

Mesenchymal Stem Cell-Secreted Exosome Promotes Chemoresistance in Breast Cancer via Enhancing miR-21-5p-Mediated S100A6 Expression

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Emerging evidence has shown the role of mesenchymal stem cell-derived exosome (MSC-exo) in inducing resistance of cancer cells to chemotherapy. However, it remains unclear whether the change of MSC-exo in response to chemotherapy also contributes to chemoresistance. In this study, we investigated the effect of a standard-of-care chemotherapeutic agent, doxorubicin (Dox), on MSC-exo and its contribution to the development of Dox resistance in breast cancer cells (BCs). We found that the exosome secreted by Dox-treated MSCs (Dt-MSC-exo) induced a higher degree of Dox resistance in BCs when compared with non-treated MSC-exo. By analysis of the MSC-exo-induced transcriptome change in BCs, we identified S100A6, a chemoresistant gene, as a top-ranked gene induced by MSC-exo in BCs, which was further enhanced by Dt-MSC-exo. Furthermore, we found that Dox induced the expression of miR-21-5p in MSCs and MSC-exo, which was required for the expression of S100A6 in BCs. Importantly, silencing of miR-21-5p expression in MSCs and MSC-exo abolished the resistance of BCs to Dox, indicating an exosomal miR-21-5p-regulated S100A6 in chemoresistance. Our study thus uncovered a novel mechanistic insight into the role of MSC-secreted exosome in the development of chemoresistance in the tumor microenvironment.

INTRODUCTION

Treatment failure due to chemoresistance remains one of the major reasons for breast cancer mortality.¹ Mesenchymal stem cells (MSCs) are a unique group of cells capable of self-renewal and multi-directional differentiation, which are also known to play a role in the tumor microenvironment. MSCs have been shown to contribute to tumorigenesis processes, including proliferation, metastasis, and drug resistance in a variety of cancers,^{2,3} mainly through the secretion of paracrine factors or the cell-cell interaction.⁴ Recently, growing evidence has shown that MSCs can also generate extracellular vesicles including exosomes (MSC-exo) to promote the tumor growth, angiogenesis, metastasis, and invasion⁵⁻⁷ through the vesicle transfer of proteins, messenger RNAs (mRNAs), or microRNAs (miRNAs) to the target cancer cells.^{8,9} Some studies have also demonstrated the

role of MSC-exo in drug resistance in cancers such as gastric cancer and multiple myeloma.^{10,11} Although the tumor microenvironment is well known to be a significant determinant of a tumor's response to chemotherapy,^{12,13} it remains unclear whether MSC-exo changes in response to chemotherapy, which is necessary to promote chemoresistance of cancer cells.

In this study, we sought to investigate whether and how doxorubicin (Dox), a standard chemotherapeutic agent in breast cancer treatment, affects MSC-exo. We have demonstrated that Dox treatment induced the expression of *miR-21-5p* in MSCs and MSC-exo, leading to the induction of *S100A6* in the breast cancer cells (BCs). Functional studies validated the role of *miR-21-5p*-mediated *S100A6* expression in chemoresistance both *in vitro* and *in vivo*, which supports a novel mechanism by which the tumor microenvironment induces chemoresistance through an MSC-secreted exosome.

Results

The Conditioned Medium of Dox-Treated MSCs Significantly Enhances the Resistance of BCs to Dox

To investigate the paracrine effect of MSCs on BCs, we treated MSCs with 1 μ M of Dox for 3 h, followed by a collection of untreated and Dox-treated MSC-conditioned medium. We further used the conditioned medium to treat breast cancer MDA-MB-231 cells in the presence of different concentrations of Dox (0, 0.5, 1, and 1.5 μ M) for 24

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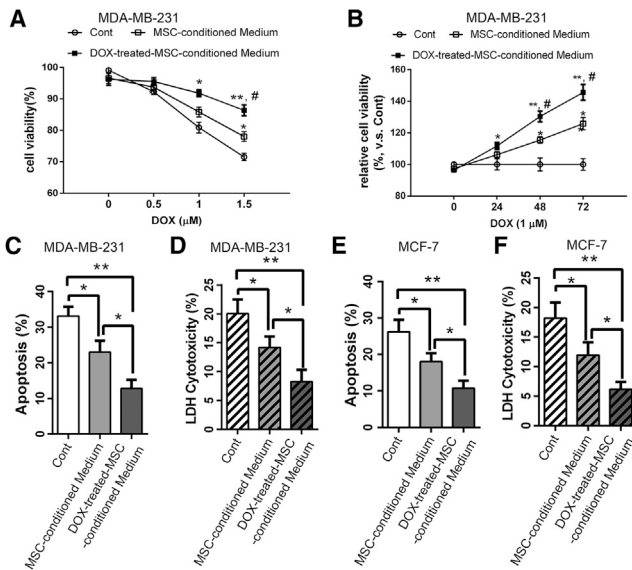


Figure 1. Conditioned Medium of Dox-Pretreated MSCs Significantly Enhances the Resistance of BCs to Dox

(A) MDA-MB-231 cells were incubated with normal medium (control [Cont]), MSC-conditioned medium, and DOX-treated MSC-conditioned medium and then treated with various concentrations of Dox (0, 0.5, 1.0, and 1.5 μM) for 24 h. Cell viability was assessed using a CCK-8 assay. (B) To determine the change in viability of the cells with time after exposure to Dox, Dox was first added to the MDA-MB-231 cells to 1 μM, and the CCK8 assay then carried out after 0, 24, 48, and 72 h. (C and E) Annexin V-APC/7-AAD double staining showed the effect of different conditioned media on the percentages of apoptotic BCs (C, MDA-MB-231 cells; E, MCF-7 cells). (D and F) LDH cytotoxicity assay measured the effect of different conditioned media on the release of LDH from damaged BCs (D, MDA-MB-231 cells; F, MCF-7 cells). Results are expressed as mean ± SD. Data were analyzed by the one-way analysis of variance (ANOVA). Significant differences between the means as determined by the Tukey post hoc test. ** $p < 0.05$, *** $p < 0.001$ in (A) and (B); * $p < 0.05$, ** $p < 0.001$ versus the cells of Cont group; and # $p < 0.05$ versus the cells treated with MSC-conditioned medium.

h, which was followed by cell viability assessment with a Cell Counting Kit-8 (CCK-8) assay (Figure 1A). The results showed that the Dox-treated MSC-conditioned medium significantly enhanced the cancer cell viability compared with untreated MSC-conditioned medium (Figure 1A). A similar effect was also observed in a time course analysis during 3 days in which Dox-treated MSC-conditioned medium supports a greater level of viability in the presence of 1 μM Dox (Figure 1B).

