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ORIGINAL RESEARCH

RETRACTED ARTICLE: Mst1-Hippo pathway triggers breast cancer apoptosis via inducing mitochondrial fragmentation in a manner dependent on JNK–Drp1 axis

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Background and objective: Mst1-Hippo pathway and mitochondrial fragmentation participate in the progression of several types of cancers. However, their roles in breast cancer requires investigation. The aim of our study is to determine whether Mst1 overexpression regulates the viability of breast cancer cells via modulating mitochondrial fragmentation.

Materials and methods: TUNEL staining, MTT assay and Western blotting were used to detect cancer cell death. Adenovirus-loaded Mst1 was transfected into cells to overexpress Mst1. Mitochondrial fragmentation was observed via immunofluorescence staining and Western blotting. Pathway blocker was used to detect whether Mst1 modulated cell death and mitochondrial fragmentation via JNK signaling pathway.

Results: Our data showed that Mst1 overexpression promoted breast cancer cell death in a manner dependent on mitochondrial apoptosis. Mitochondrial oxidative stress, energy metabolism disorder, mitochondrial cytochrome *c* translocation and mitochondrial apoptosis activation were observed after Mst1 overexpression. Furthermore, we demonstrated that Mst1 overexpression activated mitochondrial stress via triggering Drp1-related mitochondrial fragmentation, and that inhibition of Drp1-related mitochondrial fragmentation abrogated the proapoptotic effect of Mst1 overexpression on breast cancer cells. To this end, we found that Mst1 modulated Drp1 expression via the JNK signaling pathway, and that blockade of the JNK pathway attenuated mitochondrial stress and repressed apoptosis in Mst1-overexpressed cells.

Conclusion: Altogether, our results identified a tumor suppressive role for Mst1 overexpression in breast cancer via activation of the JNK–Drp1 axis and subsequent initiation of fatal mitochondrial fragmentation. Given these findings, strategies to enhance Mst1 activity and elevate the JNK–Drp1–mitochondrial fragmentation cascade have clinical benefits for patients with breast cancer.

Keywords: breast cancer, mitochondrial fragmentation, Mst1, JNK–Drp1 signaling pathway, mitochondrial dysfunction

Introduction

Breast cancer is the most commonly diagnosed cancer among women. The symptoms of breast cancer include swelling of all or part of the breast, skin irritation or dimpling, breast pain, redness, scaliness or thickening of the nipple or breast skin.[1] Although advances have been made in the diagnosis and treatment of breast cancer over the last decade, the morbidity and mortality in young patients (<40 years old) have significantly increased.[2] Moreover, tumorigenesis of the breast has not been fully elucidated. Accordingly, achieving a better understanding of the molecular mechanism underlying the development of breast cancer is essential for the design of effective treatment approaches.

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Mammalian sterile 20–like kinase-1 (Mst1), also known as STK4, KRS2, belongs to the Hippo signaling pathway.[3] Ample evidence has identified Mst1 as an activator of caspases to initiate the apoptotic signaling pathway.[4] The anticancer effects of Mst1 have also been reported in several tumors such as colorectal cancer, prostate cancer, lung cancer and liver cancer.[5] Notably, previous studies have found that Mst1 is increased in HER2-positive and triple-negative breast cancer patients treated with neoadjuvant therapy. However, the molecular mechanism by which Mst1 affects the progression of breast cancer has not been explored.[6] At the molecular level, increased Mst1 induces mitochondrial oxidative stress and mitochondrial metabolism impairment in lung cancer.[7] Moreover, higher Mst1 expression has been associated with mitophagy arrest in cardiomyocytes.[8] Increased Mst1 induces pancreatic cancer death in a manner dependent on mitochondrial cyclophilin D activity.[9] These data confirm the tumor-suppressive property of Mst1 activation on cancer survival, metabolism and invasion.[10] Interestingly, no study has explored the detailed role of Mst1 in tongue cancer.

Recently, mitochondrial fragmentation has been connected to cancer apoptosis via multiple mechanisms.[11] Several mitochondrial pathological processes are closely handled by mitochondrial fragmentation, including mitochondrial redox balance,[12] mitochondrial autophagy,[13] mitochondrial calcium management,[14] mitochondrial energy synthesis[15] and mitochondrial proapoptotic factor liberation.[16] Abnormal mitochondrial fragmentation generates massive mitochondrial debris with lower mitochondrial potential that cannot produce ATP to ensure cancer metabolism.[17] Moreover, due to mitochondrial membrane damage, fragmented mitochondria release proapoptotic factors and/or calcium into the cytoplasm/nucleus and activate the mitochondria-dependent apoptotic pathway.[18] Mechanistically, mitochondrial fragmentation is primarily regulated by mitochondrial fission factors, such as Fis1 and its ligand Drp1.[19] Interestingly, Drp1-related mitochondrial fragmentation has been associated with breast cancer apoptosis through a poorly understood mechanism. In other cancer types, Drp1-related mitochondrial fragmentation is also associated with cancer mitochondrial damage and cell apoptosis.[20] However, whether Drp1-related mitochondrial fragmentation modifies breast cancer apoptosis via regulating mitochondrial function is unknown. Therefore, the aim of our study is to explore the roles of Mst1 and Drp1-related mitochondrial fragmentation in breast cancer apoptosis with a particular focus on mitochondrial stress.

Materials and methods

Cell culture and treatment

The breast cancer cell line (MDA-MB-231 [ATCC® CRM-HTB26D™]) was used in the present study. These cells were cultured in L-DMEM (Thermo Fisher Scientific, Waltham, MA, USA) with 10% FBS (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) under 37°C/5% CO_2. After the cells reached 70%–80% confluence, adenovirus-loaded Mst1 (Ad-Mst1) was transfected into the cells. To inhibit JNK activation, SP600125 (25 μM; Selleck Chemicals, Houston, TX, USA) was added to the medium four hours.

TUNEL staining and MTT assay

Cell death was measured via a TUNEL assay using the In Situ Cell Death Detection Kit (Hoffmann-La Roche Ltd., Basel, Switzerland). The TUNEL kit stains nuclei containing fragmented DNA. After treatment, the cells were fixed with 3.7% paraformaldehyde for 30 minutes at room temperature. Subsequently, an equilibration buffer, nucleotide mix and rTdT enzyme were incubated with the samples at 37°C for 60 minutes. Then, a saline-sodium citrate buffer was used to stop the reaction. After loading with DAPI, the samples were visualized via fluorescence microscopy (BX-61; Olympus Corporation, Tokyo, Japan). In addition, the MTT assay was performed to analyze cell viability according to the methods described in a previous study.[21] The absorbance at 570 nm was determined. The relative cell viability was recorded as a ratio to that of the control group. The experiments were performed in triplicate and repeated three times with similar results.

Western blotting

Proteins (40–60 μg) were loaded for immunodetection. The samples were resolved by 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (85 V for 60 minutes). Then, the membranes were blocked with 5% nonfat dried milk in Tris-buffered saline, followed by incubation with the primary antibodies overnight at 4°C. Next, the membranes were incubated with a secondary antibody for 45 minutes at room temperature.[22] The membranes were washed with Tris Buffered Saline with Tween 20 (TBST) at least three times.[23] Then, the immunoblots were detected using an enhanced chemiluminescence substrate (Applygen Technologies, Inc. Beijing, China). The primary antibodies used in the present study were as follows: Bcl2 (1:1,000; Cell Signaling Technology, Danvers, MA, USA, #3498), Bax (1:1,000; Cell Signaling Technology, #2772), caspase-9 (1:1,000; Cell Signaling Technology, #9504), pro-caspase-3 (1:1,000; Abcam, Cambridge, MA, USA, #ab13847), cleaved

caspase-3 (1:1,000; Abcam, #ab49822), t-JNK (1:1,000; Cell Signaling Technology, #4672), p-JNK (1:1,000; Cell Signaling Technology, #9251), Drp1 (1:1,000; Abcam, #ab56788), Fis1 (1:1,000; Abcam, #ab71498), Mff (1:1,000; Cell Signaling Technology, #86668), PARP (1:1,000; Abcam, #ab32064) and Mst1 (1:1,000; Cell Signaling Technology, #3682). The experiments were performed in triplicate and repeated three times with similar results.

