (HUVEC) were purchased from American Type Culture Collection (ATCC, USA). All the cells were cultured in DMEM (Thermo Fisher Scientific, USA) supplemented with 10% FBS (HyClone, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, USA). The 231-C3 and MCF7-C3 cells are stable cell lines that express the CFP-DEVD-YFP fusion protein [18]. They were generated by transfecting a fluorescence resonance energy transfer (FRET)-based C3 biosensor into MDA-MB-231 and MCF7 cells, respectively [19].

2.2 Microfluidic circulatory system

The microfluidic circulatory system was previously described in detail [7, 8, 20]. Briefly, the system was assembled using a peristaltic pump (Ismatec, Germany) which can generate pulsatile shear flow. Tumor cells were circulated in culture medium in a micro-tubing of 500 μm in diameter and 1.5 m in length. SS was calculated using Poiseuille's equation [6]: $\tau = 4Q\eta/\pi R^3$, where τ is the SS in dyne/cm^2 , Q is the flow rate in cm^3/sec , η is the dynamic viscosity of the fluid ($0.012 \text{ dynes}\cdot\text{sec}/\text{cm}^2$), and R is the radius of the tubing ($250 \mu\text{m}$) [7, 8]. In this study, the level of SS was set to be $15 \text{ dyne}/\text{cm}^2$ (abbreviated as SS15) as it represents the average level of SS in human arteries. Control cells were seeded in 96-well plates coated with a solution of 1% Pluronic f127 (Invitrogen, USA).

2.3 Transwell migration assay

Transwell migration assay was previously described [8]. Briefly, cells were collected and re-suspended in DMEM without serum. Cell suspension was added to the upper side of a transwell chamber with a pore size of 8 μm (Corning, USA). Next, DMEM with 10% FBS was added to the lower chamber. Cells were allowed to migrate from the upper to the lower chamber for 18 h at 37°C. The membranes of the chambers were fixed with 4% paraformaldehyde. The cells that remained on the upper side of the membrane were gently removed using a cotton swab and migrated cells in the transwell chamber were stained with H&E staining solutions using the standard protocol. The membrane was then cut off and fixed on a glass slide. Migrated cells were directly imaged and counted on the slides. Quantification of migrated cells was based on three independent repeats.

2.4 Cell adhesion assay

Collagen Type I solution (Sigma-Aldrich, USA) was diluted to the final concentration of 50 $\mu\text{g/ml}$ with 0.02 M acetic acid. A 100- μl aliquot of the working solution was added to the wells of 96-well plates. After incubation at 37°C for 2 h, collagen solution was removed, and wells dried for 1 h. The wells were washed twice with fresh DMEM before use. For cell seeding, 100 μl suspension of breast cancer cells was added to each well of the collagen I-coated plates at a density of 1×10^4 cells/well. After incubation at 37°C for 20 min, images were taken using an inverted fluorescence microscope with YFP filters. Then, the medium was aspirated, and the wells were washed twice with PBS with 10-min incubations. Finally, fresh medium was added to the plates and images were taken again. At least 3 pictures were captured from various observation fields in each group. The images were processed, and the cell numbers were counted for each group. The

cell adhesion rate of each group was calculated as the ratio of cell number after wash to cell number before wash.

For monitoring cell adhesions to endothelial monolayer, HUVECs were seeded in 96-well plates at a density of 2×10^4 cells/well. After overnight incubation, an intact HUVEC monolayer was formed. A 100- μ l aliquot of breast cancer cells suspension was added to the plates at a density of 1×10^4 cells/well. The washing and imaging steps were the same as described above.

2.5 Western blot analysis

Cells were lysed with RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich, USA). The protein concentrations were determined using a Bradford assay kit (Bio-Rad, USA). SDS-PAGE gels were loaded with the same amount of proteins, and samples underwent electrophoresis at the same time. After the transfer process, the PVDF membranes were probed with primary antibodies overnight at 4°C. The next day, the blots were probed with HRP-conjugated secondary antibodies for 1 h at room temperature. The blots were developed in ECL solution, and the band intensities on the blots were measured using Image-Pro Plus.