We further used flow cytometry and the annexin V-allophycocyanin (APC)/7-aminoactinomycin D (7-AAD) double staining to evaluate the effect of MSC-conditioned medium on the apoptosis and cytotoxicity of MDA-MB-231 cells induced by Dox treatment. The results showed that Dox-induced apoptosis and cytotoxicity were significantly reduced by Dox-MSC-conditioned medium, while to a lesser extent by untreated MSC-conditioned medium (Figures 1C and 1D). A similar effect was also obtained in MCF-7 cells (Figures 1E and 1F). These findings showed that the conditioned medium from

MSCs, especially under the Dox treatment, significantly enhance the resistance of BCs to Dox.

Exosomes Secreted from Dox-Treated MSCs Significantly Enhances the Resistance of BCs to Dox

Mounting evidence has suggested a role of tumor-derived exosome in therapy failure.¹⁴ To determine whether the Dox-treated and untreated MSC-secreted exosomes (hereafter referred to as Dt-MSC-exo and MSC-exo, respectively) are responsible for the MSC-mediated chemoresistance, we extracted the exosomes from the conditioned medium of MSCs using a series of centrifugation and ultracentrifugation steps. The characteristics of exosomes were evaluated by electron microscopy, which revealed the homogeneous nature of exosomes in morphology, with sizes ranging from 50 to 100 nm (Figure 2A). Western blot further confirmed the abundant expression of the exosome-related proteins, including CD9, CD63, CD81, TSG101, and endoplasmic reticulum protein calnexin (Figure 2B). Taken together, these findings verified that the examined extracellular vesicles we isolated were indeed exosomes.

To directly evaluate the effects of MSC-exo and Dt-MSC-exo on BCs, we added them to the culture medium of MDA-MB-231 cells, followed by treatment with 1 μM Dox for 48 h. Tests with the CCK-8 assay, flow cytometry apoptotic double staining, and lactate dehydrogenase (LDH) assays showed that Dt-MSC-exo, and MSC-exo to a lesser degree, markedly enhanced the tolerance of MDA-MB-231 cells to Dox (Figure 2C).

To further ascertain the effect of the MSC-secreted exosome, we used centrifugation to remove the exosome from the MSC-conditioned medium, which was further used to treat the MDA-MB-231 cells. The result shows that Dt-MSC-conditioned medium deprived of exosome was unable to induce resistance of MDA-MB231 cells to Dox treatment, as shown by the CCK-8 cell viability assay (Figure 2D). This finding was further verified in MCF-7 cells (Figures 2E and 2F). Collectively, these results verify that the Dox enhances the capacity of MSCs to inducing BC resistance to Dox in an exosome-dependent manner.

Dox-Treated MSC-Exosome Significantly Induces *S100A6* Expression in BCs

To identify the molecular mechanism by which the MSC-derived exosome induces chemoresistance of BCs, we took advantage of a public database comprising the transcriptome change of MCF-7 cells treated with MSC-exo (GEO: GSE46950). By applying a highly stringent cut-off (>2³-fold change), we identified 75 genes whose expression exhibited a significant change in response to MSC-exo treatment.

Among the 75 candidate genes shown in Figures 3A and 3B, *S100A6*,¹⁵ *FosB*,¹⁶ *ARHGDI1B*,¹⁷ *SOD3*,¹⁸ and *ApoD*¹⁹ have been previously implicated in promoting cell survival. Further RT-PCR validation showed that only the top-ranked *S100A6* showed corresponding induction in BCs in response to exosome treatment (Figure 3C). Of notice, Dt-MSC-exo, compared with the untreated MSC-exo, induced

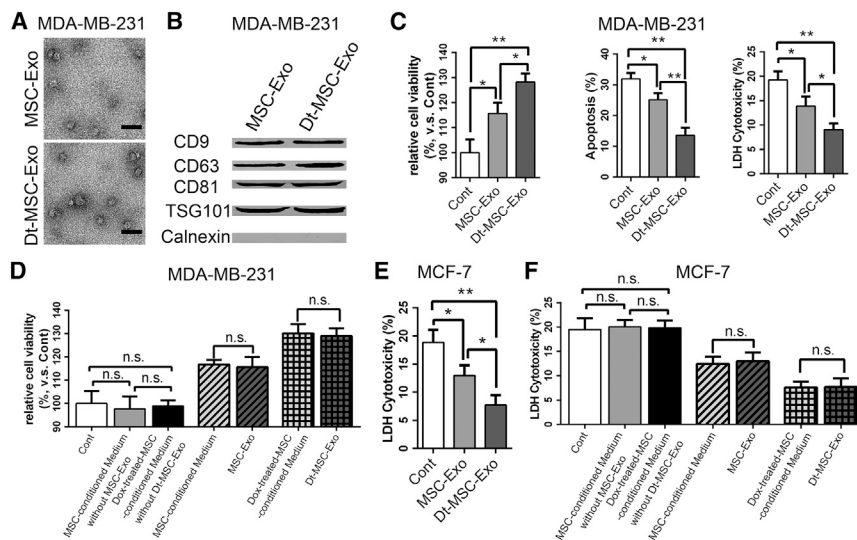


Figure 2. Exosomes Secreted by Dox-Pretreated MSCs Can Significantly Enhance BC Resistance to DOX

(A) Transmission electron microscope (TEM) images of exosomes from MSC-conditioned medium and Dox-treated MSC-conditioned medium. Scale bars, 200 nm. (B) Phenotype analysis of protein markers of CD9, CD63, CD81, TSG 101, and calnexin on vesicles by western blot. (C–F) MDA-MB-231 (C and D) and MCF-7 (E and F) cells were treated with MSC-exo and Dt-MSC-exo, and then treated with Dox (1 μ M) for 48 h: (C) CCK-8 (C, left); D, annexin V-APC/7-AAD double-staining (C, middle; F), and LDH cytotoxicity (C, right); E) assays detected the viability of the cells, percentage of apoptotic cells, and cell damage. The exosomes were first removed by centrifugation from the supernatant of MSCs and then the supernatant was added to MDA-MB-231 (D, left) and MCF-7 (F, left) cells: CCK-8 (D) and LDH cytotoxicity (F) assays detected the viability of the cells, vesicular body, and its corresponding conditioned medium on MDA-MB-231 (D, middle, right) and MCF-7 (F, middle, right) cell activity. The data are mean \pm SD, averaged from three separate experiments. * $p < 0.05$, ** $p < 0.01$. n.s., not significant.

a higher level of *S100A6* expression in both MCF-7 and MDA-MB-231 cells, while the other top candidate genes failed to respond to Dt-MSC-exo (Figures 3C and 3D). The quantitative real-time RT-PCR results were further validated by western blot (Figure 3E).

