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**Original Paper**

# Effect of Mst1 on Endometriosis Apoptosis and Migration: Role of Drp1-Related Mitochondrial Fission and Parkin-Required Mitophagy

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**Key Words**
Mitochondrial fission • Mitophagy • Migration • Apoptosis • ESCs • Mst1

**Abstract**
***Background/Aims:*** Mitochondrial homeostasis is implicated in the development and progression of endometriosis through poorly defined mechanisms. Mst1 is the major growth suppressor related to cancer migration, apoptosis and proliferation. However, whether Mst1 is involved in endometriosis apoptosis and migration via regulating the mitochondrial function remains to be elucidated. ***Methods:*** Expression of Mst1 in endometriosis was examined via western blots. Cellular apoptosis was detected via MTT and TUNEL assay. Gain of function assay about Mst1 was conducted via adenovirus over-expression. Mitochondrial functions were evaluated via mitochondrial membrane potential JC-1 staining, ROS flow cytometry analysis, mPTP opening assessment and immunofluorescence of HtrA2/Omi. The mitophagy activity were examined via western blots and immunofluorescence. ***Results:*** First, we found that Mst1 was significantly downregulated in the ectopic endometrium of endometriosis compared to the normal endometrium. However, the recovery of Mst1 function was closely associated with the inability of endometrial stromal cells (ESCs) to migrate and survive. A functional study indicated that regaining Mst1 enhanced Drp1 post-transcriptional phosphorylation at Ser616 and repressed Parkin transcription activity via p53, leading to mitochondrial fission activation and mitophagy inhibition. Excessive Drp1-related fission forced the mitochondria to liberate HtrA2/Omi into the cytoplasm. Moreover, Mst1-induced defective mitophagy evoked cellular

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oxidative stress, energy metabolism and calcium overload. Through excessive mitochondrial fission and aberrant mitophagy, Mst1 launched caspase 9-related mitochondrial apoptosis and abrogated F-actin/lamellipodium-dependent cellular migration. Notably, we also defined NR4A/miR181c as the upstream signal for Mst1 dysfunction in endometriosis. ***Conclusion:*** Collectively, our results comprehensively described the important role of the NR4A-miR181c-Mst1 pathway in endometriosis, which handled mitochondrial apoptosis and F-actin/lamellipodium-based migration via the regulation of Drp1-related mitochondrial fission and Parkin-required mitophagy, with a potential application in endometriosis therapy by limiting ESCs migration and promoting apoptosis.



## Introduction

Endometriosis is a chronic, painful, and debilitating disease in which endometrium-like glandular and stromal cells grow outside the uterine cavity. It is an inflammatory and estrogen-dependent disease that affects 6–10% of women during their reproductive years and up to 50% of women receiving fertility treatments [1, 2]. The endometrium is comprised of stromal cells and epithelial compartments. Notably, epithelial cells normally undergo anoikis, a mechanism of programmed cell death, upon detachment from the extracellular matrix [3]. Accordingly, the survival and migration of endometrial stromal cells (ESCs) outside the uterine cavity is the major cause for the development of endometriosis. Current therapeutic alternatives consist of various hormone treatments, such as GnRH-agonists (GnRH-a), aimed at increasing the apoptotic rate in eutopic and ectopic endometrial cells from women with endometriosis [4].

Mitochondria are vital for cell survival and energy metabolism. In addition, mitochondria are an important target of estrogen. The estrogen receptor-β (ERβ) localizes to the mitochondria in a ligand-dependent or ligand-independent manner. A reduction in mitochondrial function via decreasing circulating estrogen is the standard treatment for endometriosis [5]. These data indicate that mitochondria homeostasis could be considered a therapeutic target for the treatment of endometriosis. Notably, recent studies show that mitochondrial dynamics, especially Drp1-related mitochondrial fission, happen at the early stage of cellular apoptosis, such as in liver cancer, cardiomyocytes and neurocytes [6, 7]. Excessive mitochondrial fission initiates caspase 9-related mitochondrial apoptosis, leading to cell death [8, 9]. However, no evidence is available to establish the role of mitochondrial fission in the progression of endometriosis. Apart from mitochondrial fission, Parkin-required mitophagy is another housekeeper of mitochondrial homeostasis. A growing body of evidence suggests that cell migration undergoes a drastic metabolic reprogramming that requires the well-orchestrated mitophagy to remove the damaged mitochondria and sustain the mitochondrial quantity and quality [10, 11]. However, an imbalance of mitochondrial degradation processes results in alteration of cellular homeostasis, which, in turn, may limit the migration and survival of ESCs outside the uterine cavity. Based on this information, we hypothesized that mitochondrial fission and mitophagy might be involved in the development of endometriosis. If this is the case, then the molecular signaling that links fission and mitophagy to ESCs apoptosis and migration should be explored [12].

The Hippo pathway was first discovered through genetic screens in Drosophila, and many components of the Hippo pathway are highly conserved from Drosophila to mammals [13], including mammalian STE20-like kinase 1/2 (MST1/2), salvador homolog 1 (SAV1), large tumor suppressor 1/2 (LATS1/2), Yes-associated protein (YAP) and its paralog, transcriptional co-activator with PDZ-binding motif (TAZ), all of which form a kinase cascade [14]. As a transcriptional co-activator, YAP or TAZ could combine with the TEA domain family member 1 (TEAD1) in the nucleus to promote the expression of target genes. Recent studies reveal that the Hippo pathway, especially Mst1, is implicated in the regulation of tissue regeneration, adult stem cells proliferation and cancer development [15, 16]. However, the role of Mst1 in endometriosis remains unknown. Notably, careful studies from several

researchers indicate that the Hippo-pathway plays a role in mitochondrial function via Bcl-xL[17, 18], demonstrating the possible relationship between Mst1 and mitochondrial homeostasis. Furthermore, accumulating evidence describes the regulatory action of Mst1 on p53. Thus, together with the confirmed role of p53 in regulating mitochondrial fission and mitophagy [19], this evidence raises the possibility that Mst1 may govern fission and mitophagy via p53.