Immunofluorescent staining

The cells were washed twice with PBS, permeabilized in 0.1% Triton X-100, neutralized with NH_4Cl buffer and then permeabilized using 0.05% saponin/PBS (pH 7.4) for 45 minutes. Subsequently, the samples were incubated overnight with the following primary antibodies: cyt-*c* (1:500; Abcam, #ab90529), Drp1 (1:1,000; Abcam, #ab56788) and Tom-20 (1:1,000; Abcam, #ab186735). Confocal immunofluorescence images were collected using the FV10-ASW 1.7 software and an Olympus IX81 microscope.[24] The fluorescence intensity was calculated using the Image-Pro Plus 6.0 software. First, fluorescence pictures were converted to grayscale with the Image-Pro Plus 6.0 software. Then, the fluorescence intensities were separately recorded as the grayscale intensities. Mitochondria were observed in at least 100 cells, and the average length of the mitochondria was measured under an inverted microscope to quantify mitochondrial fragmentation (BX51; Olympus Corporation, Tokyo, Japan) as described in a previous study.[25] The experiments were performed in triplicate and repeated three times with similar results.[26]

Mitochondrial potential observation

The Mitochondrial Membrane Potential Detection Kit (JC-1; Beyotime Institute of Biotechnology, Haimen, China) was used to observe changes in the mitochondrial potential. Briefly, 5 mg/L of JC-1 working solution was added to the medium and incubated for 20 minutes at 37°C with CO_2. Subsequently, the cells were washed with PBS to remove the JC-1 probe, and then images were taken by fluorescence microscopy (Olympus BX-61). The ratio of red to green fluorescence was analyzed using Image-Pro Plus version 4.5 (Media Cybernetics, Inc., Rockville, MD, USA).[27]

Caspase activity detection and LDH release assay

Caspase-3 and caspase-9 activities were determined using commercial kits (Beyotime Institute of Biotechnology). The LDH release assay was used to observe cell death according to the manufacturer's guidelines.[28] The relative LDH release was recorded as the ratio to that of the control group. The experiments were performed in triplicate and repeated three times with similar results.[29]

Flow cytometry for mitochondrial ROS

Flow cytometry was used to analyze mitochondrial ROS (mROS) production. After treatment, the cells were washed three times with PBS and then resuspended in PBS using 0.25% trypsin. Subsequently, the cells were incubated with the MitoSOX red mitochondrial superoxide indicator (Molecular Probes, Eugene, OR, USA) for 15 minutes at 37°C in the dark.[30] After three washes with PBS, mROS production was analyzed via flow cytometry (Sysmex Partec GmbH, Görlitz, Germany), and the data were analyzed using the Flowmax software (version 2.3; Sysmex Partec GmbH). The experiments were performed in triplicate and repeated three times with similar results.

Transfection

The pcDNA3.1315-Mst1 vector was designed and purchased from Genechem Biosciences, Inc. (Rockville, MD, USA). Then, the plasmid was transfected into 293 T cells using Lipofectamine 2000. After 48 hours, the supernatant was collected to obtain Ad-Mst1. Subsequently, MDA-MB-231 cells were infected with Ad-Mst1 for 6 hours at 37°C with 5% CO_2. Western blotting was performed to verify the overexpression efficiency.

Statistical analyses

All results presented in this study were obtained from at least three independent experiments. The statistical analyses were performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). All results in the present study were analyzed by one-way ANOVA, followed by Tukey's test. $P<0.05$ was considered statistically significant.

Results

Mst1 overexpression promotes apoptosis in breast cancer cells

To investigate the role of Mst1 in breast cancer apoptosis, Ad-Mst1 was transfected into the MDA-MB-231 cells. The overexpression efficiency was confirmed via Western blotting (Figure 1A and B). Then, cell viability was measured using the MTT assay.[32] Compared to that of the control group, Ad-Mst1 transfection significantly reduced the cell viability in MDA-MB-231 cells (Figure 1C). To evaluate whether the reduction in cell viability was attributable to increased apoptosis, TUNEL staining was performed. As shown in Figure 1D and E, compared to that of the control

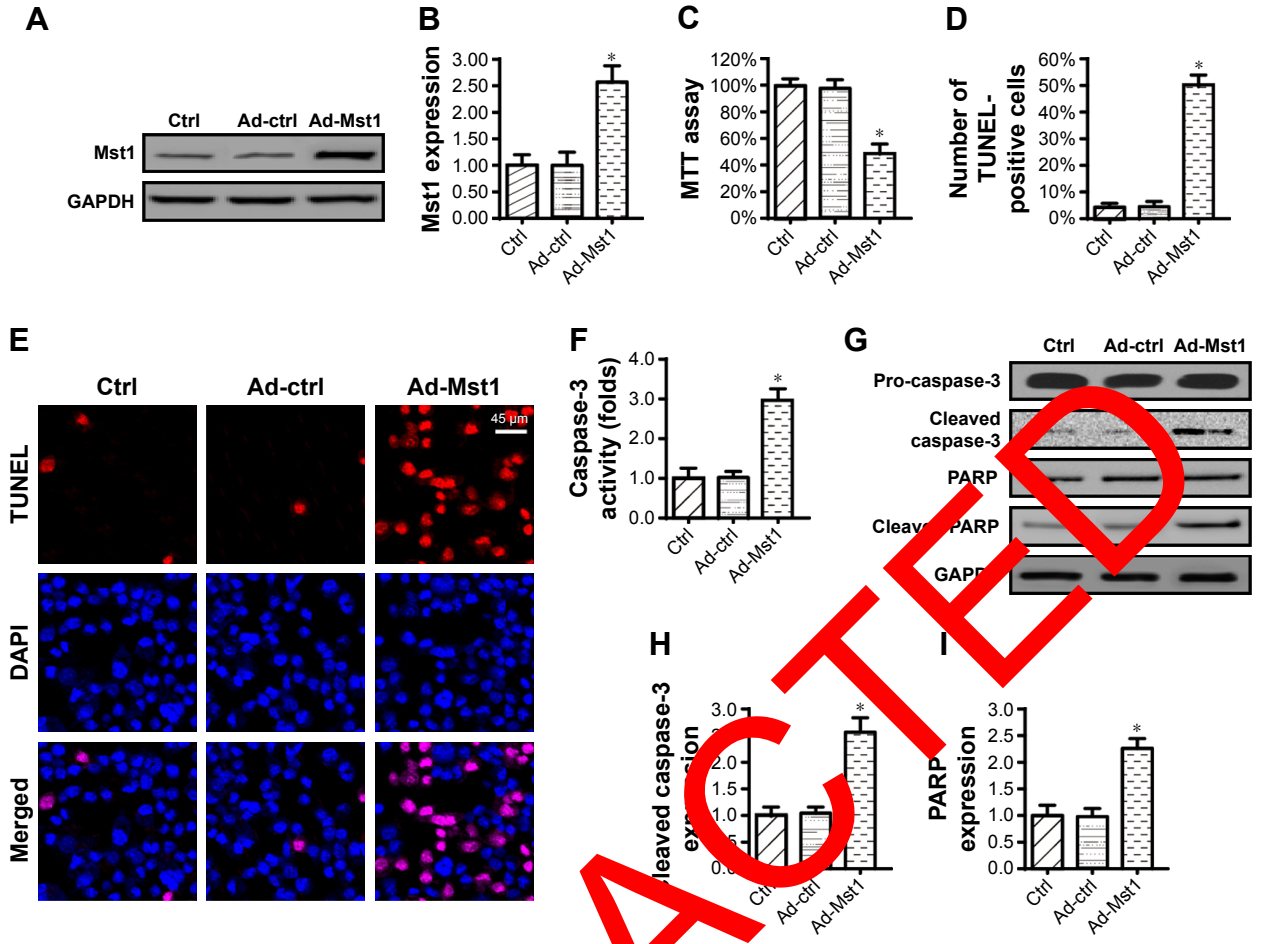

Figure 1 Mst1 overexpression induces apoptosis in breast cancer cells in vitro.
Notes: (**A**, **B**). Ad-Mst1 was transfected into MDA-MB-231 breast cancer cells. Western blotting was used to observe the overexpression efficiency. (**C**) The MTT assay was used for cell viability detection. Ad-Mst1 was transfected into MDA-MB-231 breast cancer cells. (**D**, **E**) TUNEL staining for apoptotic cells. Red dots were recorded, and the ratio of TUNEL-positive cells was evaluated to reflect cell apoptosis. (**F**) Caspase-3 activity was measured via ELISA. Ad-Mst1 was transfected into MDA-MB-231 breast cancer cells. (**G–I**) Western blotting was performed to analyze the expression of proapoptotic proteins, such as cleaved caspase-3 and PARP. *$P<0.05$ vs Ad-ctrl group.
Abbreviation: Ad-Mst1, adenovirus-loaded Mst1.

group, overexpression of Mst1 elevated the ratio of TUNEL-positive cells, suggesting that Mst1 overexpression promoted cell apoptosis in MDA-MB-231 cells. This result was further confirmed by evaluating caspase-3 activity. As shown in Figure 1F, Mst1 overexpression increased caspase-3 activity in MDA-MB-231 cells. In addition to caspase-3 activation, cleaved caspase-3 and PARP (the substrate of caspase-3 activation) expression was obviously upregulated in response to Ad-Mst1 transfection (Figure 1G–I). Altogether, these data indicated that Mst1 overexpression promoted apoptosis in breast cancer cells.