***S100A6* Upregulation by Dt-MSC-exo Confers Increased Cell Viability and Dox Resistance**

Previous studies have reported an anti-apoptotic effect of *S100A6* in breast cancer.²⁰ Next, we performed *S100A6* knockdown to evaluate the functional role of *S100A6* in MSC-exo-induced chemoresistance. Quantitative real-time RT-PCR and western blot analyses confirmed that small interfering RNA (siRNA)-mediated *S100A6* knockdown has successfully abolished the increased *S100A6* expression by MSC-exo or Dt-MSC-exo (Figures 4A and 4B). As expected, *S100A6* knockdown in MCF-7 and MDA-MB-231 cells abolished the Dt-MSC-exo-induced resistance to Dox (Figures 4C and 4E) and promoted an apoptotic response to Dox (Figures 4D and 4F).

To test the possibility that MSC-exosome-derived *S100A6* plays a role in causing resistance to BCs, we performed *S100A6* knockdown in MSCs and Dox-treated MSCs and collected its exosome for incubation with BCs. Although *S100A6*-siRNA significantly inhibited the expression of *S100A6* in MSCs (Figures 5A–5C), BCs treated with MSC-exo or Dt-MSC-exo showed no significant difference in *S100A6* expression (Figures 5D and 5E). This result has excluded a possible role of the exosomal source of *S100A6* mRNA that could be transmissible to cancer cells.

Dox-Induced miR-21-5p Expression in MSCs and MSC-exo Is Required for the *S100A6* Induction in BCs

It has been previously shown that miR-21-5p can induce the expression of *S100A6*.²¹ We next investigated whether MSC-exosomal miR-

21-5p is responsible for the induction of *S100A6* in cancer cells. Indeed, we show that miR-21-5p was present in both MSCs and MSC-exo, which was significantly induced by Dox treatment (Figures 6A and 6B). Transfection of MDA-MB-231 and MCF-7 cells with a miR-21-5p antagonist abrogated the increased expression of miR-21-5p by MSC-exo and Dt-MSC-exo (Figure 6C), resulting in the abolishment of *S100A6* induction by MSC-exo or Dt-MSC-exo (Figure 6D). Importantly, miR-21-5p antagonist also inhibited the expression of miR-21-5p in MSCs and MSC-exo (Figures 6E and 6F). As such, miR-21-5p antagonist-treated MSC-exo was unable to induce mRNA and protein expression of *S100A6* in BCs (Figure 6G). Consequently, miR-21-5p antagonist-treated Dt-MSC-exo was unable to improve the BC viability (Figure 7A) or inhibit the Dox-induced apoptosis (Figure 7B). To investigate whether Dox can induce expression of miR-21-5p or *S100A6* in BCs, we treated MCF-7 cells and MDA-MB-231 cells in the presence of different concentrations of Dox (0, 0.5, 1, and 1.5 μ M) for 12 h and in a time-dependent manner (0, 6, 12, and 18 h) with 1 μ M of Dox. Quantitative real-time RT-PCR and western blot analyses showed that Dox treatment in BCs had no significant effect on the enhancement of miR-21-5p and *S100A6* (Figure S1). This observation suggests a transmittable effect of miR-21-5p from exosome to BCs and indicates that inhibition of miR-21-5p expression in Dt-MSC-exo could significantly alleviate the Dox resistance.

MSC-exo Delivers miR-21-5p, Leading to *S100A6* Expression and Dox Resistance *In Vivo*

We next used an MDA-MB-231 tumor xenograft model to validate the *in vivo* capacity of the MSC-exo in regulating the tumor growth response to Dox. To this end, we silenced the *S100A6* in MDA-MB-231 cells (Figure 8A) for xenograft tumor formation and injected MSC-exo or Dt-MSC-exo into the xenograft tumor. The result shows

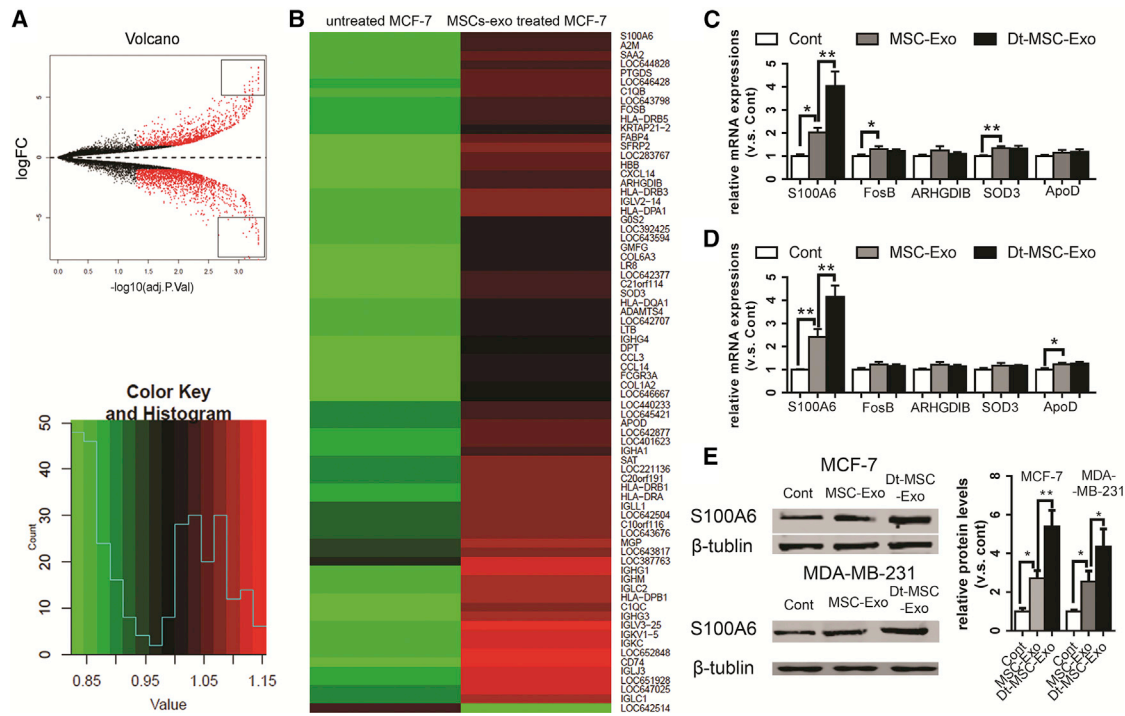


Figure 3. Screening for Genes Affected by Exosomes Secreted by Dox-Pretreated MSCs and Identifying the Increase of S100A6 Expression in BCs