Given the important role of Mst1 in tissue regeneration, cancer development and mitochondrial homeostasis, the regulatory machinery of Mst1 activation has gained much attention. MiR-181c is identified as the transcriptional repressor of Mst1. The overexpression of miR-181c induces the inactivation of Mst1, leading to pancreatic cancer cell survival and migration. However, whether miR-181c is also involved Mst1 regulation in endometriosis is incompletely understood. Accordingly, this study was undertaken to explore the effect and mechanism of Mst1 in the etiology of endometriosis, particularly focusing on Drp1-related mitochondrial fission, Parkin-required mitophagy, the p53 pathway and the upstream regulatory signaling for Mst1.

## Materials and Methods

*Ethics statement*

The present study was conducted in accordance with the Declaration of Helsinki and the guidelines of the Ethics Chinese PLA general Hospital, Beijing, China. The all experimental protocol was approved by Ethics Committee of Chinese PLA general Hospital, Beijing, China.

*Cell culture*

The human endometrial stromal cells (ESCs) were purchased from the American Type Culture Collection (ATCC) Company (Manassas, VA, USA) and cultured in RPMI medium (Thermo Scientific, Rockford, IL) supplemented with 10% fetal bovine serum (FBS, HyClone, USA), 1% L-glutamine and 0.5% gentamycin (Sigma, Cat. No. G1272) at 37 °C in an atmosphere of 5% $CO_2$ [20]. To inhibit mitochondrial fission, Mdivi1 was used for 2 hours. To reduce or enhance the activity of miR-181c, the pharmacological blocker and mimic of miR-181c were used, respectively.

*Cell migration and scratch assay*

Cell migration was analyzed using a transwell chamber assay (24-wells, 8-μm pore size with polycarbonate membrane) as previously described [21]. For the scratch assay, cells were cultured in a serum-free medium for 24 h and then disrupted with pipette tips. Wound healing was observed for 48 h, and photographs were taken every 24 h [22].

*Western blot analysis*

For the analysis of the protein levels, cells were lysed with RIPA supplemented with PMSF. The protein concentration was analyzed using the BCA protein assay. The protein (50 μg) was separated by 10% SDS-PAGE and then transferred to PVDF [23]. The membranes were blocked with 5% nonfat milk for 1 h at room temperatures and then incubated with primary antibodies: GAPDH (1:2000), caspase3 (1:2000), XIAP (1:1000), caspase9 (1:1000), survivin (1:1000), Bax (1:1000), Bad (1:1000), Parkin (1:1000), p62 (1:1000) and LC3II (1:1000) from Cell Signaling Technology, U.S.A.; Beclin1 (1:1000), Atg5 (1:1000), p53 (1:1000), VDAC (1:1000) and F-actin (1:1000) from Abcam, USA) overnight at 4°C. The membranes were washed in TBST for 15 min and were then incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, U.S.A.) for 1 h at room temperature. Blots were detected through an enhanced chemiluminescence (ECL) substrate kit (Thermo USA) [24].

*Mitochondrial membrane potential (ΔΨm), ATP production and mPTP opening and TUNEL assay*

The mitochondrial transmembrane potential was analyzed using a JC-1 Kit (Beyotime, Shanghai, China) according to the manufacturer's protocol [25]. The cellular ATP levels were measured using a firefly luciferase-based ATP assay kit (Beyotime). The opening of the mPTP was visualized as a rapid dissipation of tetramethylrhodamine ethyl ester fluorescence as described in a previous study [26].

TUNEL assay was used to detect the apoptosis of ESCs, per the manufacturer's instructions [27]. TUNEL staining was performed with fluorescein-dUTP (Invitrogen, U.S.A.) for apoptotic cell nuclei, and DAPI was used to stain all cell nuclei. Cells in which the nucleus was stained with fluorescein-dUTP were defined as TUNEL positive. The slides were then measured under a confocal microscope [28].

*Determination of caspase3 activity and MTT assay*

Caspase3 activity kit (Beyotime Institute of Biotechnology, China) was used to detect the activity of caspase3 according to the manufacturer's protocol. The assay was repeated 3 times. MTT assay was used to examine cell viability [29]. ESCs were seeded in a 96-well plate. Then, 20 μl MTT (5 mg/ml) was added to the medium for 4 h. Next, the supernatant was discarded, and 100 μl DMSO was added to each well for 10 min. Finally, the optical density (OD) was measured at A490 nm [30].

*Immunofluorescence*

After treatment, the cells in all groups were firstly washed under PBS for about three times, and then permeabilized for 15 min at with 0.1% Triton X-100 at the room temperature. Then, the cells were washed three times in PBS again [31]. Subsequently, the primary antibodies were used to label the cellular proteins overnight at 4°C. Then, cells were washed under PBS, which were loaded with secondary antibodies for about 1 h at room temperature [32]. The primary antibodies used in the present study were as follows: Tomm20 (1:500, Abcam, #ab78547), LAMP1 (1:200, Abcam, #ab24170), HtrA2/Omi (1:500, Abcam, #ab133504), p53 (Ser15) (1:500, Cell Signaling Technology, #9284), Mst1 (1:500, Abcam, #ab3336), Parkin (1:500, Abcam, #ab15954), F-actin (1:500, Abcam, #ab205), Tubulin (1:500, Abcam, #ab18207), LC3II (1:500, Abcam, #ab48394) and Drp1 (1:500, Abcam, #ab184247) [33].