Mst1 enhancement activates mitochondrial apoptosis

Subsequently, we explored the mechanism by which Mst1 overexpression induced apoptosis in breast cancer cells. Previous studies have suggested that Mst1 is the upstream mediator of mitochondrial homeostasis.[16,33] Based on this information, we investigated whether Mst1 overexpression activated mitochondria-dependent apoptosis in breast cancer. The early feature of mitochondrial apoptosis is a reduction in the mitochondrial membrane potential that reflects mitochondrial functions, such as ATP production and oxidative stress.[34] Using JC-1 staining, which is a probe of the mitochondrial membrane potential, we found that Mst1 overexpression reduced the mitochondrial potential compared to that of the control group (Figure 2A and B). In light of the central role of the mitochondrial potential in ATP production,[35] we measured total ATP production in the Mst1-overexpressed cells. Compared to that of the control group, Mst1 overexpression significantly alleviated ATP production in MDA-MB-231 cells (Figure 2C). We also found that mitochondrial ROS production was significantly increased in response to Mst1 overexpression

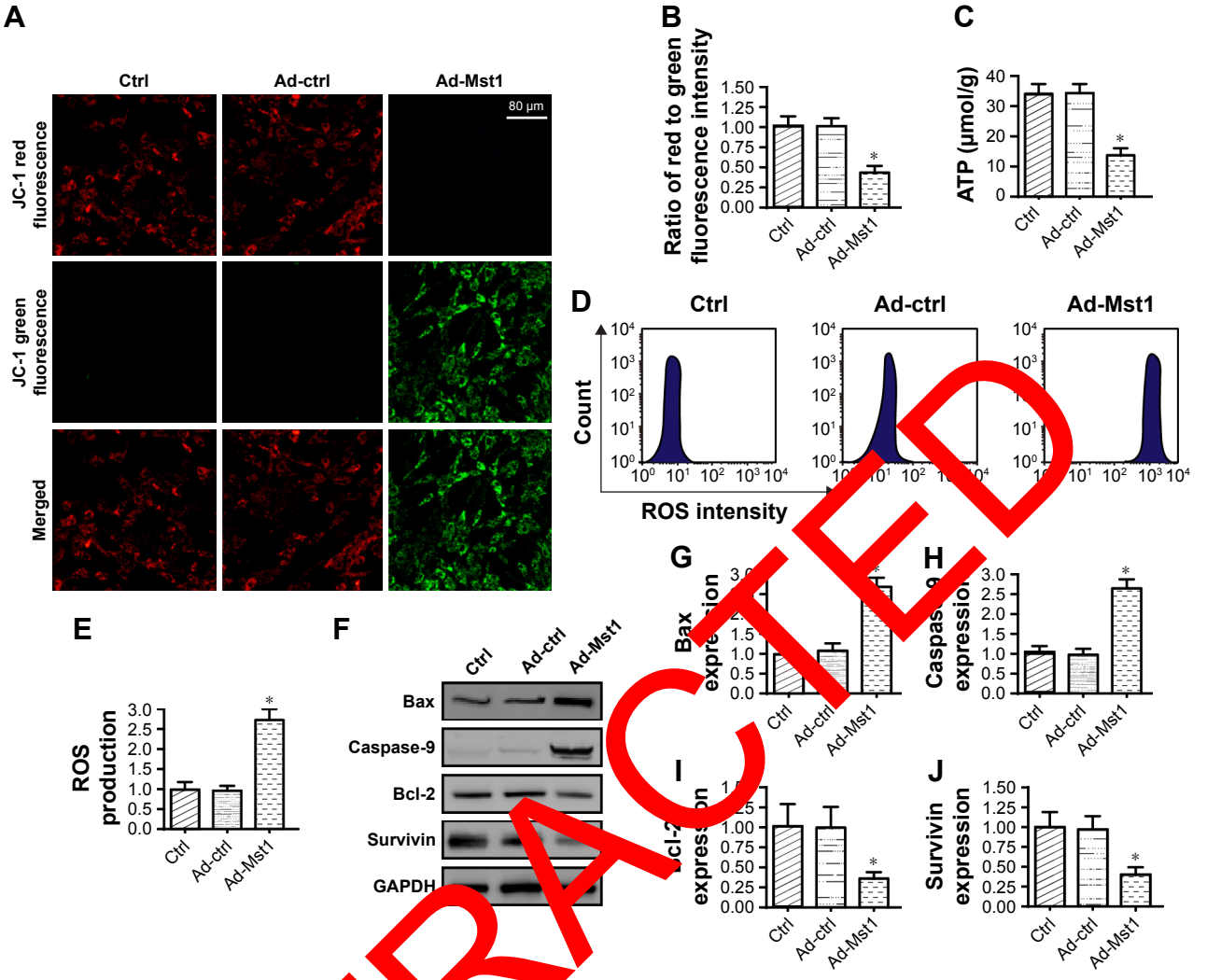

Figure 2 Mitochondrial apoptosis is activated [illegible] Mst1 overe[illegible]ression.
Notes: (**A**, **B**) Mitochondrial membrane p[illegible]ial was observe[illegible]ing JC-1 staining. (**C**) ATP production was measured. Ad-Mst1 was transfected into MDA-MB-231 breast cancer cells. (**D**, **E**) Mitochondrial ROS [illegible]oduc[illegible] was analyzed [illegible]g flow cytometry. The relative ROS content was recorded as a ratio to that of the control group. (**F–J**) Western blotting was performed to analyze [illegible] expression of mitochondrial apoptotic proteins. Ad-Mst1 was transfected into MDA-MB-231 breast cancer cells. *$P<0.05$ vs Ad-ctrl group.
Abbreviation: Ad-Mst1, ade[illegible]rus-loaded Mst1.

as assessed [illegible] flow c[illegible]ometry (Figure 2D and E), indicating th[illegible] Mst1 overexp[illegible]ion induced mitochondrial oxidati[illegible] injur[illegible].

The la[illegible]ature of mitochondrial apoptosis is the activation of mito[illegible]ondrial apoptotic proteins.[36] With Western blotting, we found that the proapoptotic factors related to mitochondrial apoptosis, such as caspase-9 and Bax, were significantly upregulated in the Mst1-overexpressed cells (Figure 2F–J). Conversely, the antiapoptotic proteins related to mitochondrial apoptosis, such as Bcl-2 and survivin, were obviously downregulated in response to Mst1 overexpression (Figure 2F–J). Altogether, our data illustrated that Mst1 overexpression was accompanied by activation of mitochondrial apoptosis in tongue cancer.

Mst1 triggers Drp1-related mitochondrial fragmentation

Next, experiments were conducted to analyze the mechanism by which Mst1 overexpression activated mitochondrial apoptosis. Previous studies have found a causal role of mitochondrial fragmentation in initiating the caspase-9–related mitochondrial apoptosis pathways in several cancer types.[37] Based on this information, we examined whether Mst1 overexpression induced mitochondrial apoptosis in a manner dependent on mitochondrial fragmentation. To answer this question, the mitochondrial morphology was observed in response to Mst1 overexpression in MDA-MB-231 cells. As shown in Figure 3A and B, compared to that of the control group, Mst1 overexpression generated significant massive