(A and B) Differentially expressed proliferation-related genes in untreated MCF-7 cells and MSC-exo-treated MCF-7 cells are displayed in a volcano plot (A) and heatmap (B). Quantitative PCR analysis of exosome-treated BCs. (C and D) MCF-7 (C) and MDA-MB-231 (D) cells treated with MSC-exo and Dt-MSC-exo. The relative levels of S100A6, FOSB, ARHGDI B, SOD3, and ApoD in the Cont, MSC-exo, and Dt-MSC-Exo. (E) Western blot analysis. Images are representative of three independent experiments. Data are expressed as the mean \pm SD of the relative values from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

that the application of MSC-exo and Dt-MSC-exo promoted tumor growth in response to Dox treatment, while S100A6 knockdown inhibited the MSC-exo-induced tumor growth (Figures 8B and 8C). Caspase-3, known as the death enzyme, has an important role in the controlled execution of programmed cell death.²² Correspondingly, western blot shows that tumors treated with MSC-exo showed reduced active caspase-3 expression, while tumor with S100A6 knockdown showed increased expression of active caspase-3 (Figure 8D).

To evaluate the exosomal effect of miR-21-5p *in vivo*, MSCs were treated with miR-21-5p antagonist, followed by isolation of exosomes used to treat the xenograft tumors. The results showed that the miR-21-5p antagonist-treated Dt-MSC-exo markedly inhibited xenograft tumor growth (Figures 8E–8G). Western blot also confirmed the reduction of S100A6 by miR-21-5p antagonist and increased the expression of active caspase-3 (Figure 8H). Taken together, these data demonstrated that Dt-MSC-exo could significantly enhance BC resistance to Dox through exosomal miR-21-5p-mediated S100A6 expression.

DISCUSSION

The importance of the microenvironment in tumor development and progression is widely recognized. The tumor microenvironment is composed of both tumor cells and stromal cells. MSCs, one of the

pivotal components of the tumor microenvironment, is a focus of research on the progression of tumors.^{23,24} Notably, evidence suggests that MSCs release cytokines and growth factors that influence the behavior of tumors in a paracrine-dependent manner.^{25–27} Previous studies have demonstrated that tumor progression, enhancement of metastatic potential, chemoresistance, and resistance to radiotherapy may be attributed to MSCs.^{28–30} For instance, head and neck squamous carcinoma cells are resistant to paclitaxel when co-cultured with bone marrow-derived MSCs.³¹ MSCs can also utilize autophagy to recycle macromolecules and synthesize antiapoptotic factors to facilitate growth and survival of surrounding tumor cells.³² In colorectal carcinoma, NRG1 released by MSCs activates the phosphatidylinositol 3-kinase (PI3K)/AKT pathway to stimulate the growth of tumor cells.³³ Platinum-based chemotherapy in breast cancer also induces MSCs to secrete unique fatty acids that confer chemoresistance.³⁴ Adipose-derived MSCs enhanced BCRP protein expression and secreted interleukin (IL)-8, leading to reduced Dox sensitivity in triple-negative breast cancer.³⁵ In this study, we investigated how Dox, a common chemotherapeutic agent in breast cancer treatment, affects the role of MSC-exo in the development of Dox resistance by BCs.

In our present study, we found that MSC-exo induced resistance in BCs to Dox. However, exosomes secreted by Dox-treated MSCs

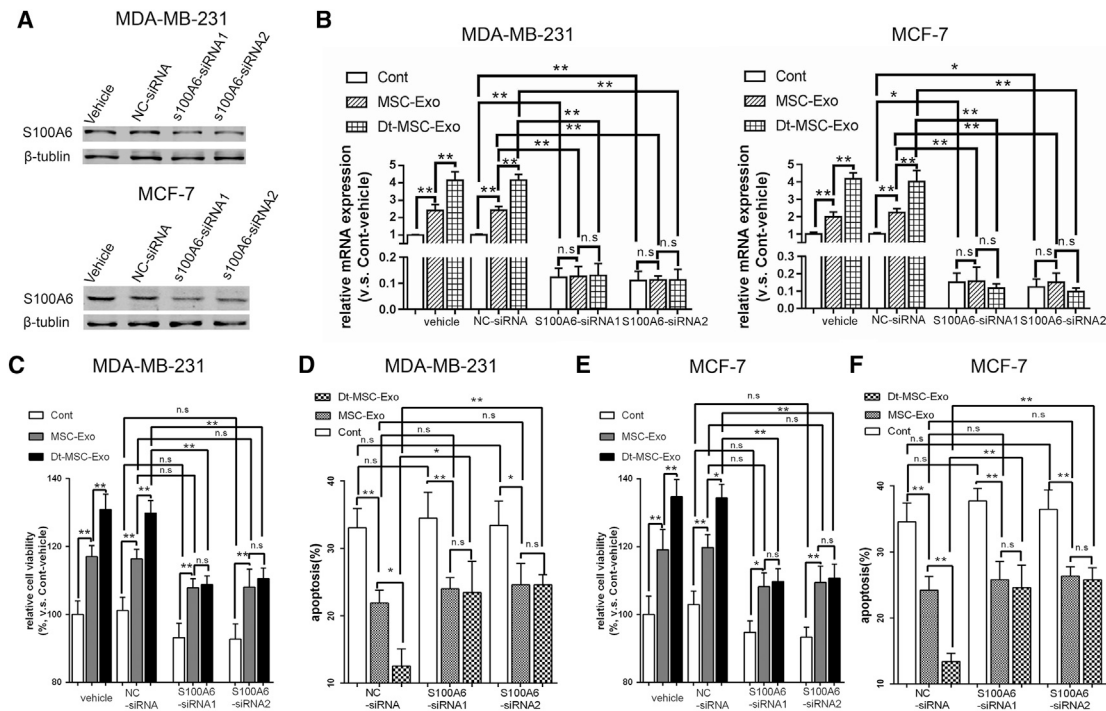


Figure 4. Downregulation of S100A6 in BCs Lessens the Induction of Dox Tolerance by Dt-MSC-exo

MDA-MB-231 cells and MCF-7 cells were transfected with S100A6 siRNAs and negative control siRNA (nc-siRNA). (A and B) The protein (A) and mRNA (B) expression of S100A6 in MDA-MB-231 cells and MCF-7 cells after treatment with Dt-MSC-exo and MSC-exo (B). (C–F) Analysis by CCK8 viability (C and E) and apoptotic double-staining (D and F) assays showing no significant difference between Dt-MSC-exo and MSC-exo in the tolerance of BCs to Dox. Compared with nc-siRNA, treatment with S100A6 siRNAs resulted in more apoptotic cells in MSC-exo and Dt-MSC-exo. (C and D) MDA-MB-231 cells; (E and F) MCF-7 cells. The data are mean ± SD, averaged from three separate experiments. *p < 0.05, **p < 0.01. n.s., not significant.