*Isolation of mitochondrial-enriched fraction*

Cells were washed with cold PBS and incubated on ice in lysis buffer (Beyotime, China) for 30 minutes. The cells were subsequently scraped, and the homogenates were spun at 800 x g for 5 minutes at 4°C. The supernatants were centrifuged at 10, 000 x g for 20 min at 4°C to acquire the pellets, which were spun again [6, 24]. The final pellets were suspended in lysis buffer containing 1% Triton X-100 and were noted as mitochondrial-rich lysate fractions [34].

*Mitochondrial respiratory assays*

Mitochondrial respiration was initiated by adding glutamate/malate to a final concentration of 5 and 2.5 mmol/L, respectively. State 3 respiration was initiated by adding ADP (150 nmol/L); state 4 was measured as the rate of oxygen consumption after ADP phosphorylation. The respiratory control ratio (state 3/state 4) and the ADP/O ratio (number of nmol ADP phosphorylated to atoms of oxygen consumed) were calculated as previously described [35, 36].

*ROS, cytoplasmic calcium ($[Ca^{2+}]c$) and mitochondrial calcium ($[Ca^{2+}]m$) detection*

The contents of $[Ca^{2+}]c$ was imaged with Fluo-2 (Molecular Probes). Samples were then directly examined by confocal microscopy using the ×40 1.42 NA oil immersion objective. For quantification of the concentrations change of $[Ca^{2+}]c$, flow cytometry was used [37]. For analysis of $[Ca^{2+}]m$, Rhod-2 (Molecular Probes) was used and the images were captured by confocal microscopy. Fluorescence intensity of Furo-2 and Rhod-2 was measured by excitation wavelengths of 340 and 550 nm, and emission wavelengths of 500 and 570 nm, respectively. Data (F/F0) were obtained by dividing fluorescence intensity (F) by (F0) at resting level (t = 0) which was normalized by control groups [9, 19].

*RNA isolation and quantitative RT-PCR (qPCR)*

After treatment, cellular RNA was isolated via Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription of RNA was performed with Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Switzerland)[38]. About one μg of RNA was reversely transcribed into cDNA for each sample. Synthesized cDNA was amplified on the LightCycler® 480 System with SYBR Green I Master (Roche Applied Science, Mannheim, Germany)[39]. The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 72°C for 35 sec, for telomere PCR. The experiments were repeated three times with triplicates of each sample. Fold change of Mst1 and Parkin mRNA expression was normalized by GAPDH as an internal control [40].

*Mst1 and Parkin overexpression by adenovirus*

To carry out the gain-of function assay about Mst1 and Parkin, we overexpressed the Mst1 and Parkin in ESCs via adenovirus-based technology. Firstly, the pCDH-mCMV-Mst1 plasmid was purchased from Vigene Bioscience. Then, the plasmid was transfected into 293 T cells via lipofectamine 2000 for about 48-96 hours in an incubator with 5% $CO_2$ at 37°C [41]. Subsequently, collection of the supernatant to obtain the adenovirus which was identified via PCR. After amplification of the obtained Mst1-adenovirus (Ad-Mst1), the ESCs were transfected by Ad/Mst1. The transfection and overexpression efficiency were measured via western blots.

*The small RNA interference assay*

To evaluate the functional role of p53, Mst1 and HtrA2/Omi, siRNAs were used to reduce their expression. The selective siRNAs were purchased from Yangzhou Ruibo Biotech Co., Ltd. After treatment, cells were washed under Opti-Minimal Essential Medium without serum or antibiotics [42]. Then, the cells were cultured for about 1-2 days to 30-40% confluence. Then, the siRNA was transfected into cells via Lipofectamine 2000 transfection reagent according to the manufacturer's protocol [43]. After 48-96 hours, the cells were collected and the knockdown efficiency were determined via western blot analysis [44].

*Statistical analysis*

All results were expressed as mean±SD. We performed statistical analysis using one-way ANOVA test. A value of $P< 0.05$ was considered statistically significant.

## Results

*Mst1 is downregulated in ectopic endometrium and negatively correlates with ESCs survival and migration*

To investigate the functional relevance of Mst1 during endometriosis, we performed qPCR and western blots to detect the level of Mst1 in ectopic and normal endometrium from women with endometriosis. In this study, 8 cases of tissue samples were collected and detected using Mst1 primers or an anti-Mst1 antibody. The Mst1 mRNA and protein expressions were significantly lower in the ectopic endometrium compared to the normal endometrium (Fig. 1 A-C). Furthermore, we conducted immunohistochemistry to observe the change in Mst1. Mst1 immune expression was distributed strongly in the nucleus and cytoplasm of the epithelial and stromal cells in the normal endometrium, whereas Mst1 protein expression was weak in the ectopic endometrium (Fig. 1D-E). These results suggested that Mst1 was downregulated in endometriosis.