2.6 SiRNA-mediated silencing of MnSOD

Two Silencer Select siRNAs against MnSOD (SiRNA No.1 ID: s13268, and No.2 ID: s13269) and Silencer Select negative control siRNA were purchased from Ambion (Thermo Fisher Scientific, USA). Breast cancer cells were seeded in 60-mm petri dishes and allowed to attach overnight. Cells were washed with OptiMEM (Life Technologies) before transfection with 20 nM of siRNAs using Lipofectamine RNA/iMAX (Life Technologies) according to the manufacturer's instructions. The negative control group was transfected with non-targeting siRNA (Thermo Fisher

Scientific, USA). After incubation for 8 h, the transfection medium was replaced with fresh culture medium. At 72 h post-transfection, cells were collected for circulatory treatment.

2.7 Overexpression of MnSOD

The plasmid that overexpresses MnSOD (pMnSOD) was purchased from Origene (USA). The pMnSOD was constructed in a pCMV6-Entry vector, and the MnSOD was tagged with Myc-DDK at the C-terminal. Breast cancer cells were seeded in 60-mm petri dishes and allowed to attach overnight. Cells were washed with OptiMEM (Life Technologies) before transfection with 1 µg of plasmid using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions. The negative control group was transfected with the empty vector. After incubation for 8 h, the transfection medium was replaced with fresh culture medium. At 72 h post-transfection, cells were collected and subjected to circulatory treatment.

2.8 Immunoprecipitation

Immunoprecipitation was performed using the Pierce Classic IP Kit (Thermo Fisher Scientific, USA). Briefly, the 231-C3 cells were collected after circulation and lysed using the IP Lysis/Wash Buffer with protease inhibitors (1:100). For pre-clearing, the lysate was incubated with Control Agarose Resin in a spin column at 4°C for 1 h with gentle end-over-end mixing. After centrifugation, the pre-cleared lysate in a flow-through was collected and mixed with primary anti-LMW-PTP antibodies to form the immune complex. After overnight incubation at 4°C, the mixture was loaded into the spin column containing pre-washed Protein A/G Agarose and incubated for 1 h with gentle end-over-end mixing. The agarose resin was washed with three column volumes of IP Lysis/Wash Buffer and one volume of conditioning buffer. Then, Elution Buffer was added to

the column, and after 10-min incubation at room temperature, the column was centrifuged to collect the eluate.

2.9 Protein tyrosine phosphatase (PTP) activity assay

RediPlate EnzChek Tyrosine Phosphatase assay kit was purchased from Thermo Fisher Scientific. The experiment was conducted based on the product instruction. Briefly, 80 µl of reaction buffer was added into the plate wells to solubilize the PTPase substrate. The samples from immunoprecipitation were diluted 1:50 using the reaction buffer, and 20 µl of the final solution was added into the wells and gently mixed. To the blank control wells, 20 µl of empty reaction buffer was added. The plates were incubated at 37°C for 20 min and a fluorescence plate reader was used to measure fluorescence (excitation 358 nm, emission 452 nm). In the final quantification, the fluorescence readings were normalized to static group.

2.10 Data analysis

All data are presented as the mean $\pm$ SD of three independent experiments. The significance between two groups were assessed by the two-tailed Student's t-test in all experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered statistically significant.

3. Results

3.1 MnSOD mediates SS-promoted breast cancer cell migration

In our last report, we found that SS could stimulate tumor cell migration by elevating intracellular levels of ROS [8]. Previous studies conducted in our laboratory have identified mitochondrial superoxide as the original form of ROS generated by SS [7]. MnSOD converts superoxide to less toxic hydrogen peroxide and protects mitochondria and tumor cells [7, 21]. Interestingly, another difference between superoxide and hydrogen peroxide is that hydrogen peroxide can easily diffuse through the mitochondrial membrane, while superoxide cannot [9, 22]. Most of the ROS targets locate inside the cytosol, including ERK1/2. This indicates that hydrogen peroxide, but not superoxide, is able to trigger signaling pathways and regulate cellular functions. Thus, we proposed that MnSOD might play important roles in SS-promoted cell migration by converting superoxide to hydrogen peroxide to activate ERK1/2 in the cytosol.

To validate the function of MnSOD on SS-promoted tumor cell migration, we overexpressed MnSOD in breast cancer cell line MCF7-C3 by transfecting a plasmid (pMnSOD) that overexpresses MnSOD. Our previous work showed that MCF7-C3 cells had a relatively low level of MnSOD [7] and did not respond to SS regarding the migration capacity [8]. In the negative control group, the MCF7-C3 cells were transfected with an empty vector. As the blank control, we used normal MCF7-C3 cells without any treatments. The MCF7-C3 cells were collected 72 h after transfection and were circulated under SS15 for 6 h. The circulated MCF7-C3 cells were either lysed for Western blot analysis or collected and subjected to transwell migration assay.