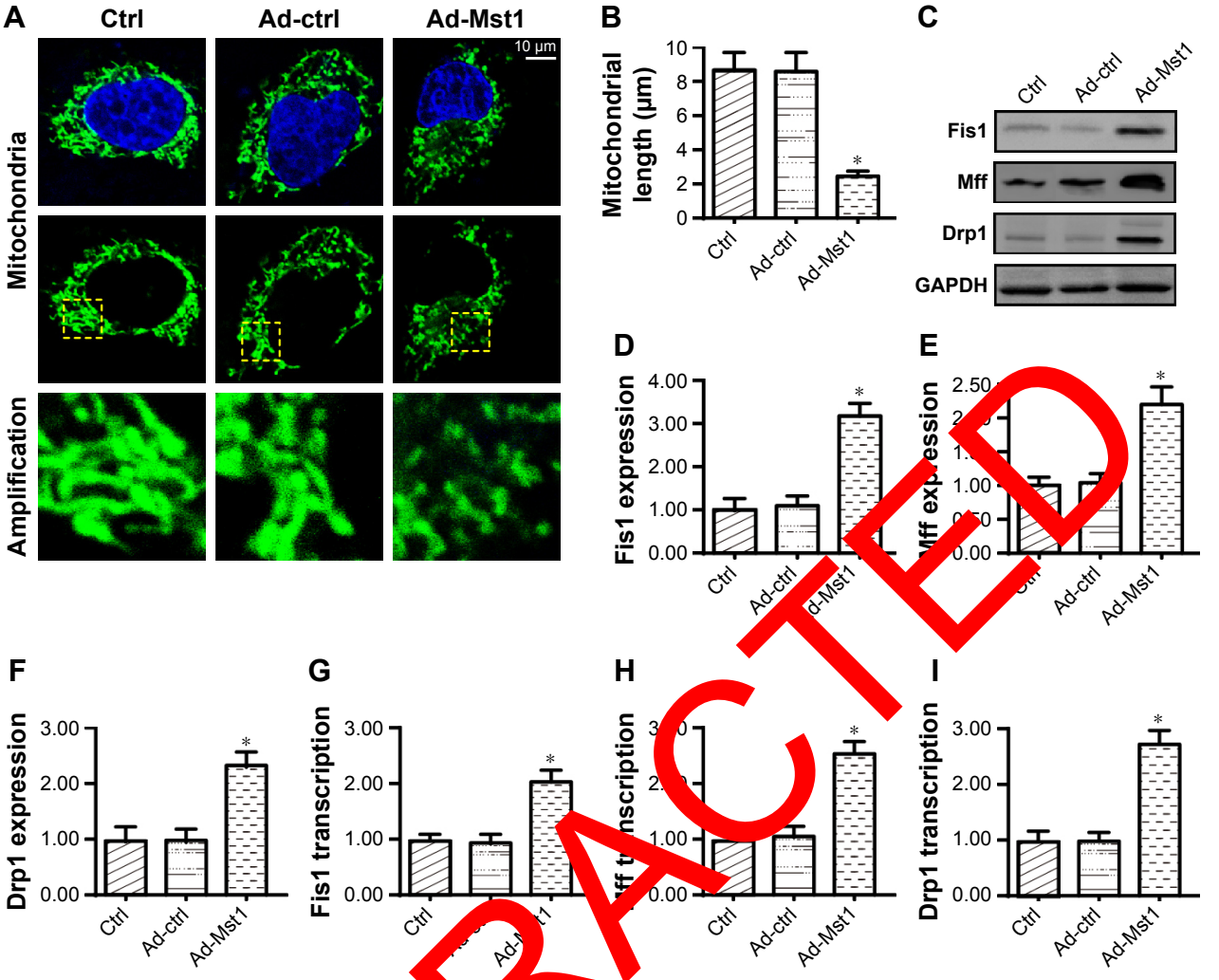

Figure 3 Mst1 overexpression activates mitochondrial fragmentation in a manner dependent on Drp1.
Notes: (**A**, **B**) Immunofluorescence for mitochondria using a mitochondrial-specific antibody Tom-20. The average length of the mitochondria was measured, and this parameter was used to quantify mitochondrial fragmentation. (**C–F**) Western blotting was applied to evaluate the expression of pro-fission proteins, including Drp1, Mff and Fis1. Relative Drp1 expression was recorded in response to Mst1 overexpression. (**G–I**) qPCR assay was used to evaluate the transcription of pro-fission factors. *$P<0.05$ vs Ad-ctrl group.
Abbreviations: Ad-Mst1, adenovirus-mediated Mst1; qPCR, quantitative PCR.

fragmentation of the mitochondria. Subsequently, the mitochondrial length was measured to quantify mitochondrial fragmentation according to the method described in previous reports.[32] As shown in Figure 3A and B, compared to that in the control group, the length of the mitochondria decreased to ~2.3 μm after Mst1 was overexpressed, reconfirming activation of mitochondrial fragmentation under Mst1 overexpression.

Based on previous findings, Drp1 is an indispensable factor that regulates mitochondrial division.[38] In the present study, using the Western blotting assay, we found that Drp1 expression was significantly increased in response to Mst1 overexpression in MDA-MB-231 cells (Figure 3C–F). As a consequence of Drp1 upregulation, the expression of pro-fission proteins, such as Drp1 and Mff, was mostly increased in the Mst1-overexpressed cells compared to those in the control group (Figure 3C–F). Similar results were obtained using quantitative PCR assay (Figure 3G–I). This information highlighted the promotive effects of Mst1 overexpression on mitochondrial fragmentation.

Inhibition of Drp1-related mitochondrial fragmentation reduces mitochondrial stress and promotes cell survival in breast cancer cells

The above data validated the regulatory effects of Mst1 on Drp1-related mitochondrial fragmentation. However, whether Drp1-related mitochondrial fragmentation is

required for the mitochondrial damage and cell apoptosis induced by Mst1 overexpression is unknown. To answer this question, Drp1 was deleted using siRNA. The knockdown efficiency was confirmed via Western blotting (Figure 4A and B). Then, mitochondrial stress was determined by analyzing ROS production and cyt-*c* liberation. The feature of mitochondrial stress is cyt-*c* liberation into the cytoplasm/nucleus due to mitochondrial membrane damage.[39] Once translocated from the mitochondria into the nucleus,[40] cyt-*c* interacts with caspase-9 and activates caspase-9–related mitochondrial apoptosis. Using the Western blotting assay, we found that cytoplasmic cyt-*c* (cyto cyt-*c*) expression was increased in response to Mst1 overexpression in MDA-MB-231 cells (Figure 4C and D), confirming that Mst1 overexpression