resulted in greater resistance of BCs to Dox than did those from untreated MSCs. We found that MSC-exo augmented the level of S100A6, a chemoresistance gene, in the BCs, whereas Dt-MSC-exo led to a greater increase in S100A6 level in the BCs than in MSC-exo. Our study also showed that Dox augmented miR-21-5p expression in MSCs and the increase of the miR-21-5p level in MSC-exo.

miR-21-5p is an important oncogenic miRNA that was recently reported to be highly upregulated in multiple tumors.^{36–38} The molecular mechanism of miR-21-5p action has been well characterized. miR-21-5p promotes tumor growth and metastasis by targeting several tumor suppressors, including PTEN, PDCD4, Bcl2, and tissue inhibitor of metalloproteinase 3 (TIMP3) in breast cancer, suggesting that miR-21-5p may be involved in regulating angiogenesis.^{36,39,40} S100A6 has been shown to be overexpressed in many human tumors, including pancreatic cancer,^{41–43} melanocytic tumors,^{44,45} colorectal cancer,^{46,47} and breast cancer.²⁰ Previous studies have shown that S100A6 can promote the proliferation and migration of MCF-7 BCs and inhibit its apoptosis. Therefore, miR-21-5p and S100A6 are involved in regulating those genes that cause apoptosis in many diseases. Studies have shown that miR-21-5p promotes the expression of S100A6.²¹ In the present study, we showed that silencing miR-21-5p in MSCs eliminated the significant difference

between MSC-exo and Dt-MSC-exo in their ability to elevate S100A6 level and promote Dox resistance in BCs. These results suggested that Dox can enhance MSC-exo-induced Dox resistance of BCs by increasing S100A6 content in BCs as a result of promoting the elevation of the miR-21-5p level in MSC-exo. Other potential miR-21 target genes also may be responsible for the effects of Dt-MSC-exo on breast cancer chemo-resistance, which need further in-depth study. Thus, the expression of miR-21-5p in MSCs is a prominent factor in oncology therapies employing Dox-treated MSCs. Note that in addition to overexpression in MSCs, previous studies have demonstrated that miR-21-5p is upregulated in numerous types of cancer and has been associated with cellular proliferation, migration, invasion, and apoptosis, which are the main processes underlying cancer pathogenesis.^{48,49} This suggests that when studying the use of MSCs for tumor therapy, it may be insufficient to consider only miR-21-5p expression in MSCs; the effect of pretreatment of MSCs with chemotherapy drugs on the tumor microenvironment also should be considered.

Growing evidence suggests that MSC-exo transfers proteins, mRNA, and miRNA to recipient cells, where these molecules exert various effects on the growth, metastasis, and drug response of different tumor cells. It has been reported that MSC-exo induces drug resistance in

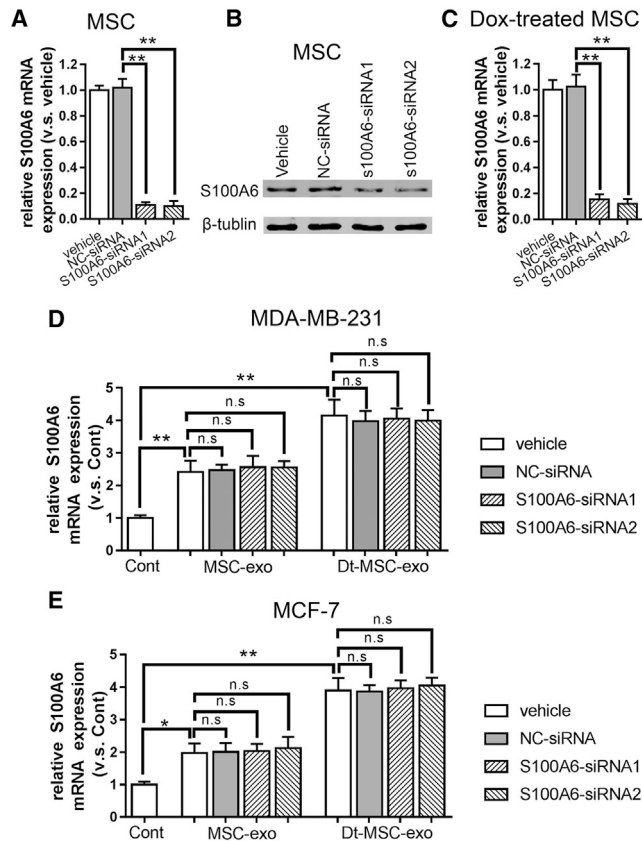


Figure 5. Downregulation of S100A6 in MSCs Has No Significant Effect on the Enhancement of S100A6 by MSC-exo and Dt-MSC-exo in BCs

(A–C) The mRNA (A and C) and protein (B) expression of S100A6 in MSCs (A and B) and Dox-treated MSCs (C) after treatment with S100A6-siRNA. (D and E) Downregulation of S100A6 in MSCs: relative expression of S100A6 in MSC-exo and Dt-MSC-exo treated with S100A6-siRNA in MDA-MB-231 (D) and MCF-7 (E) cells. The data are mean \pm SD, averaged from three separate experiments. * $p < 0.05$, ** $p < 0.01$. n.s., not significant.

different tumor cells. MSC-exo triggered the activation of calcium/calmodulin-dependent protein kinases (CaM-Ks) and Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) kinase cascade in gastric cancer cells.¹⁰ MSC-exo not only increases multiple myeloma cell growth but also induce resistance to bortezomib, a proteasome inhibitor. MSC-exo inhibits the reduction of Bcl-2 expression caused by bortezomib and reduces the cleavage of caspase-9, caspase-3, and PARP.¹¹ In some studies, MSC-exo has been found to mediate drug efflux and the transfer of drug resistance to recipient cells, by transferring protein (MRP2, ATP7A, and ATP7B) and miRNAs (miR-100, miR-222, miR-30a, and miR-17). The miRNAs transferred to receptor cells can change the cell cycle and affect cell apoptosis, thus reducing the susceptibility to drugs. Consistent with previous results, this study demonstrated that MSC-exo was sufficient to confer Dox resistance in BCs both *in vivo* and *in vitro* and that Dt-MSC-exo resulted in a higher level of resistance to Dox in BCs than in MSC-exo. Therefore,

the finding that Dt-MSC-exo increases chemotherapy resistance could be utilized to predict whether Dox should be used for treatment. Further studies are needed to explore whether MSC-exo treated with other chemotherapy drugs will also change the tumor microenvironment and lead to chemoresistance.