Based on the poor expression of Mst1 in endometriosis and its well-established ability to regulate cell apoptosis and migration, we hypothesized that Mst1 may control the survival and mobilization of endometrial cells. To test this hypothesis, we performed an adenovirus-based overexpression (Ad/Mst1) of Mst1 in a human endometrial stromal cell line (ESCs). The overexpression efficiency is shown in Fig. 1F-H. Then, the transfected ESCs were subjected to MTT and TUNEL assays. The data showed that the Ad/Mst1-ESCs had a reduced cellular viability compared to the control group (Fig. 1I-K). Moreover, we also conducted a transwell assay to evaluate the role of Mst1 on ESCs migration. As illustrated in Fig. 1L-M, the overexpression of Mst1 alleviated the number of migrated cells when compared to the control group. Altogether, the above data demonstrated that Mst1 was dysregulated in ectopic endometrium, and the recovery of Mst1 expression impaired ESCs migration and viability.

*Regaining Mst1 activates Drp1-related mitochondrial fission in ESCs*

Mitochondrial fission is necessary for cell apoptosis and migration. In our experiments, we used the RFP-mitochondria probe to label mitochondria and observed the mitochondrial fission under Mst1 overexpression. As shown in Fig. 2A, compared to the control group, the Ad/Mst1-treated ESCs had more mitochondrial fragmentations. However, this tendency was

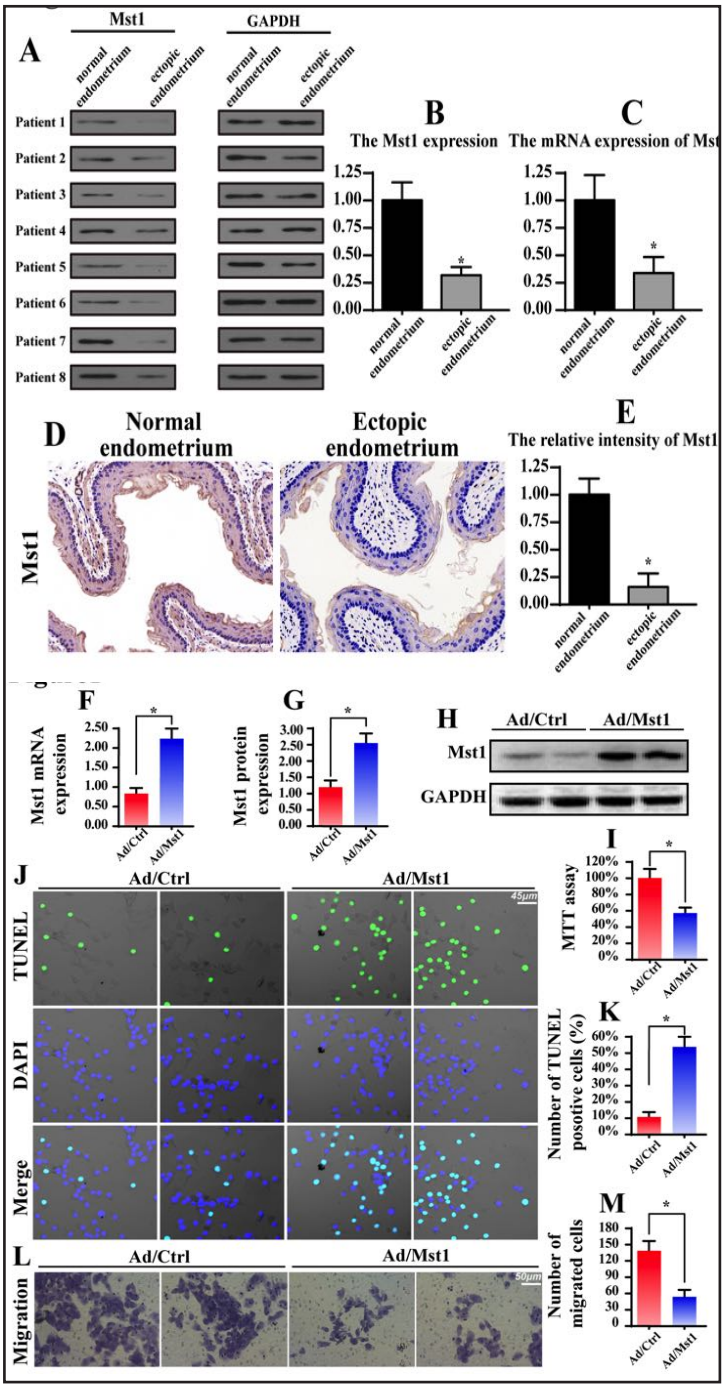


**Fig. 1.** Recovery of Mst1 promotes ESCs apoptosis and migration inhibition in vitro. A-B. Western blotting for Mst1 protein in ectopic endometrium and normal endometrium. Eight randomly selected pairs of patients with ectopic endometrium and normal endometrium are presented. C. The change of Mst1 mRNA in ectopic endometrium and normal endometrium. D-E. The immunohistochemistry of Mst1 ectopic endometrium and normal endometrium. F. Adenovirus-based overexpression (Ad/Mst1) of Mst1 in a human endometrial stromal cell line (ESCs). Ad/Mst1 enhanced the mRNA expression of Mst1 in ESCs. G-H. A western blot assay was used to evaluate the overexpression efficiency of Ad/Mst1 in ESCs. I. A MTT assay was applied to measure the role of Ad/Mst1 in ESC viability. J. ESCs, stably expressing Ad/Mst1 or Ad/Ctrol, underwent TUNEL detection. Representative photographs are presented. K. The relative number of TUNEL positive cells. L. A transwell assay was used to detect the migratory response of the ESCs transfected with Ad/Mst1 or Ad/Ctrl. Representative photographs are presented. M. The relative number of migratory cells was counted. *P<0.05.

inhibited by Mdivi1, an inhibitor of fission. To quantify the fission, we measured the length of the mitochondria. The average mitochondrial length in the control group was 7.91±2.13 μm (Fig. 2B). However, the overexpression of Mst1 reduced the mitochondrial length to 2.09±0.96 μm. Notably, Mdivi1 reversed the length of the mitochondria despite transfection with Ad/Mst1.