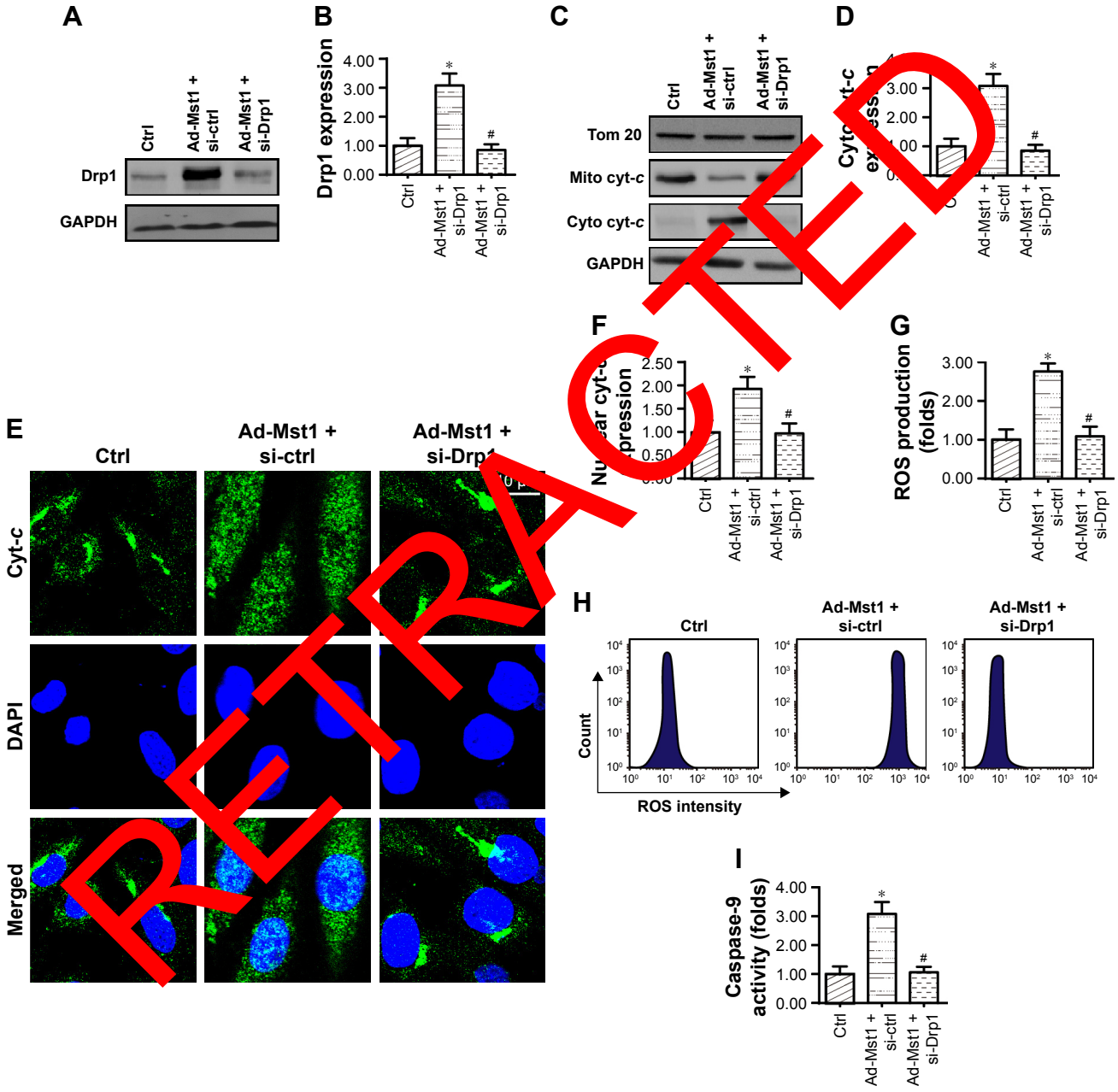

Figure 4 Loss of Drp1-related mitochondrial fragmentation attenuates mitochondrial damage and cell apoptosis.
Notes: (**A**, **B**) Drp1 siRNA was transfected into MDA-MB-231 breast cancer cells. Western blotting was used to analyze the knockdown efficiency. (**C**, **D**) Western blotting was used to detect cyt-*c* translocation from mitochondria into the cytoplasm. (**E**, **F**) Immunofluorescence for cyt-*c* liberation. Relative nuclear cyt-*c* expression was recorded as a ratio to that of the control group. Drp1 siRNA was transfected into MDA-MB-231 breast cancer cells. (**G**, **H**) Flow cytometry was performed to analyze the mitochondrial ROS levels in response to Drp1 deletion. (**I**) Caspase-9 activity was measured using ELISA. *$P<0.05$ vs Ad-ctrl group; #$P<0.05$ vs Ad-Mst1 + si-Drp1 group.
Abbreviation: Ad-Mst1, adenovirus-loaded Mst1.

contributed to cyt-*c* migration to the cytoplasm. This result was further supported by immunofluorescence assay (Figure 4E and F). However, Mst1 overexpression-mediated cyt-*c* liberation was negated by Drp1 silencing. Subsequently, mitochondrial ROS was analyzed by flow cytometry. As shown in Figure 4G and H, compared with that of the control group, Mst1 overexpression promoted ROS overloading, and this effect was abolished by the Drp1 siRNA. This information verified the necessary role played by Drp1-related mitochondrial fragmentation in aggravating Mst1-mediated mitochondrial stress.

To assess cell apoptosis, a caspase-9 activity assay was performed. As shown in Figure 4I, compared to that of the control group, caspase-9 activity was significantly increased in response to Mst1 overexpression. However, loss of Drp1 abrogated the proapoptotic property of Mst1 overexpression in breast cancer cells. Altogether, these results supported the functional importance of Drp1-related mitochondrial fragmentation in triggering Mst1-related mitochondrial stress and cancer cell apoptosis.

Mst1 increases Drp1 via activating the MAPK–JNK signaling pathway

To this end, we investigated the molecular mechanisms by which Mst1 modulated Drp1 in breast cancer cells. According to the previous study, the MAPK–JNK signaling pathway was involved in mitochondrial fragmentation management via modifying the expression of profission factors, such as Mff.[41] In the present study, we evaluated whether Mst1 affected Drp1 expression via the MAPK–JNK pathway. Western blotting demonstrated that phosphorylated JNK (p-JNK) was significantly increased in response to Mst1 overexpression, which was indicative of JNK activation under Mst1 overexpression (Figure 5A–C). Subsequently, SP600125, which is a JNK pathway blocker, was added to the Mst1-overexpressed cells. Then, the p-JNK and Fis1 expression levels were observed. As shown in Figure 5A–C, after blockade of the JNK pathway, p-JNK expression was obviously downregulated and was accompanied by a decrease in Drp1 expression. This result was further verified by immunofluorescence. Compared to that of the control group, abundant Drp1 expression was observed in the Mst1-overexpressed group (Figure 5D–F). However, the Drp1 levels were rapidly downregulated in response to SP600125 (Figure 5D–F), reconfirming that the MAPK–JNK signaling pathway was involved in Drp1 expression.

The MAPK–JNK signaling pathway also affects mitochondrial function and cell survival

Lastly, we explored whether the MAPK–JNK signaling pathway participated in mitochondrial stress and cancer cell apoptosis. To address this question, a pathway blocker was used. As shown in Figure 6A, compared to that of the control group, Mst1 overexpression elevated caspase-9 activity, and this effect was nullified by SP600125. Total ATP production was also repressed by Mst1 overexpression, and this effect was reversed by SP600125 (Figure 6B). These results indicated that inhibition of the JNK pathway alleviated Mst1-mediated mitochondrial stress. To assess cell survival, the MTT and LDH release assays were performed. As shown in Figure 6C, compared to that of the control group, cell viability as assessed by MTT was significantly reduced in response to Mst1 overexpression and was reversed to near-normal level by SP600125 treatment. Moreover, cell death as evaluated by the LDH release assay was enhanced by Mst1. However, SP600125 treatment attenuated Mst1-overexpressed cell death (Figure 6D). Similarly, the number of TUNEL-positive cells was significantly increased in the Mst1-overexpressed cells, and this effect was abolished by SP600125 (Figure 6E and F). Altogether, these results illustrated that the MAPK–JNK pathway was involved in Mst1-related mitochondrial stress and cell apoptosis in breast cancer cells.

Discussion

In the present study, our results identified the antitumor effects and mechanisms of Mst1 overexpression in breast cancer. MDA-MB-231 breast cancer cell line was used in the present study, and adenovirus targeting Mst1 was transfected into MDA-MB-231 cells. Using an array of functional investigations, we demonstrated that Mst1 overexpression promoted cell apoptosis via inducing mitochondrial stress. Our results further highlighted the necessary role of Drp1-related mitochondrial fragmentation in mediating mitochondrial stress, because inhibition of Drp1-related mitochondrial fragmentation sustained mitochondrial function and repressed cancer cell apoptosis. To this end, the molecular analysis illustrated that Mst1 modulated Drp1 via the MAPK–JNK pathway, which was also involved in Mst1-mediated mitochondrial stress and cancer death. Collectively, this study is the first to explore the actions of Mst1 and Drp1-related mitochondrial fragmentation in breast cancer cell viability. Based on our results, the Mst1–JNK–Drp1 signaling pathway