In conclusion, our study demonstrated that MSC-exo induces resistance of BCs to Dox and that Dt-MSC-exo can lead to a greater resistance of BCs to Dox than MSC-exo. Dox enhances MSC-exo-induced Dox resistance of BCs by increasing the S100A6 content of BCs as a result of promoting an increase in the level of miR-21-5p level in MSC-exo. Most importantly, silencing miR-21-5p in MSCs eliminated the significant differences between MSC-exo and Dt-MSC-exo in inducing the elevation of the S100A6 level of BCs and the development of Dox resistance of BCs *in vitro* and *in vivo*. This finding provides a novel understanding of the role of Dox-treated MSCs in the tumor microenvironment and may aid in the development of novel therapeutic strategies to circumvent Dox-treated MSCs-related drug resistance in patients with breast cancer.

MATERIALS AND METHODS

Cell Culture and Culture Conditions

Human bone marrow-derived MSCs were isolated and cultured as described previously.⁵⁰ MDA-MB-231 cells and MCF-7 cells were obtained from ATCC (Manassas, VA, USA). All cells were cultured in complete DMEM containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C in humidified air with 5% CO₂. Dox was purchased from Selleck Chemicals (USA). To collect Dox-treated MSC-conditioned medium, MSCs were treated with 1 μ M Dox for 3 h, after which the culture medium was replaced with fresh DMEM/F-12. After a 24-h incubation, Dox-treated MSC-conditioned medium was collected and filtered through a 0.22- μ m filter. The MSC-conditioned medium was obtained by collection through 0.22- μ m filtration of the supernatant media from MSCs treated without Dox. BCs were cultured with a control medium, MSC-conditioned medium, and Dox-treated MSC-conditioned medium.

Exosomes Extraction and Purification

The MSC-conditioned medium consisted of DMEM supplemented with 10% FBS from which bovine exosomes and protein aggregates had been removed by ultracentrifugation at 100,000 \times g for 16 h at 4°C. When the MSCs reached 80%–90% confluence, they were cultured in the conditioned medium for 48 h, and then the supernatants containing exosomes were harvested. The exosomes were purified by a modification of the procedure of differential centrifugation and purification on a sucrose cushion described by Qu et al.⁵¹ In brief, the supernatants were centrifuged for 20 min at 2,000 \times g to separate cellular debris. The clarified supernatant was then concentrated through centrifugation twice for 30 min at 1,000 \times g using a 100-kDa molecular weight cutoff (MWCO) hollow-fiber membrane (Millipore, Bedford, MA, USA). The concentrated supernatant was collected and diluted in PBS. The diluted supernatant was transferred to an ultracentrifuge tube, underlaid with a 30% sucrose/D2O cushion

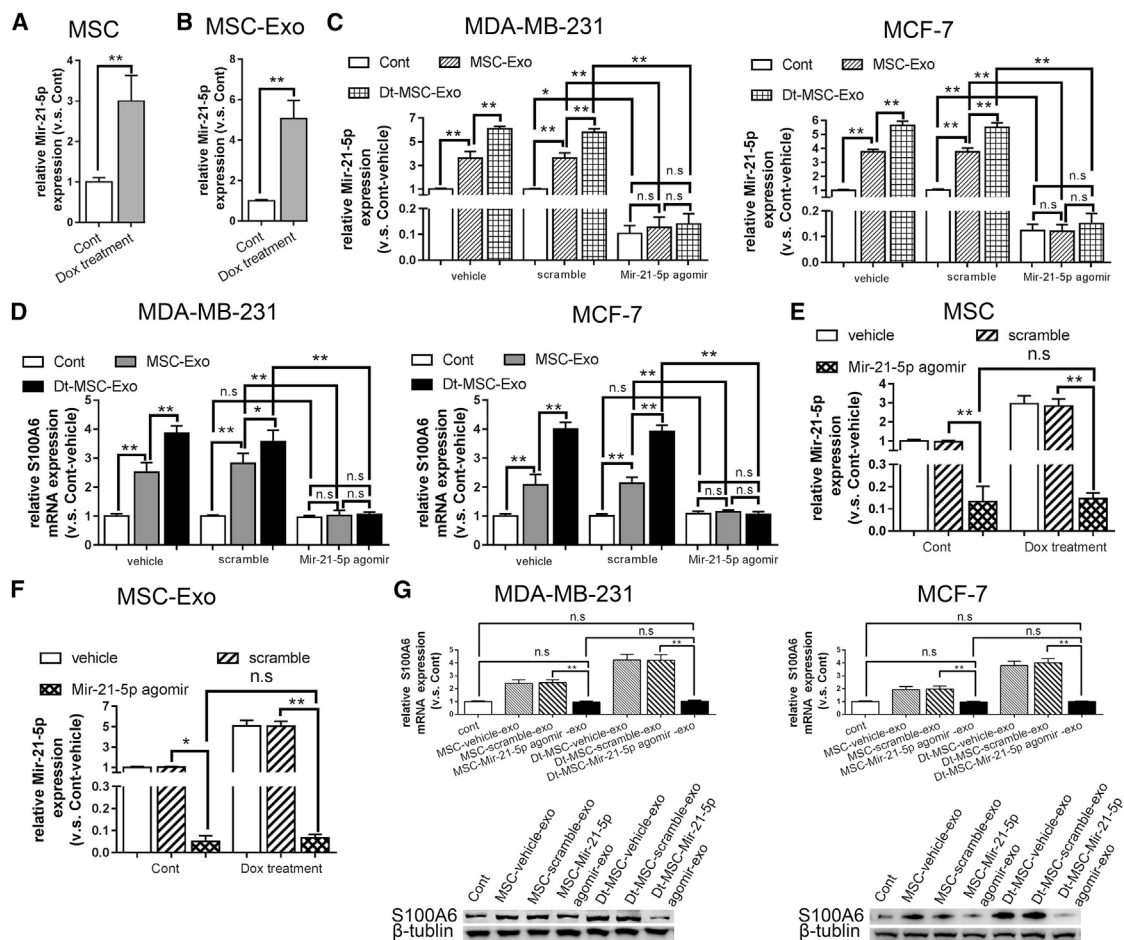


Figure 6. MSC-exo and Dt-MSC-exo Promote the Expression of S100A6 in BCs by Delivering miR-21-5p

(A and B) Relative expression of miR-21-5p in MSCs (A) and MSC-exo (B). The Dox-treated group displayed a higher content than did the control group. (C and D) MDA-MB-231 cells and MCF-7 cells were transfected with miR-21-5p antagonist or negative control scrambled RNA. Relative expression of miR-21-5p (C) and S100A6 (D) in MDA-MB-231 cells (left) and MCF-7 cells (right) treated with miR-21-5p antagonist. Two independent experiments showed similar results. (E and F) Reduced miR-21-5p expression in MSCs (E) and MSC-exo (F). (G) The mRNA and protein expression of S100A6 in MSC-exo and Dt-MSC-exo measured after treatment with miR-21-5p antagonist. Two independent experiments showed similar results. The data are mean \pm SD, averaged from three separate experiments. * $p < 0.05$, ** $p < 0.01$. n.s., not significant.