Fig. 1. SS15 enhanced ERK1/2 phosphorylation and cell migration in MnSOD-overexpressing MCF7-C3 cells. MCF7-C3 cells were collected 72 h after transfection with plasmid overexpressing MnSOD (pMnSOD)

and circulated under SS15 or kept under static conditions for 6 h. (A) The circulated MCF7-C3 cells were collected and lysed for Western blot analysis. Levels of phosphorylated ERK1/2, total ERK1/2 and MnSOD were detected and analyzed. GAPDH was used as the internal control for equal loading. (B) The circulated cells were collected and subjected to an 18-h transwell migration assay. Upper panel shows representative images of migrated cells on transwell membranes in different groups. Scale bar, 100 μ m. Lower panel shows the quantification of migrated cells. * $p < 0.05$. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

The Western blot results confirmed that MnSOD was expressed at a high level in the transfected cells (Fig. 1A). Interestingly, we found that in the MnSOD-overexpressing MCF7-C3 cells, the level of phosphorylated ERK1/2 (pERK1/2) was increased after 6-h circulatory treatment under SS15. However, for MCF7-C3 cells with an endogenous expression level of MnSOD in both negative control and blank control groups, there was not much difference in the levels of pERK1/2 between the cells under static conditions and the cells circulated under SS15 (Fig. 1A). Consistent with the Western blot results, the transwell migration assay showed that after SS15 treatment, overexpression of MnSOD increased the migration capacity of MCF7-C3 cells compared to negative control. In contrast, overexpression of MnSOD did not significantly increase the transmigration ability of MCF7-C3 cells under the static conditions (Fig. 1B). In addition, for the MnSOD-overexpressing MCF7-C3 cells, migration was significantly increased by SS15 treatment compared to the static group (Fig. 1B).

To further verify the function of MnSOD, two efficacy-validated siRNAs targeting MnSOD mRNA (named siMnSOD-1 and siMnSOD-2) were transfected into engineered MDA-MB-231-C3 (231-C3) cells to knock down MnSOD expression. The transfected 231-C3 cells were then collected and circulated under SS15 or kept under static conditions for 6 h, which were then either lysed for Western blot analysis or subjected to transwell migration assay. In Western blot analysis,

we detected the levels of pERK1/2, total ERK1/2 and MnSOD. Apparently, reducing MnSOD levels prevented the increase of phosphorylated ERK1/2 upon circulatory treatment, but did not affect the total protein levels of ERK1/2. In contrast, for cells kept under static conditions, no significant reduction of pERK1/2 levels were observed after the levels of MnSOD were reduced (Fig. 2A).

In transwell migration assay for 231-C3 cells circulated under SS15 for 6 h, both siMnSOD-1 and siMnSOD-2 groups displayed substantially decreased cell migration compared with the negative group (Fig. 2B). For cells under static conditions, although the reduction in MnSOD level suppressed the migration of 231-C3 cells to some extent, the difference was not significant between the siMnSOD-1 and siMnSOD-2 groups and the control and negative groups (Fig. 2B). This is in a good agreement with our previous findings in which pretreatment of 231-C3 cells with antioxidants reduced SS-promoted cell migration [8]. These observations suggest that ROS and MnSOD are the key signaling molecule and the enzyme that can enhance cell migration and invasiveness in response to external stresses, such as fluidic SS, but not under static conditions.

Fig. 2. Knock-down of MnSOD in 231-C3 cells suppressed SS-promoted ERK1/2 phosphorylation and cell migration. The 231-C3 cells were collected 72 h after transfection with siRNAs (siMnSOD-1 and siMnSOD-2) and circulated under SS15 or kept under static conditions for 6 h. (A) Cells were collected and lysed for

Western blot analysis. Levels of phosphorylated ERK1/2, total ERK1/2 and MnSOD were detected and analyzed. GAPDH was used as the internal control for equal loading. (B) Cells were collected and subjected to an 18-h transwell migration assay. Upper panel shows representative images of migrated cells on transwell membranes in different groups. Scale bar, 100 μ m. Lower panel shows the quantification of migrated cells. ** $p < 0.01$. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