Given that fission is activated by Drp1, which shuttles from the cytoplasm on the surface of the mitochondria in the process of mitochondrial fission, we therefore assessed whether Mst1 regulates the Drp1 translocation on mitochondria. First, western blots was used to detect the cellular location of Drp1. Ad/Mst1 enhanced the expression of mito-Drp1

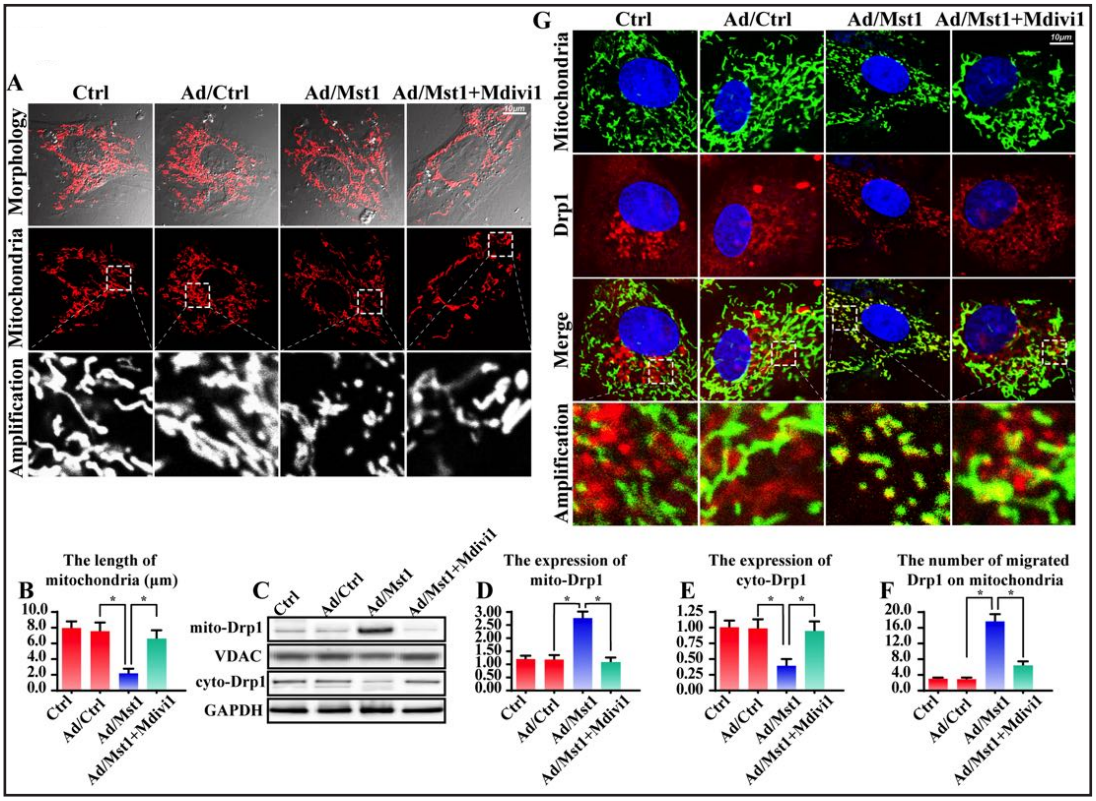


**Fig. 2.** Mst1 regulates Drp1-related mitochondrial fission. A. The morphology of the mitochondria was labeled by an RFP-mitochondria probe. Representative photographs are presented. In the amplified panel, Mst1 induced mitochondria to divide into several fragmentations. B. The length of the mitochondria was measured to reflect the mitochondrial fission. C. Western blots were used to detect the expression of proteins related to fission. VDAC and GAPDH were the loading control for mitochondrial proteins and cytoplasmic proteins, respectively. D-E. The relative expression of mitochondrial Drp1 (mito-Drp1) and cytoplasmic Drp1 (cyto-Drp1) were normalized to the loading control. F-G. The immunofluorescence of Drp1 and mitochondria. In the amplified panel, Drp1 preferred to interact with the fragmented mitochondria, which was regulated by the Mst1. The number of overlaps between Drp1 and mitochondria was counted. *P<0.05.

and reduced the content of cyto-Drp1 compared to the control group (Fig. 2 C-E). These phenotypic changes were rescued by Mdivi1. To provide more solid evidence for the Drp1-related mitochondrial fission, we co-stained the mitochondria and Drp1 at the same time. As shown in Fig. 2F-G, Ad/Mst1 stimulated the overlap of Drp1 and mitochondria. Moreover, in the amplified panel of Fig. 2G, the fragmented mitochondria were labeled by more Drp1. Notably, Mdivi1 blocked the action of Ad/Mst1 on Drp1-related mitochondrial fission.

*Mst1-mediated fission induces ESCs apoptosis and migration arrest via HtrA2/Omi*

To determine whether Mst1-mediated fission was associated with ESC apoptosis and migration inhibition, we first evaluated the mitochondrial apoptosis pathway. Mitochondrial apoptosis is characterized by the mitochondrial potential dissipation, mPTP opening and pro-apoptotic factor liberation from the mitochondria into the cytoplasm, which are followed by the activation of caspase 9 and caspase 3. As shown in Fig. 3A-B, Ad/Mst1 reduced the membrane potential compared to the control group. Moreover, Ad/Mst1 also increased the mPTP opening rate (Fig. 3C). However, the inhibition of fission via Mdivi1 reversed the mitochondrial potential and reduced the mPTP opening.