(density 1.210 g/cm³) and followed by ultracentrifugation at 100,000 \times g for 1 h at 4°C. Gradient fractions were collected from the bottom of the tube and washed three times with PBS by centrifugation at 1,000 \times g for 30 min in a 100-kDa MWCO Ultrafree-15 capsule. Finally, the protein content of the concentrated exosomes was determined using a bicinchoninic acid (BCA) protein assay kit. CD9, CD63, CD81, TSG101, and calnexin (Abcam, Cambridge, UK) were measured by western blot. The aliquots were passed through 0.22- μ m microcentrifuge filters and stored at -80°C.

In Vivo Study

Sixty BALB/c 6-week-old female nude mice were purchased from Shanghai SLAC Laboratory Animal (Shanghai, P.R. China). MDA-MB-231 cells were stably transfected with S100A6-shRNA or scramble-shRNA (negative control group). MDA-MB-231 cells (2 \times 10⁶) stably expressing S100A6-shRNA or scramble-shRNA

were subcutaneously injected into the right axilla of the mice and were randomly divided into three groups (n = 5) each. Mice were administered Dox (4 mg/kg; intravenously [i.v.]) every 3 days when tumors reached a volume of 150–200 mm³. The mice carrying xenograft tumor of MDA-MB-231 cells infected with S100A6-shRNA were divided into three groups (5 mice/group) randomly. Three groups respectively received an intratumoral injection containing (1) PBS (100 mg/mL), (2) MSC-exo (100 mg/mL), and (3) Dt-MSC-exo (100 mg/mL). Similarly, mice carrying a xenograft tumor of MDA-MB-231 cells infected with scramble-shRNA were also divided into three groups (5 mice/group) randomly and given the same treatment as for the S100A6-shRNA group. The mice were examined every 2 days and sacrificed at 12 days after Dox treatment. The tumor tissue was excised from mice, weighed, and the tumor volumes were calculated by the modified ellipsoidal formula $V = \frac{1}{2}(\text{length} \times \text{width}^2)$.

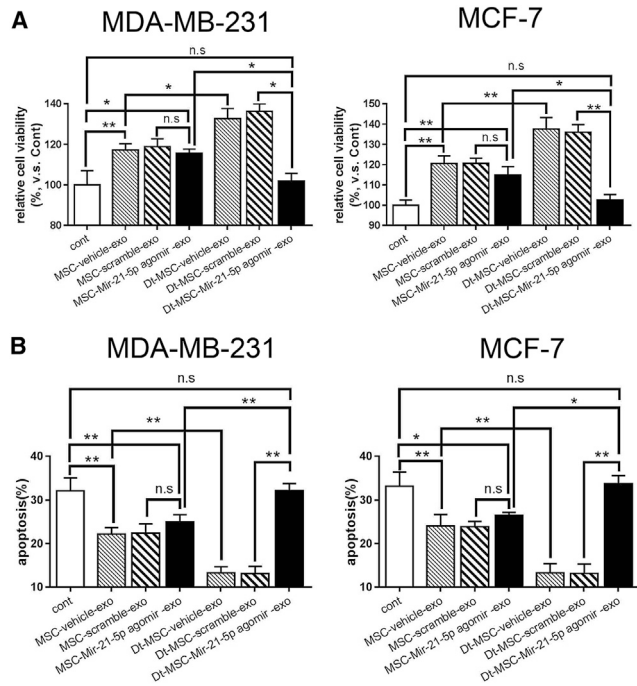


Figure 7. Downregulation of miR-21-5p in MSC-exo Inhibited the Promotion of Tolerance of BCs to Dox

(A and B) Cell viability was assessed using a CCK-8 assay (A), and apoptosis was assessed using the apoptotic double-staining assay (B). The data show means \pm SD (n = 3). *p < 0.05, **p < 0.01, compared with control. n.s., not significant.

miR-21-5p Antagomir Intervention

The miR-21-5p antagomir (RiboBio Guangzhou, P.R. China) was used to antagonize the miR-21-5p expression specifically. Cells were treated with 120 nM miR-21-5p antagomir for 24 h to achieve sufficient miR-21-5p inhibition (verified by quantitative real-time RT-PCR as described below). Scrambled RNA was used as a negative control. Cells were changed with fresh medium supplemented with 120 nM miR-21-5p antagomir and cultured for 24 h. The medium was collected for exosome enrichment. The miR-21-5p expression was analyzed by a Hairpin-it miRNA qPCR quantitation kit.

Stable shRNA Transfection

Lentiviral vector (LV)-shRNA vectors targeting human S100A6 were purchased from Shanghai GenePharma (Shanghai, P.R. China) and were used for stable shRNA transfection. For lentiviral infection, MDA-MB-231 cells and MCF-7 cells were plated on six-well tissue culture plates at a density of 2×10^5 cells and transfected the next day with lentiviral particles in the presence of 5 μ g/mL Polybrene (Sigma). The transfected clones were selected using 6 μ g/mL puromycin (Invitrogen) and were then harvested via trypsinization within cloning rings. A scrambled non-specific shRNA was transfected in parallel with the S100A6 shRNA as control experiments. S100A6 expression was then verified via immunofluorescence and western blot analyses.

Transfection of S100A6-Targeting siRNA

Three siRNAs for silencing human S100A6 (si-S100A6) and a negative control siRNA (si-NC) were obtained from RiboBio (Guangzhou, P.R. China). The sequences of the human S100A6-specific siRNA were 5'-GCGAAUGUGCGUUGUGUAATT-3' (siRNA-1), 5'-GUGGCCAUCUCCACAAGUTT-3' (siRNA-2), and 5'-CCUCUCU GAGUCAAUCCATT-3' (siRNA-3); the sequence 5'-UUC UCC GAA CGU GUC ACG UTT-3' served as the negative control (scrambled non-specific siRNA). Cells were transfected with si-S100A6 or si-NC using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen).