3.2 MnSOD mediates SS-promoted tumor cell adhesion

In addition to cell migration, cell adhesion is another important cellular function that determines the efficiency of metastasis. Tumor cell adhesion to ECM is important for invasion and colonization in secondary organs, and tumor cell adhesion to endothelial monolayer is an inevitable step before extravasation [16, 17, 23-25]. As both tumor cell extravasation and invasion to secondary organs occur after circulation of tumor cells in the bloodstream, we investigated the effects of SS on the adhesion of tumor cells to both ECM and endothelium. The 231-C3 cells were introduced into the microfluidic system and circulated under SS of 5, 15 and 30 dynes/cm² for 6 h. After circulation, cells were immediately collected and re-suspended in fresh DMEM medium. The circulated 231-C3 cells were then subjected to cell adhesion assays as described in Materials and Methods.

Fig. 3. SS stimulated the adhesion of 231-C3 cells. (A and C) SS promoted tumor cell adhesion to ECM. The 231-C3 cells were circulated under SS5, SS15 and SS30 for 6 h and then seeded on collagen I-coated plates. Cells were washed with PBS after incubation for 20 min. Panel A shows representative fluorescent images of cells remaining on plates after PBS wash. Scale bar, 100 μ m. Panel C shows the quantified rates of cell adhesion to ECM. (B and D) SS promoted tumor cell adherence to endothelial monolayer. HUVECs were seeded in 96-well plates and incubated for 24 h to establish an intact HUVEC monolayer. The 231-C3 cells were circulated under SS5, SS15 and SS30 for 6 h and then seeded on HUVEC monolayer. Cells were washed with PBS after incubation for 20 min. Panel B shows representative fluorescent images of cells remaining on HUVEC monolayer after PBS wash. Scale bar, 100 μ m. Panel D shows the quantified rates of cell adhesion on HUVEC monolayer. (E and F) MDA-MB-157 and BT-549 cells were labeled with green fluorescence using Calcein Green. The stained cells were then circulated under SS15 for 6 h and subjected to the adhesion assay. (E) SS15 stimulated adhesion of MDA-MB-157 cells to ECM and HUVEC monolayer. Left panel shows representative images of cells remaining on ECM and HUVEC monolayer. Right panel is the quantification of the adhesion rate. (F) SS15 stimulated adhesion of BT-549 cells to ECM and HUVEC monolayer. Left panel shows representative images of cells remaining on ECM and HUVEC monolayer. Right panel is the quantification of the adhesion rate. * $p < 0.05$, ** $p < 0.01$. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

The results showed that after SS treatment, more 231-C3 cells remained on both the plates coated with collagen and the plates with HUVEC monolayer (Fig. 3A and B). For the cells kept under static conditions, there were approximately 26% of cells remaining on collagen I-coated plates (Fig. 3C) and 39% of cells on HUVEC monolayer (Fig. 3D). A venous level of SS (5 dynes/cm², SS5) did not significantly change the percentage of adherent cells on collagen I-coated plates, which was approximately 24%, and slightly increased the adhesion rate of tumor cells on HUVEC monolayer to 48%. However, SS15 significantly increased the percentage of adherent cells to 66% on collagen I-coated plates (Fig. 3C) and to 82% on HUVEC monolayer (Fig. 3D). However, raising SS level from 15 to 30 dynes/cm² only slightly increased cell adhesion rates for both substrates (Fig. 3C and D). These results showed that SS could promote the adhesion capacity of 231-C3 cells.

To verify the general implication of arteriosus SS in increasing cell adhesion, two other triple-negative breast cancer cell lines, namely, MDA-MB-157 and BT-549, were circulated under SS15 for 6 h and subjected to the adhesion assay. In good agreement with the results obtained on 231-C3 cells, the adhesions of MDA-MB-157 and BT-549 were also significantly increased on both ECM and HUVECs after circulatory treatment under SS15 (Fig. 3E and F).

As MnSOD regulates SS-promoted tumor cell migration, we predicted that MnSOD might also influence the effects of SS on tumor cell adhesion. We knocked down MnSOD gene expression in 231-C3 cells, circulated the cells under SS15 and subjected them to cell adhesion assay. The results showed that decreasing MnSOD expression significantly suppressed tumor cell adhesion to both ECM (Fig. 4A) and endothelium (Fig. 4B). In particular, siMnSOD-1 decreased tumor cell adhesion to ECM from 67% to 40% and reduced tumor cell adhesion to HUVEC monolayer from 72% to 36%. The other siRNA, siMnSOD-2 produced very similar reductive effects on adhesion of 231-C3 cells to both ECM and HUVECs. As expected, for cells under static conditions, knocking down of MnSOD had minor effects on adhesion of 231-C3 cells to both ECM (Fig. 4A) and HUVEC (Fig. 4B), which is consistent with our previous results on cell migration.