As a consequence of the mPTP opening, the mitochondria-contained pro-apoptotic factors (cytochrome c, second mitochondria-derived activator of caspases and HtrA2/Omi) are released into the cytoplasm and even into the nuclear space, where they interact with

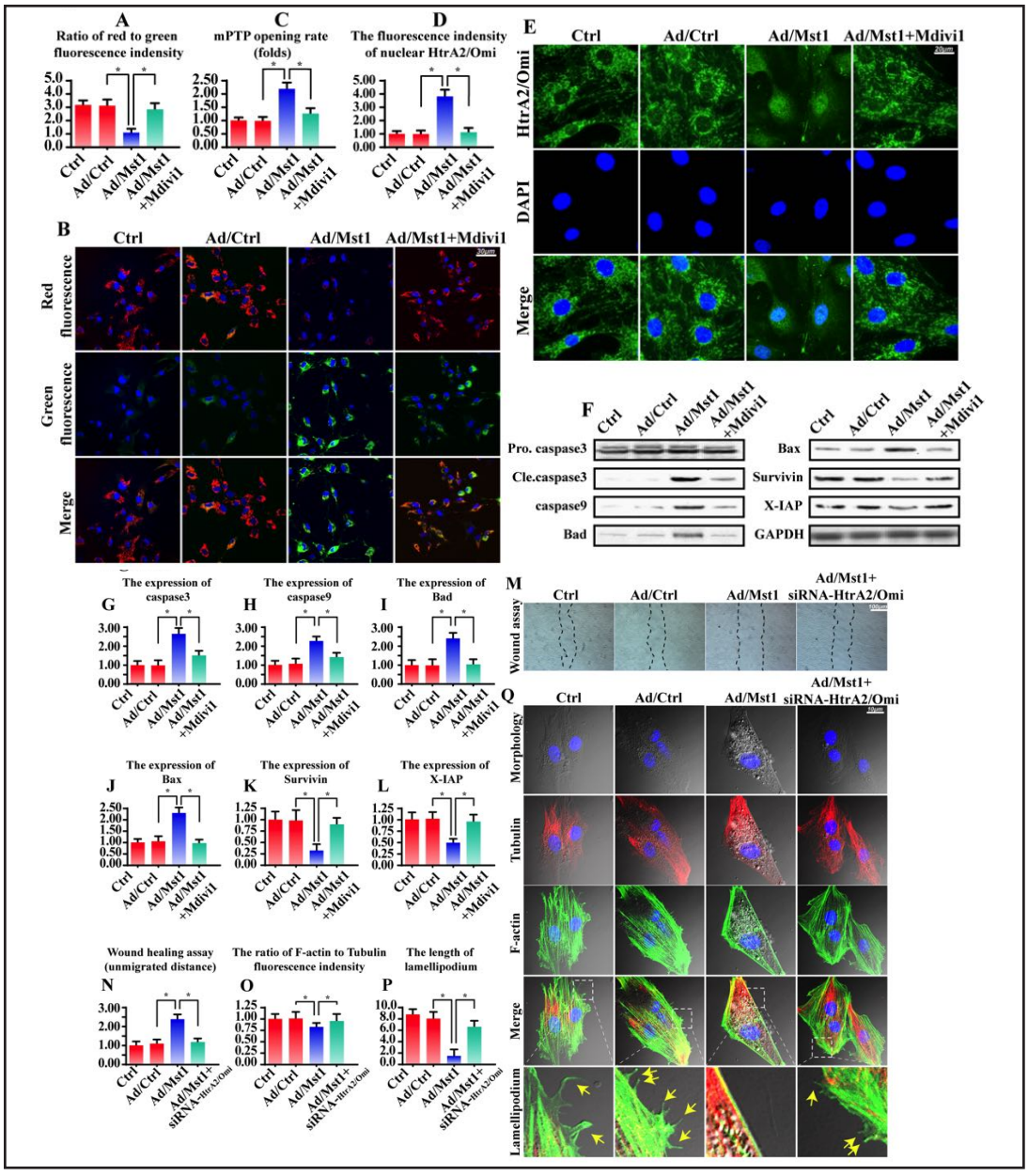


**Fig. 3.** Mst1-mediated mitochondrial fission impairs the ESCs viability and migration by activating the mitochondrial apoptosis pathway and releasing HtrA2/Omi. A-B. Mitochondrial membrane potential changes via JC1 staining. The ratio of the red to green fluorescence intensity was measured to quantify the mitochondrial potential. C. An assessment of mPTP opening was performed using the established calcein cobalt loading procedure by incubating the cells with calcein-acetoxymethyl ester (calcein-AM). The immunofluorescence intensity of calcein-AM was measured to reflect the mPTP opening. D-E. The immunofluorescence of HtrA2/Omi in the cellular location. Mst1-mediated fission caused HtrA2/Omi leakage into the cytoplasm and nucleus. The fluorescence intensity of nuclear HtrA2/Omi was evaluated. F. Western blots were used to detect the protein changes related to mitochondrial apoptosis. G-L. The relative expression of caspase 3, caspase 9, Bax, Bad, Survivin and X-IAP. M-N. A wound-healing assay was carried out to evaluate the mobilization of the ESCs transfected with Ad/Mst1 or Ad/Ctrl. O-Q. The immunofluorescence of F-actin and tubulin. In the amplified panel, F-actin-based lamellipodium outside of the cell was measured. O. The F-actin expression was normalized to tubulin, which is another element of the cytoskeleton but has no role in the cellular migration. Mst1 upregulation reduced F-actin expression. P. The length of the lamellipodium was determined. *P<0.05.