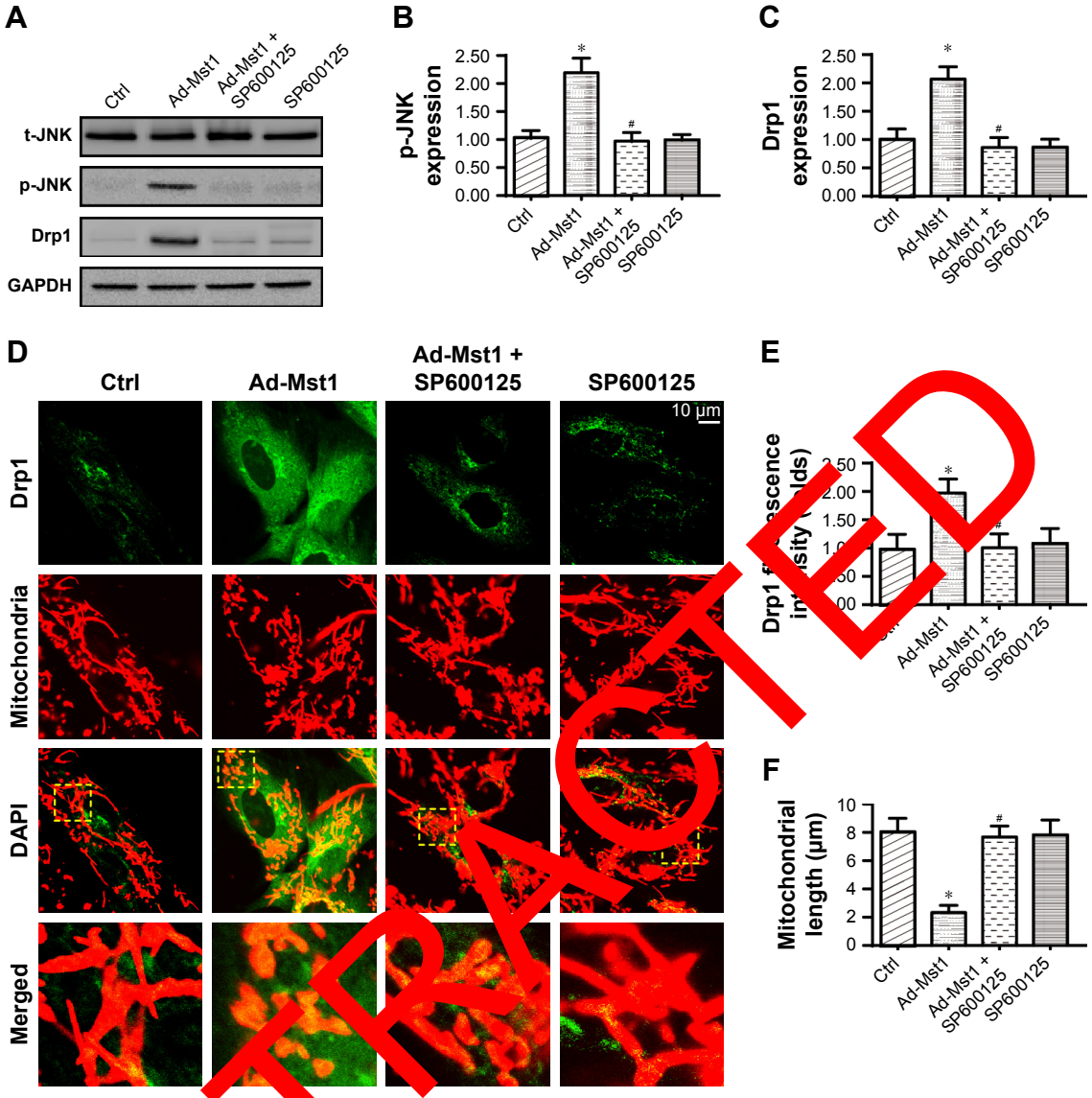

Figure 5 Mst1 regulates Drp1 via the JNK signaling pathway.
Notes: (**A**–**C**) Western blotting was used to analyze p-JNK and Drp1 expression. The JNK blocker SP600125 was used to prevent JNK activation in Mst1-overexpressed cells. (**D**–**F**) Immunofluorescence assay for Drp1 and mitochondria. The relative fluorescence intensity of Drp1 was measured. *$P<0.05$ vs Ad-ctrl group; #$P<0.05$ vs Ad-Mst1 group.
Abbreviation: Ad-Mst1, adenovirus-loaded Mst1.

should be considered as a potential target to treat breast cancer via inducing cell apoptosis and mitochondrial stress. Notably, more investigations in animal research and clinical practice are required to obtain more complete elucidation of the properties of Mst1-modified Drp1 mitochondrial fragmentation in breast cancer death.

Strong evidence supports the proapoptotic role of mitochondrial fragmentation in initiating the mitochondrial apoptosis pathway in various cancer types. For example, in lung cancer,[42] ovarian cancer,[43] breast cancer, liver cancer,[44] pancreatic cancer[45] and colorectal cancer,[46] mitochondrial fragmentation has been well recognized as a major proapoptotic factor that acts via regulation of mitochondrial homeostasis,[47] endoplasmic reticulum stress and the inflammatory response. Mechanistically, excessive mitochondrial fragmentation produces massive mitochondrial debris that contains a lower mitochondrial membrane potential and fragmentary DNA.[48] These damaged mitochondria cannot generate sufficient ATP to fuel cell metabolism, but instead liberate proapoptotic factors into the cytoplasm/nucleus to initiate the mitochondria-related apoptosis pathway.[18,49] For example, in endothelial oxidative injury, mitochondrial

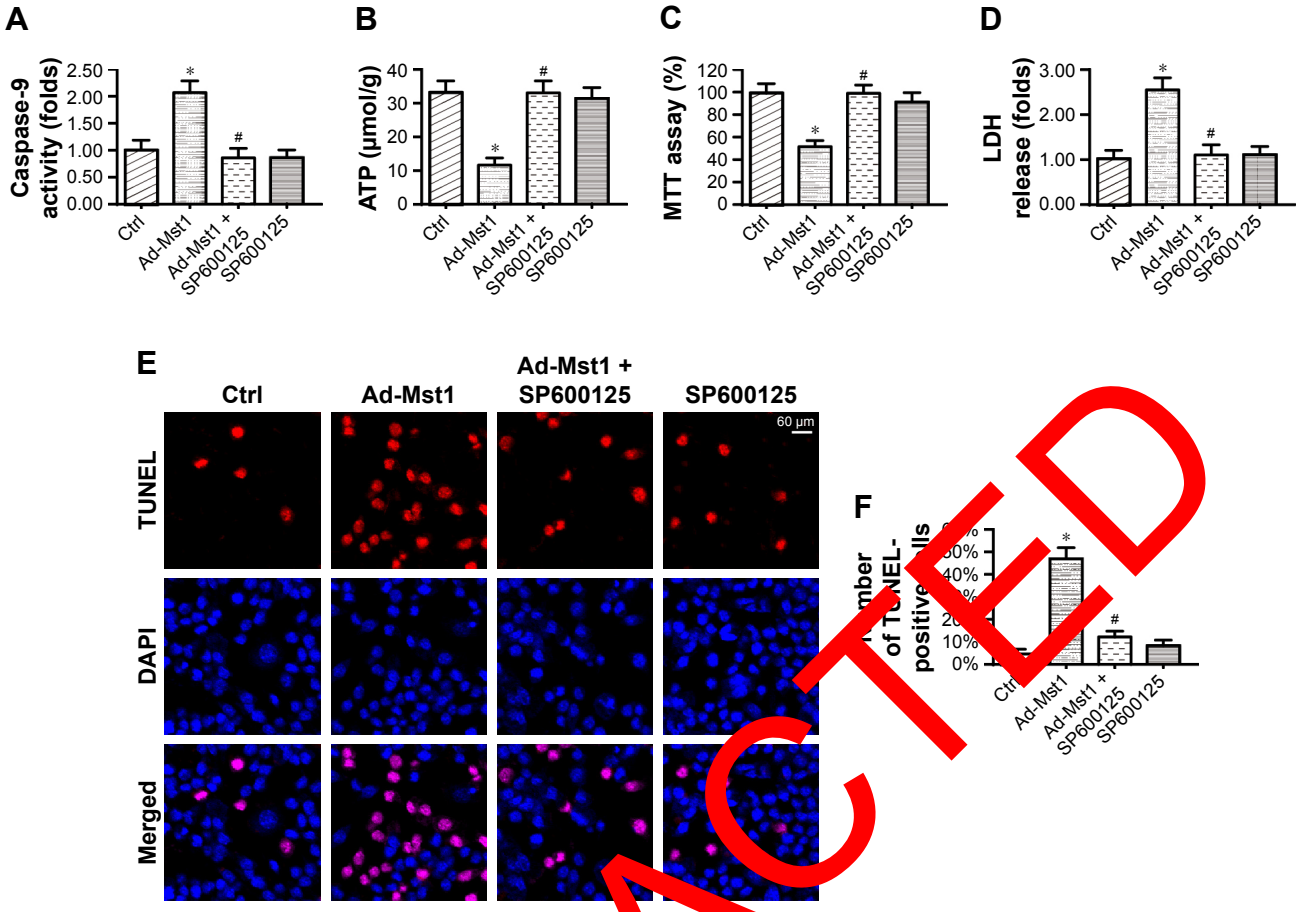

Figure 6 The JNK signaling pathway participates in mitochondrial stress and cell ap[illegible]tos[illegible]
Notes: (**A**) Caspase-9 activity was measured by ELISA. SP600125 w[illegible]o inhibit [illegible] activation. (**B**) ATP production was determined in response to JNK inhibition using SP600125. (**C**) Cell viability was measured by the MTT assay, a[illegible]SP600125 was used [illegible] inhibit JNK activation. (**D**) The LDH release assay was performed to analyze cell death with ELISA. (**E**, **F**) TUNEL staining for apoptotic cells [illegible] the numb[illegible] of apoptot[illegible]ells were recorded in response to SP600125. *$P<0.05$ vs Ad-ctrl group; #$P<0.05$ vs Ad-Mst1 group.
Abbreviation: Ad-Mst1, adenovirus-loaded Mst1.

fragmentation impairs mitochondrial [illegible]NA transcription and replication, and consequently represses mitochondrial respiratory complex expression,[36] leading to ATP depletion. Additionally, in cardiac [illegible]chemia/reperfusion injury,[50] abnormal mitochondrial fragmentation interrupts mitophagy[51] and promotes mitochondrial [illegible]ium overloading,[52] which obligates cardiomyocytes to undergo death.[53] In rectal cancer,[16] aberrant mitochondrial fragmentation mediates cellular oxidative stress,[48] which blunts cancer migration. In the present study, our results indicated that mitochondrial fragmentation was associated with mitochondrial apoptosis and mitochondrial ROS overproduction; inhibition of mitochondrial fragmentation sustained mitochondrial metabolism and attenuated mitochondrial damage, finally promoting cancer cell survival. Therefore, our results combined with those of previous studies lay the foundation for understanding the molecular features of mitochondrial fragmentation in mitochondrial damage and substantiate the sufficiency and necessity of mitochondrial fragmentation in inducing cancer death.[54]

In the present study, we provided a piece of evidence to support the regulatory effects of Drp1 in inducing mitochondrial fragmentation in breast cancer. This finding was similar to those of previous studies. For example, IL-2–induced oxidative injury and cell apoptosis in liver cancer was associated with Drp1 activation and subsequent mitochondrial fragmentation.[55] Moreover, Drp1-related mitochondrial fragmentation was involved in the apoptosis and migration of human rectal cancer in a manner dependent on HtrA2/Omi signaling pathway.[16] In bladder cancer cells, activation of Drp1-related mitochondrial fragmentation impairs cancer invasion and attenuates tumor chemoresistance.[56] Importantly, a recent study[48] has reported that the sensitivity of breast cancer to IR-783 (anticancer drug) is closely regulated by Drp1-related mitochondrial fragmentation. In the present study, we observed a direct role of Drp1-related

mitochondrial fragmentation in initiating mitochondrial apoptosis in breast cells. This finding helps further understanding of the causal action of Drp1 in breast cancer cell viability.[57] From a therapeutic perspective, clinicians should bear in mind that activation of Drp1-related mitochondrial fragmentation is of utmost importance when designing antitumor therapies for breast cancer cells.