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conditions for 6 h. (A) Cells were collected and seeded on collagen I-coated 96 well plates and were washed after 20-min incubation. Upper panel shows representative fluorescent images of cells remaining on plates after PBS wash. Scale bar, 100 μ m. Lower panel shows the quantified cell adhesion rates. (B) Cells were collected and seeded on established HUVEC monolayer in 96-well plates and were washed after 20-min incubation. Upper panel shows representative fluorescent images of cells remaining on plates after PBS wash. Scale bar, 100 μ m. Lower panel shows the quantified adhesion rates. * $p < 0.05$, ** $p < 0.01$. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

3.3 The SS-promoted tumor cell adhesion is mediated by cellular ROS

Our previous work revealed that SS was able to simulate the generation of superoxide in mitochondria, which was converted to hydrogen peroxide (H_2O_2) by MnSOD. H_2O_2 was then diffused from mitochondria to the cytosol and acted as a signaling molecule to stimulate cell migration [8]. In this study, we have found that MnSOD could also regulate SS-promoted tumor cell adhesion (Fig. 4), which suggests the importance of cellular ROS in the related pathway. To investigate the role of SS-generated ROS in tumor cell adhesion, we pre-treated 231-C3 cells with two antioxidants: 20 μ M propyl gallate (PG) or 5 mM N-acetyl-cysteine (NAC) for 1 h before circulating the cells under SS15. The results of cell adhesion assay showed that PG decreased the adhesion rates of circulated 231-C3 cells from 69% to 45% on ECM (Fig. 5A) and from 82% to 41% on HUVEC monolayer (Fig. 5C), while NAC decreased cell adhesion rate to 34% and 39%, respectively.

To explore the importance of high ROS levels in promoting cell adhesion, 231-C3 cells were treated with 50 μ M H_2O_2 for 6 h under static conditions. Cells were then collected and subjected to cell adhesion assay. The exogenous ROS significantly increased cell adhesion rates from 21%

to 58% on ECM (Fig. 5B) and from 30% to 70% on endothelial monolayer (Fig. 5D). This result supports our hypothesis that SS uses ROS elevation as a mean to stimulate cell adhesion.

Fig. 5. Cellular ROS mediated SS-promoted tumor cell adhesion. (A) Antioxidants impaired the effects of SS on tumor cell adhesion to ECM. The 231-C3 cells were circulated under SS15 with or without pretreatment of 20 μ M PG or 5 mM NAC. Cells were then collected and seeded on collagen I-coated plates. Cells were washed with PBS after 20-min incubation. Upper panel shows representative fluorescent images of cells that remained on plates after PBS wash. Scale bar, 100 μ m. Lower panel shows the quantified cell adhesion rates. (B) Hydrogen

peroxide stimulated tumor cell adherence to ECM. The 231-C3 cells were seeded in non-adhesive 96-well plates with or without 50 μM H_2O_2 for 6 h. Cells were collected and seeded onto collagen-I coated plates and washed with PBS. Upper panel shows representative images of 231-C3 cells remaining on the plates after PBS wash. Scale bar, 100 μm . Lower panel shows the quantified cell adhesion rates. (C) Antioxidants impaired the effects of SS on tumor cell adhesion to endothelial monolayer. The 231-C3 cells were circulated under SS15 with or without pretreatment of antioxidants. Cells were then collected and seeded on HUVEC monolayer. Cells were washed with PBS after 20-min incubation. Upper panel shows representative fluorescent images of cells that remained on plates after PBS wash. Scale bar, 100 μm . Lower panel shows the quantified cell adhesion rates. (D) Hydrogen peroxide stimulated tumor cell adherence to endothelial monolayer. The 231-C3 cells were seeded in non-adhesive 96-well plates with or without 50 μM H_2O_2 for 6 h. Cells were collected and seeded onto a well-established HUVEC monolayer and washed with PBS. Top panel shows representative images of 231-C3 cells that remained on HUVEC monolayer after PBS wash. Scale bar, 100 μm . Lower panel shows the quantified cell adhesion rates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