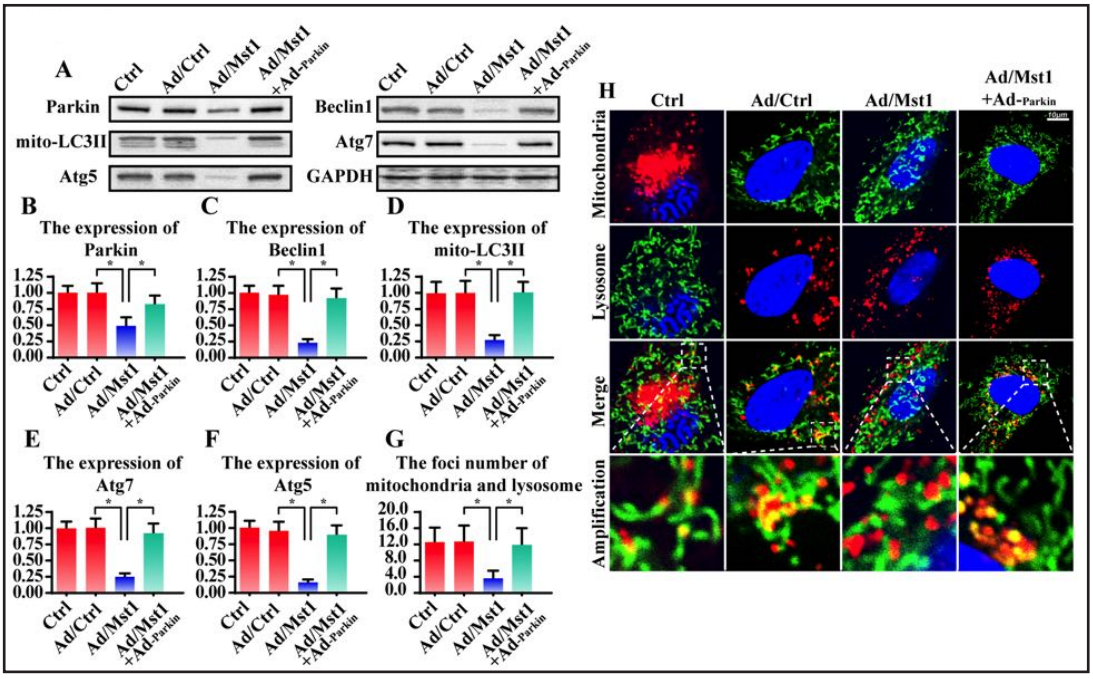


**Fig. 4.** Mst1 controls mitophagy activity via Parkin. A-F. Mst1 overexpression reduced the mitophagy markers. Western blots were used to detect the expression of Parkin, mitochondrial LC3II (mito-LC3II), Atg5, Beclin1 and Atg7. In addition, Parkin was overexpressed via an adenovirus-based overexpression of Parkin (Ad-Parkin). G-H. The mitophagy was observed via immunofluorescence of the mitochondria and lysosome. The number of the overlaps between the mitochondria and lysosome was counted to quantify the mitophagy activity. *P<0.05.

and activate caspase 9/3. Through an immunofluorescence assay of HtrA2/Omi, we found that Ad/Mst1 evoked HtrA2/Omi diffusion into the cytoplasm and even into the nucleus (Fig. 3D-E). This effect was concomitant with an increase in the expression of the down-stream pro-apoptotic proteins (Fig. 3F-L). However, Mdivi1 cancelled these effects. Altogether, the above data confirmed that Mst1-mediated fission was responsible for ESCs apoptosis via the activation of the caspase 9-involved mitochondria apoptosis pathway.

Notably, several studies have reported that HtrA2/Omi inhibits cancer migration via impairing F-actin-based lamellipodium. The arrangement and dynamic behavior of F-actin filaments supply the protrusive force for cell movement via forming lamellipodium, which drives cell migration in some direction. To explore whether Mst1-mediated fission blunted ESCs migration via HtrA2/Omi, siRNA technology was used to silence HtrA2/Omi in the Ad/Mst1-treated ESCs. Then, the wound healing assay was utilized to evaluate ESCs mobilization. First, the Ad/Mst1-treated ESCs showed reduced mobility compared to the control group (Fig. 3M-N). However, the knockdown of HtrA2/Omi reversed the capacity of the Ad/Mst1-treated ESCs to migrate. Considering the important role of F-actin-based lamellipodium in ESC migration, an immunofluorescence assay of F-actin was carried out. In addition, to demonstrate whether F-actin was specifically regulated by Mst1 and fission, we also co-stained tubulin, another element of the cytoskeleton, which makes up the microtubule and promotes intracellular transport but has no role in cellular migration. As shown in Fig. 3O-Q, after the overexpression of Mst1, F-actin was unclear, inordinate and fragmented, which was accompanied by an obvious decline in the ratio of F-actin to tubulin, and this was indicative of F-actin degradation. Furthermore, Ad/Mst1 also reduced the length of the lamellipodium (Fig. 3O-Q). However, these configuration changes were rescued by the knockdown of HtrA2/Omi or application of Mdivi1. Notably, no changes were observed in the tubulin structure. These data indicated that Mst1 governed the lamellipodium-based migration of ESCs in a fission-dependent manner.