Herein, we reported that Mst1 modulated Drp1-related mitochondrial fragmentation via the MAPK–JNK pathway. The relationship between the JNK pathway and Drp1-related mitochondrial fragmentation has been extensively explored. In acute myocardial ischemia reperfusion injury and chronic heart fibrosis, activated JNK promotes mitochondrial fragmentation and cardiomyocyte death. Similarly, in liver cancer, rectal cancer, gastric cancer,[49] endometriosis[58] and cervical cancer,[59] the JNK pathway has been identified as the upstream factor for mitochondrial fragmentation activation. In agreement with previous studies, we also found that inhibition of the JNK pathway repressed Drp1 expression and attenuated mitochondrial fragmentation. These results define the JNK pathway as a tumor suppressor that acts by triggering mitochondrial fragmentation with potential implications for new approaches to breast cancer therapy.

The primary limitation in the present study is t[illegible] only used one type of cell line to explore the influen[illegible] of Mst1 on breast cancer cell viability. Additiona[illegible]xperim[illegible] using more cell lines and/or human sa[illegible]les ar[illegible] require[illegible] to provide more evidence to support t[illegible]action [illegible]Mst1 on breast cancer death.

Conclusion

Altogether, the present s[illegible]dy identified t[illegible] Mst1–JNK–Drp1-mitochondrial fragm[illegible]ntation axis as a potential candidate target for new therap[illegible]s aga[illegible]st breast cancer. Overexpression of Mst1 activ[illegible]d the [illegible]K pathway and then upregulated Drp1 exp[illegible]ssion. [illegible]creased [illegible]1 expression triggered mitochondr[illegible] fragm[illegible]tation, which exacerbated mitochondrial damage, u[illegible]ately initiating the mitochondria-dependent apoptosis pat[illegible]ay in breast cancer. Although our present study presents a new signaling pathway responsible for breast cancer cell death, additional investigations using animal studies or human samples are needed to validate our concept and help transform basic research findings into clinical application.

Data sharing statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Bikfalvi A. History and conceptual developments in vascular biology and angiogenesis research: a personal view. *Angiogenesis*. 2017;20(4):463–478.
2. Blackburn NJR, Vulesevic B, M[illegible]ll B, et al. M[illegible]ylglyoxal-derived advanced glycation end products [illegible]ntribute to nega[illegible]e cardiac remodeling and dysfunction pos[illegible]yocard[illegible] infarction. [illegible]sic Res Cardiol*. 2017;112(5):57.
3. Abeysuriya RG, Lo[illegible]ey SW, R[illegible]binson [illegible]ostnova S. A unified model of melatonin, [illegible]ulfato[illegible]elatonin, and sleep dynamics. *J Pineal Res*. 2018;64([illegible]e124[illegible]
4. Abdulmah[illegible]W, Patel D[illegible]abadi M[illegible], et al. HMGB1 redox during sepsis. [illegible]*Biol*. 2017;13:[illegible]7.
5. Al M[illegible]nun Bi[illegible]n A, Lang F. Stimulation of eryptosis by afatinib. *Cell Physiol Bioch[illegible]*. 2018;47(3):1259–1273.
6. [illegible]ez-Sánchez N, [illegible]z-Chamorro I, Díaz-Sánchez M, et al. Melatonin reduces inflammatory response in peripheral T helper lymphocytes from relap[illegible]g–remitting multiple sclerosis patients. *J Pineal Res*. 2017;63(4):[illegible]2442.
7. Zhang W, L[illegible]K, Pei Y, Ma J, Tan J, Zhao J. MST1 regulates non-small [illegible]ncer A549 cell apoptosis by inducing mitochondrial damage via ROCK1/F-actin pathways. *Int J Oncol*. 2018;53(6):2409–2422.
8. [illegible]gelova PR, Barilani M, Lovejoy C, et al. Mitochondrial dysfunction in parkinsonian mesenchymal stem cells impairs differentiation. *Redox Biol*. 2018;14:474–484.
9. Chen SH, Li DL, Yang F, Wu Z, Zhao YY, Jiang Y. Gemcitabine-induced pancreatic cancer cell death is associated with MST1/cyclophilin D mitochondrial complexation. *Biochimie*. 2014;103:71–79.
10. Buijs N, Oosterink JE, Jessup M, et al. A new key player in VEGF-dependent angiogenesis in human hepatocellular carcinoma: dimethylarginine dimethylaminohydrolase 1. *Angiogenesis*. 2017;20(4):557–565.
11. Zhou H, Wang S, Hu S, Chen Y, Ren J. ER–mitochondria microdomains in cardiac ischemia–reperfusion injury: a fresh perspective. *Front Physiol*. 2018;9:755.
12. Zhou H, Shi C, Hu S, Zhu H, Ren J, Chen Y. BI1 is associated with microvascular protection in cardiac ischemia reperfusion injury via repressing Syk-Nox2-Drp1-mitochondrial fission pathways. *Angiogenesis*. 2018;21(3):599–615.
13. Jin Q, Li R, Hu N, et al. DUSP1 alleviates cardiac ischemia/reperfusion injury by suppressing the Mff-required mitochondrial fission and Bnip3-related mitophagy via the JNK pathways. *Redox Biol*. 2018;14:576–587.
14. Zhu H, Jin Q, Li Y, et al. Melatonin protected cardiac microvascular endothelial cells against oxidative stress injury via suppression of IP3R-[Ca^{2+}]c/VDAC-[Ca^{2+}]m axis by activation of MAPK/ERK signaling pathway. *Cell Stress Chaperones*. 2018;23(1):101–113.
15. Yan H, Qiu C, Sun W, et al. YAP regulates gastric cancer survival and migration via SIRT1/Mfn2/mitophagy. *Oncol Rep*. 2018;39(4):1671–1681.
16. Li H, He F, Zhao X, et al. YAP inhibits the apoptosis and migration of human rectal cancer cells via suppression of JNK–Drp1–mitochondrial fission–HtrA2/Omi pathways. *Cell Physiol Biochem*. 2017;44(5):2073–2089.
17. Zhou H, Wang J, Hu S, Zhu H, Toanc S, Ren J. BI1 alleviates cardiac microvascular ischemia–reperfusion injury via modifying mitochondrial fission and inhibiting XO/ROS/F-actin pathways. *J Cell Physiol*. 2018;234(8):5056–5069.