3.4 SS promotes tumor cell adhesion by activating FAK

Different components of the focal adhesion complex including focal adhesion kinase (FAK), Src and paxillin are known ROS targets, among which FAK is the most-studied one [26]. FAK plays important roles in both attachment and detachment of cells by regulating assembly and disassembly of focal adhesions [27, 28]. Several studies have proved that the phosphorylation level of FAK is strongly associated with adhesion and spreading of cells on ECM [29, 30]. We hypothesized that the SS-generated cellular ROS may promote tumor cell adhesion by activating FAK. To determine the activity of FAK, we examined its phosphorylation level at the tyrosine-397 residue, which is the key phosphorylation site of FAK activation [26, 31].

The 231-C3 cells were pre-treated with or without 20 μM PG or 5 mM NAC for 1 h before circulation under SS15 for 6 h. The circulated cells were allowed to adhere to collagen I-coated dish for 20 min and then collected for Western blot analysis. The results showed that

phosphorylation at tyrosine-397 of FAK experienced a significant, 5.2-fold increase in cells circulated under SS15 compared to cells under static conditions, where the increase value was suppressed by the application of PG to 2.8-fold and by adding NAC to 2-fold (Fig. 6A).

To test the role of ROS in activating FAK, 231-C3 cells were treated with 50 μ M H₂O₂ for 6 h under static conditions. The cells were then allowed to attach to collagen I-coated dish for 20 min and collected for Western blot analysis. H₂O₂ treatment drastically elevated FAK phosphorylation level by 6.9-fold in the cells under static conditions (Fig. 6B). These results established a positive correlation between SS, ROS and FAK activation.

The mechanisms of ROS in activating FAK have been reported by others [30, 32]. One of the mechanisms is to inhibit the activity of low-molecular-weight protein tyrosine phosphatase (LMW-PTP), which can de-phosphorylate FAK [29, 30, 33]. We used anti-LMW-PTP antibody to pull down LMW-PTP proteins from four groups of 231-C3 cells: static, SS15, SS15+NAC and SS15+PG (Fig. 6C) and evaluated their activity levels using PTP activity assay (Thermo Fisher Scientific). The results showed that SS15 reduced the activity of LMW-PTP in 231-C3 cells, while the antioxidants NAC and PG prevented the reduction of LMW-PTP activities caused by SS15 treatment (Fig. 6D). Taken together, we found that SS-generated cellular ROS could activate FAK, possibly by reducing LMW-PTP activities.

To further investigate the role of FAK in SS-enhanced cell adhesion to ECM, a well-established inhibitor of FAK, 1,2,4,5-benzenetetraamine tetrahydrochloride (Y15) [34, 35], was used. The 231-C3 cells were pre-treated with Y15 at 10 μ M for 1 h before circulation for 6 h. The cells were collected and seeded into 96-well plates coated with collagen I to assess the adhesion capacity. For cells under static conditions, treatment with Y15 decreased cell adhesion on collagen I-coated

plates from 27% to 9%. On the other hand, for cells circulated under SS15, Y15 decreased cell adhesion from 68% to 12% (Fig. 6E). For both the cells circulated under SS15 and the cells kept under static conditions, the FAK inhibitor Y15 almost totally blocked cell attachment to ECM, implying the indispensable function of FAK in mediating cell-ECM interaction, as well as its important role in SS-enhanced cell adhesion. These results support the hypothesis that SS-promoted tumor cell adhesion to ECM is mediated, at least partly, by the ROS-enhanced FAK activation.

Although many different types of integrins are found to play vital roles in tumor cell-endothelium interactions, the function of focal adhesions is highly neglected. All integrins are associated with the cytoskeleton through focal adhesions and the connections of membrane molecules with cytoskeleton are very important for establishing stable cell adhesion to the substrates, including the endothelium. As we have found that SS promoted tumor cell adhesion to ECM by enhancing FAK activation, we proposed that the SS-enhanced adhesion to endothelial monolayer was also caused by the increased activities of FAK.

To test this hypothesis, we pre-treated 231-C3 cells with Y15 for 1 h before circulation for 6 h. The cell adhesion assay showed that Y15 decreased adhesion to HUVEC monolayer from 34% to 18% for cells under static conditions, and decreased adhesion of circulated cells to HUVEC monolayer from 79% to 25% (Fig. 6F). These results indicated the importance of FAK in mediating tumor cell adhesion to endothelium, which has never been reported before. Most importantly, we demonstrated that the SS-enhanced tumor cell adhesion to endothelium is highly associated with the SS-induced FAK activation.