Quantitative Real-Time RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). The concentration and purity of RNA were determined by measuring the absorbance at 260 nm and the absorbance ratio of 260/280 nm in a Take3 micro-volume plate (BioTek, USA). Subsequently, quantitative real-time RT-PCR was performed using SYBR Green master mix (Invitrogen) on the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA). The method to quantify mRNA and miRNA was performed as described previously.⁵² Expression of mRNA was normalized to endogenous GAPDH mRNA levels.

Western Blot

Exosomes were directly used for protein analysis. The protein concentration of cells and exosomes was determined using a protein assay kit (Bio-Rad), and samples were separated on SDS polyacrylamide gels for western blot analysis. Anti-S100A6 (ab181975) and anti-active caspase-3 (ab2302) antibodies were purchased from Abcam (Cambridge, UK), and anti- β -tubulin antibody was purchased from Sigma-Aldrich. β -tubulin was used as an internal loading control.

Cell Viability/Cytotoxicity Assay

Cell viability was assessed by CCK-8 assays (Dojindo Laboratories, Japan) according to the manufacturer's recommendations. Cytotoxicity was assessed by LDH leakage (Dojindo Laboratories, Japan) according to the recommendations of the manufacturer.

Apoptosis Assay

An annexin V/propidium iodide (PI) apoptosis detection kit (BD Biosciences) was used to evaluate apoptosis according to the manufacturer's instructions. MDA-MB-231 cells and MCF-7 cells cultured with MSC-exo (50 μ g/mL) and Dt-MSC-exo (50 μ g/mL) for 60 h were harvested. Cells were incubated with 5 μ L of annexin V-fluorescein isothiocyanate (FITC) for 15 min. Subsequently, PI staining was performed. Cell apoptosis was detected by flow cytometry (LSR II, BD Biosciences).

Statistical Analysis

Statistical analysis was performed by GraphPad Prism (v5.01; GraphPad, La Jolla, CA, USA). All experimental data *in vitro* were obtained from at least three independent experiments and expressed as the mean \pm standard deviation. A Student's t test was used to compare

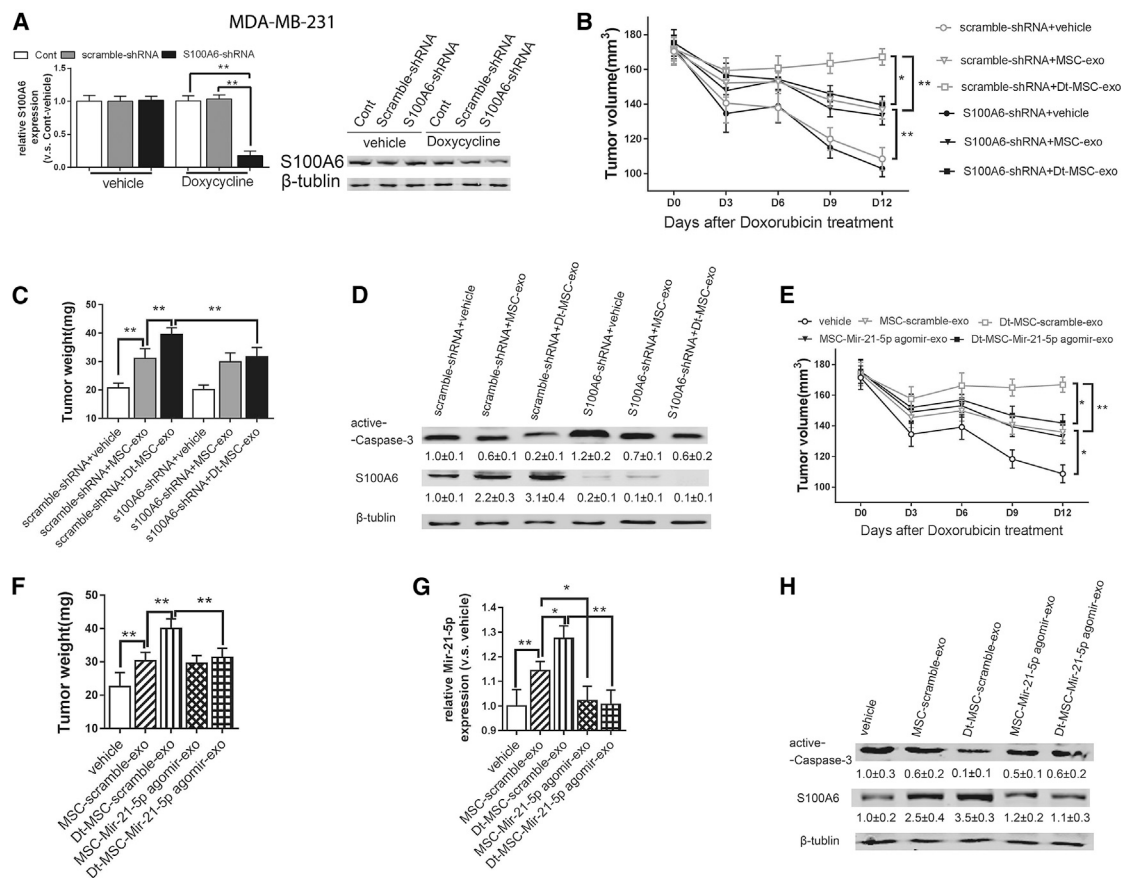


Figure 8. In Vivo Antitumor Activity of MSC-exo and Dt-MSC-exo

(A) MDA-MB-231 cells were stably transfected with Dox-conditioned S100A6-shRNA or its control scramble-shRNA, and the mRNA and protein expression levels of S100A6 in the control group and the Dox-induced group were measured. (B–D) Downregulation of S100A6 in BC cells: tumor volumes (B) and weight (C) of the xenografts in each group are shown. The measurements were initiated at day 12 post-injection. (D) Protein expression of S100A6 and active-caspase 3. (E–H) Downregulation of miR-21-5p content in MSC-exo: tumor volume (E), weight (F), and expression levels of miR-21-5p are shown. (H) Protein expression of S100A6 and active-caspase 3. Data are expressed as the mean \pm SD of the relative values from three independent experiments. * $p < 0.05$, ** $p < 0.01$.

experimental and relative control groups. Multiple comparisons were performed using one-way analysis of variance followed by the Tukey post hoc test. $p < 0.05$ was considered to indicate a statistically significant difference.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omto.2020.10.008>.

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AUTHOR CONTRIBUTIONS

Y.Liu and Q.Y. designed the total project. T.L., Q.L., Y.J., L.N., and J.T. performed the *in vitro* experiments. A.T., L.D., W.Z., and W.X. performed the *in vivo* experiments. Y.Liu supervised the study and wrote the manuscript. Y.Lu and Q.Y. revised the paper. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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