18. Zhao Q, Ye M, Yang W, et al. Effect of MST1 on endometriosis apoptosis and migration: role of Drp1-related mitochondrial fission and Parkin-required mitophagy. *Cell Physiol Biochem*. 2018;45(3):1172–1190.
19. Losón OC, Song Z, Chen H, Chan DC. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. *Mol Biol Cell*. 2013;24(5):659–667.
20. Antoniou C, Chatzimichail G, Xenofontos R, et al. Melatonin systemically ameliorates drought stress-induced damage in *Medicago sativa* plants by modulating nitro-oxidative homeostasis and proline metabolism. *J Pineal Res*. 2017;62(4):e12401.
21. Gadicherla AK, Wang N, Bulic M, et al. Mitochondrial Cx43 hemichannels contribute to mitochondrial calcium entry and cell death in the heart. *Basic Res Cardiol*. 2017;112(3):27.
22. Ackermann M, Kim YO, Wagner WL, et al. Effects of nintedanib on the microvascular architecture in a lung fibrosis model. *Angiogenesis*. 2017;20(3):359–372.
23. Erland LAE, Yasunaga A, Li ITS, Murch SJ, Saxena PK. Direct visualization of location and uptake of applied melatonin and serotonin in living tissues and their redistribution in plants in response to thermal stress. *J Pineal Res*. 2019;66(1):e12527.
24. Areti A, Komirishetty P, Akuthota M, Malik RA, Kumar A. Melatonin prevents mitochondrial dysfunction and promotes neuroprotection by inducing autophagy during oxaliplatin-evoked peripheral neuropathy. *J Pineal Res*. 2017;62(3):e12393.
25. Zhou H, Zhu P, Wang J, Zhu H, Ren J, Chen Y. Pathogenesis of cardiac ischemia reperfusion injury is associated with CK2α-disturbed mitochondrial homeostasis via suppression of FUNDC1-related mitophagy. *Cell Death Differ*. 2018;25(6):1080–1093.
26. Cobley JN, Close GL, Bailey DM, Davison GW. Exercise redox biochemistry: conceptual, methodological and technical recommendations. *Redox Biol*. 2017;12:540–548.
27. Fernández Vázquez G, Reiter RJ, Agil A. Melatonin increases brown adipose tissue mass and function in Zücker diabetic fatty rats: implications for obesity control. *J Pineal Res*. 2018;64(4):e12472.
28. Biernacki M, Ambrożewicz E, Gęgotek A, Toczek M, Bielawska K, Skrzydlewska E. Redox system and phospholipid metabolism in the kidney of hypertensive rats after FAAH inhibitor URB597 administration. *Redox Biol*. 2018;15:41–50.
29. Li S, Xie R, Jiang C, Liu M. Schizandrin a alleviates LPS-induced [illegible] in human keratinocyte cell HaCaT through a microRNA-[illegible]-dependent regulation. *Cell Physiol Biochem*. 2018;4[illegible]:2229–22[illegible].
30. Brazão V, Colato RP, Santello FH, et al. Effects of melatonin on thymic and oxidative stress dysfunctions during *Trypanosoma cruzi* infection. *J Pineal Res*. 2018;65(3):e12510.
31. Ba X, Boldogh I. 8-Oxoguanine DNA glycosylase 1: beyond repair of the oxidatively modified base lesions. *Redox Biol*. 2018;14:669–678.
32. Cai SY, Zhang Y, Xu YP, [illegible] HsfA1a up-regulates melatonin biosynthesis to confer cadmium tolerance in tomato plants. *J Pineal Res*. 2017;62(2):e12387.
33. Zhou H, Yu Y, Wang [illegible] Ma Q, Chen Y. Melatonin therapy for diabetic cardiomyopathy: a mechanism involving Syk-mitochondrial complex I-SERCA pathway. *Cell Signal*. 2018;47:88–100.
34. Fuhrmann DC, Brüne B. Mitochondrial composition and function under the control of hypoxia. *Redox Biol*. 2017;12:208–215.
35. Zhou H, Du W, Li Y, et al. Effects of melatonin on fatty liver disease: the role of NR4A1/DNA-PKcs/p53 pathway, mitochondrial fission, and mitophagy. *J Pineal Res*. 2018;64(1):e12450.
36. Zhou H, Hu S, Jin Q, et al. Mff-dependent mitochondrial fission contributes to the pathogenesis of cardiac microvasculature ischemia/reperfusion injury via induction of mROS-mediated cardiolipin oxidation and HK2/VDAC1 disassociation-involved mPTP opening. *J Am Heart Assoc*. 2017;6(3):pii:e005328.
37. Cao Z, Fang Y, Lu Y, et al. Melatonin alleviates cadmium-induced liver injury by inhibiting the TXNIP–NLRP3 inflammasome. *J Pineal Res*. 2017;62(3):e12389.
38. Camaré C, Pucelle M, Nègre-Salvayre A, Salvayre R. Angiogenesis in the atherosclerotic plaque. *Redox Biol*. 2017;12:18–34.
39. Zhou H, Li D, Zhu P, et al. Melatonin suppresses platelet activation and function against cardiac ischemia/reperfusion injury via PPARγ/FUNDC1/mitophagy pathways. *J Pineal Res*. 2017;63(4):e12438.
40. Li R, Xin T, Li D, Wang C, Zhu H, Zhou H. Therapeutic effect of sirtuin 3 on ameliorating nonalcoholic fatty liver disease: the role of the ERK-CREB pathway and Bnip3-mediated mitophagy. *Redox Biol*. 2018;18:229–243.
41. Zhou H, Zhu P, Guo J, et al. Ripk3 induces mitochondrial apoptosis via inhibition of FUNDC1 mitophagy in cardiac IR injury. *Redox Biol*. 2017;13:498–507.
42. Zhou H, Li D, Zhu P, et al. Inhibitory effect of melatonin on necroptosis via repressing the Ripk3-PGAM5-CypD-mPTP pathway attenuates cardiac microvascular ischemia–reperfusion injury. *J Pineal Res*. 2018;65(3):e12503.
43. Chen LY, Renn TY, Liao WC, et al. Melatonin successfully rescues hippocampal bioenergetics and improves cognitive function following drug intoxication by promoting Nrf2-ARE signaling activity. *J Pineal Res*. 2017;63(2):e12417.
44. Cao J, Wei R, Yao S. Matrine has pro-apoptotic effects on liver cancer by triggering mitochondrial fission and activating Mst1-JNK signalling pathways. *J Physiol Sci*. Epub 2018 Aug 28.
45. Cameron AR, Logie L, Patel K, et al. Metformin selectively targets redox control of complex I energy transduction. *Redox Biol*. 2018;14:187–197.
46. Choi GH, Lee HY, Back K. Chloroplast overexpression of rice caffeic acid *O*-methyltransferase increases melatonin production in chloroplasts via the 5-methoxytryptamine pathway in transgenic rice plants. *J Pineal Res*. 2017;63(1):e12412.
47. Carloni S, Riparini G, Buonocore G, Balduini W. Rapid modulation of the silent information regulator 1 by melatonin after hypoxia–ischemia in the neonatal rat brain. *J Pineal Res*. 2017;63(3):e12434.
48. Chang [illegible] Salante-Alcalde D, Bhuiyan MS, et al. Cardiac-specific inactivation of LPP3 in mice leads to myocardial dysfunction and heart failure. *Redox Biol*. 2018;14:261–271.
49. Yan H, Xiao F, Zou J, et al. NR4A1-induced increase in the sensitivity of a human gastric cancer line to TNFα-mediated apoptosis is associated with the inhibition of JNK/Parkin-dependent mitophagy. *Int J Oncol*. 2018;52(2):367–378.
50. Zhou H, Wang J, Zhu P, et al. NR4A1 aggravates the cardiac microvascular ischemia reperfusion injury through suppressing FUNDC1-mediated mitophagy and promoting Mff-required mitochondrial fission by CK2α. *Basic Res Cardiol*. 2018;113(4):23.
51. Zhou H, Zhang Y, Hu S, et al. Melatonin protects cardiac microvasculature against ischemia/reperfusion injury via suppression of mitochondrial fission-VDAC1-HK2-mPTP-mitophagy axis. *J Pineal Res*. 2017;63(1):e12413.
52. Zhou H, Ma Q, Zhu P, Ren J, Reiter RJ, Chen Y. Protective role of melatonin in cardiac ischemia–reperfusion injury: from pathogenesis to targeted therapy. *J Pineal Res*. 2018;64(3):e12471.
53. Choi SI, Lee E, Akuzum B, et al. Melatonin reduces endoplasmic reticulum stress and corneal dystrophy-associated TGFBIp through activation of endoplasmic reticulum-associated protein degradation. *J Pineal Res*. 2017;63(3):e12426.
54. Domínguez Rubio AP, Correa F, Aisemberg J, et al. Maternal administration of melatonin exerts short- and long-term neuroprotective effects on the offspring from lipopolysaccharide-treated mice. *J Pineal Res*. 2017;63(4):e12439.
55. Chen T, Dai SH, Li X, et al. Sirt1–Sirt3 axis regulates human blood–brain barrier permeability in response to ischemia. *Redox Biol*. 2018;14:229–236.
56. Zhang Y, Zhou H, Wu W, et al. Liraglutide protects cardiac microvascular endothelial cells against hypoxia/reoxygenation injury through the suppression of the SR-Ca(2+)-XO-ROS axis via activation of the GLP-1R/PI3K/Akt/survivin pathways. *Free Radic Biol Med*. 2016;95:278–292.

57. Feng D, Wang B, Wang L, et al. Pre-ischemia melatonin treatment alleviated acute neuronal injury after ischemic stroke by inhibiting endoplasmic reticulum stress-dependent autophagy via PERK and IRE1 signalings. *J Pineal Res*. 2017;62(3):e12395.
58. Wang H, Zhao X, Ni C, Dai Y, Guo Y. Zearalenone regulates endometrial stromal cell apoptosis and migration via the promotion of mitochondrial fission by activation of the JNK/Drp1 pathway. *Mol Med Rep*. 2018;17(6):7797–7806.
59. Chen L, Liu L, Li Y, Gao J. Melatonin increases human cervical cancer HeLa cells apoptosis induced by cisplatin via inhibition of JNK/Parkin/mitophagy axis. *In Vitro Cell Dev Biol Anim*. 2018;54(1):1–10.