Fig. 6. SS-generated cellular ROS stimulated tumor cell adhesion by activating FAK. (A) The 231-C3 cells were circulated under SS15 for 6 h with or without pretreatment of 20 μ M PG or 5 mM NAC. Cells were seeded on ECM for 20 min, then collected and subjected to Western blot analysis. Levels of phosphorylated and total FAK were examined. (B) The 231-C3 cells were seeded in non-adhesive 96-well plates with or without 50 μ M H₂O₂ for 6 h. Cells were collected and seeded on ECM for 20 min, then collected and subjected to Western blot analysis. Levels of phosphorylated and total FAK were examined. GAPDH was used as internal control for equal loading. (C and D) SS15 suppressed the activities of LMW-PTP in 231-C3 cells. (C) Immunoprecipitation was conducted to isolate LMW-PTP from the lysates of 231-C3 cells under four treatment conditions: static, SS15, SS15+NAC and SS15+PG. The efficiency of immunoprecipitation was examined using Western blot analysis. The blots of the extracts in different groups were probed with anti-LMW-PTP antibody. (D) The activities of LMW-PTP were evaluated based on the RediPlate Assay. In the final quantification, the activity levels of each group were normalized to the static group. (E) The 231-C3 cells were circulated under SS15 for 6 h with or without pretreatment of FAK inhibitor Y15, then seeded in plates coated with collagen I. Left panel shows representative fluorescent images of cells remaining on ECM after PBS wash. Scale bar, 100 μ m. Right panel is the quantified result of cell adhesion rates. (F) HUVECs were seeded in 96-well plates and incubated for 24 h to establish an intact HUVEC monolayer. The 231-C3 cells were circulated under SS15 for 6 h with or without pretreatment of FAK inhibitor Y15, then seeded on HUVEC monolayer. Left panel shows representative fluorescent images of cells remaining on HUVEC monolayer after PBS wash. Scale bar, 100 μ m. Right panel is the quantified results of cell adhesion rate. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with the indicated group. # p < 0.05, ## p < 0.01 compared with SS15 group. All quantification data are presented as the mean $\pm$ SD of three independent experiments.

4. Discussion

The antioxidant enzymes are components of cellular antioxidant system, which is responsible for maintaining cellular redox hemostasis [21, 36, 37]. As one of the most important antioxidant enzymes, mitochondrial MnSOD is well-studied for its function in reducing mitochondrial oxidative stress [38]. By catalyzing the dismutation of mitochondrial superoxide, MnSOD protects the mitochondria and cells as an anti-apoptotic protein [39]. Mutations in MnSOD have been associated with cancer [40, 41]. However, the functions of MnSOD in cancer progression are very complicated, as it could play different roles at different stages of metastasis [7, 22, 40, 42]. Here, for the first time, we report that MnSOD regulates tumor cell responses to SS at the stage of tumor cell circulation. We identified the importance of MnSOD in SS-promoted tumor cell migration. MnSOD converts SS-generated superoxide into hydrogen peroxide, which could activate cytosol proteins including ERK1/2 and stimulates tumor cell migration.

In this report, we also revealed the effects of SS on tumor cell adhesion to ECM. The interaction of tumor cells with ECM in the surrounding microenvironment is of vital importance for survival and growth of tumor cells, especially at the stage of developing distant metastases [15, 44]. Recent studies reported that the initiation of metastases is a very inefficient process, where most tumor cells are unable to colonize and proliferate in secondary organs, which is determined by the interactions of tumor cells with constituents of the local microenvironment, including ECM components and host stromal cells [45-48]. Therefore, the efficiency of tumor cell adhesion and spreading on ECM indicate how tumor cells adapt to the local microenvironment in distant organs after extravasation. This study has revealed that SS is able to promote tumor cell adhesion and spreading on ECM, which means that circulation of tumor cells within the bloodstream assists their survival and growth at the stage of colonization. We explored the underlying mechanism and

identified the SS→ROS→FAK→cell adhesion pathway that regulated this effect of SS. The details on how SS-generated ROS enhanced FAK phosphorylation remains unclear. We hypothesize that the action of ROS on FAK activation is mediated by PTPs. The PTPs in tumor cells suppress the activities of FAK through dephosphorylation [49, 50]. We found that, as the direct targets of ROS, the activities of LMW-PTPs were inhibited by SS. During circulation in shear flow, the cellular ROS could oxidize PTPs and suppress their activities, and the tumor cells are in a ready-to-adhere status. After the tumor cells attach to ECM, the integrin-ligand interaction triggers the phosphorylation of FAK, the lower levels of PTP activities in circulated tumor cells could lead to higher levels of FAK phosphorylation compared to cells without SS treatment. However, the association between lower activities of LMW-PTP and enhanced FAK activation needs further investigation.

We also found that SS promoted tumor cell adhesion to endothelial monolayer through the same mechanism. Recent studies exploring the interactions between tumor cells and the endothelium mainly focused on the membrane molecules [17, 25, 51], while the importance of membrane protein-cytoskeleton connections was neglected. Integrins of tumor cells have been found to play important roles in the adherence to endothelium [17, 52, 53]. Here, for the first time, we report that FAK, which is one of the key components of focal adhesion complex, is highly associated with the stability of tumor cell-endothelium interactions.

Furthermore, we identified the importance of MnSOD in SS-promoted tumor cell adhesion. Taken our previous findings together, we found that by regulating the existing forms and distributions of cellular ROS, MnSOD not only protects tumor cells during circulation but also plays important roles in enhancing tumor cell migration and adhesion. The signaling pathway is

summarized in Fig. 7. In short, SS stimulates the generation of mitochondrial superoxide, which will be accumulated inside mitochondria and induce apoptosis. MnSOD converts mitochondrial superoxide into hydrogen peroxide, which could diffuse out to cytosol to activate ERK1/2 to promote tumor cell migration and activate FAK to promote tumor cell adhesion.

Fig. 7. Proposed mechanism that regulates tumor cells responses to fluidic SS. MnSOD converts SS-generated superoxide into hydrogen peroxide, which activates cytosol proteins including ERK1/2 and FAK to stimulate tumor cell migration and adhesion.

The above findings provide new insights into the therapeutic strategies for suppressing metastasis. First, different types of ROS may have different effects on tumor cells which are determined by the properties and distributions of ROS. Taking our studies as examples, at the stage of tumor cell circulation during metastasis, mitochondrial oxidative stress (superoxide) and cytosolic oxidative stress (hydrogen peroxide) play different roles. As a result, application of

antioxidants that specifically target hydrogen peroxide could suppress SS-promoted tumor cell invasiveness without reducing SS-induced cell death during circulation. On the other hand, the blind use of antioxidants without considering the ROS targets may protect the tumor cells from SS-induced cell damage and facilitate metastasis. Thus, a better understanding of the existing forms and distributions of cellular ROS in tumor cells has significant meaning.

Second, instead of targeting a specific type of ROS, modulating the cellular antioxidant system could be another efficient way to suppress metastasis. By converting a damaging molecule superoxide into a signaling molecule hydrogen peroxide, MnSOD not only protects the tumor cells under SS but also regulates the pro-metastatic effects of SS. Interestingly, compared to non-metastatic cancer cell lines, the metastatic cancer cell lines usually have higher levels of MnSOD [7], which makes them more resistant to SS and endows them with enhanced aggressiveness after surviving the circulation. MnSOD, as a component of cellular antioxidant system, can function both as a prognostic marker and a therapeutic target. Decreasing the expression or inhibiting the function of MnSOD could facilitate SS-mediated cell death, as well as inhibit SS-promoted cell migration and extravasation.

Finally, a comprehensive understanding of the mechanisms regulating the enhanced aggressiveness of circulating tumor cells would provide different potential therapeutic targets. In addition to ROS and MnSOD, ERK1/2 and FAK kinases can also be used as the targets for suppressing metastasis. As the seeds for distant metastases, CTCs elimination during circulation and extravasation inhibition of CTCs can be two promising approaches to suppress metastasis.

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6. Author contributions

K.Q. Luo, S. Ma and A. Fu conceived the study and designed the experiments; K.Q. Luo and S. Lim supervised the project; S. Ma performed most of the experiments and analyzed the data; G.G.Y. Chiew and S. Ma conducted the immunoprecipitation and PTP assay experiments and analyzed the data; A. Fu designed the microfluidic system; and S. Ma wrote the manuscript, which was revised and edited by K.Q. Luo and approved by all authors.

7. Conflict of interest

None.

8. Reference

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