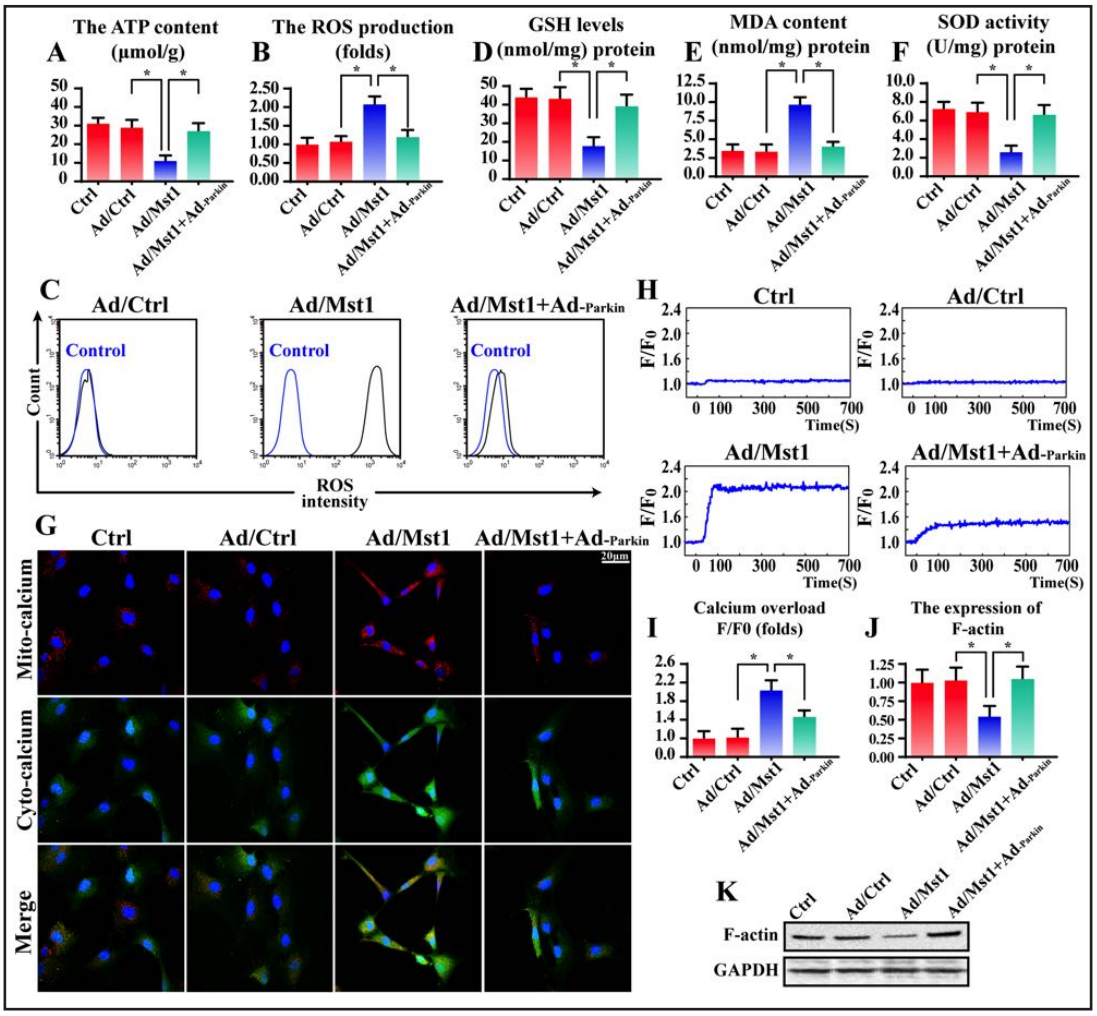


**Fig. 5.** Inhibition of Parkin-required mitophagy by Mst1 impaired mitochondrial energy metabolism, induced cellular oxidative injury and evoked cytoplasmic calcium overload. A. The change in cellular ATP content. Mst1 reduced the ATP production, which was reversed by Parkin overexpression. B-C. Cellular ROS production was detected via flow cytometry. D-F. The change in the anti-oxidant factors GSH and SOD. MAD, a lipid peroxide, was measured to reflect the cellular oxidative stress. G. The co-immunofluorescence of $[Ca^{2+}]c$ and $[Ca^{2+}]m$. The contents of $[Ca^{2+}]c$ were imaged with Fluo-2. For the $[Ca^{2+}]m$ analysis, Rhod-2 was used, and the images were captured by confocal microscopy. Mst1 controlled $[Ca^{2+}]c$ and $[Ca^{2+}]m$ overload in a mitophagy-dependent manner. H-I. The cytoplasmic calcium map was analyzed via Fluo-2 staining by an excitation wavelength of 340 nm and an emission wavelength of 500 nm. The data (F/F0) were obtained by dividing the fluorescence intensity (F) by (F0) at resting level (t = 0), which was normalized to the control groups. J-K. The relative expression of cellular F-actin, which is the necessary element in cell mobilization. The recovery of Parkin inhibited Mst1-mediated F-actin degradation into G-actin. *P<0.05.

*Mst1 represses Parkin-required mitophagy in ESCs*

Apart from fission, mitophagy is also involved in mitochondrial quantity and quality control, which have the ability to block cellular apoptosis via the removal of damaged mitochondria. Therefore, we wanted to know whether Mst1 played a role in mitophagy. Western blot assays interrogating the mitophagy parameters revealed that Ad/Mst1 reduced mito-LC3II, Beclin1, p62 and Atg5 (Fig. 4A-F). Moreover, Ad/Mst1 also strongly repressed the expression of Parkin, which is a receptor or activator of mitophagy (Fig. 4A-B). To provide more solid evidence for the action of Mst1 on mitophagy, we co-stained the

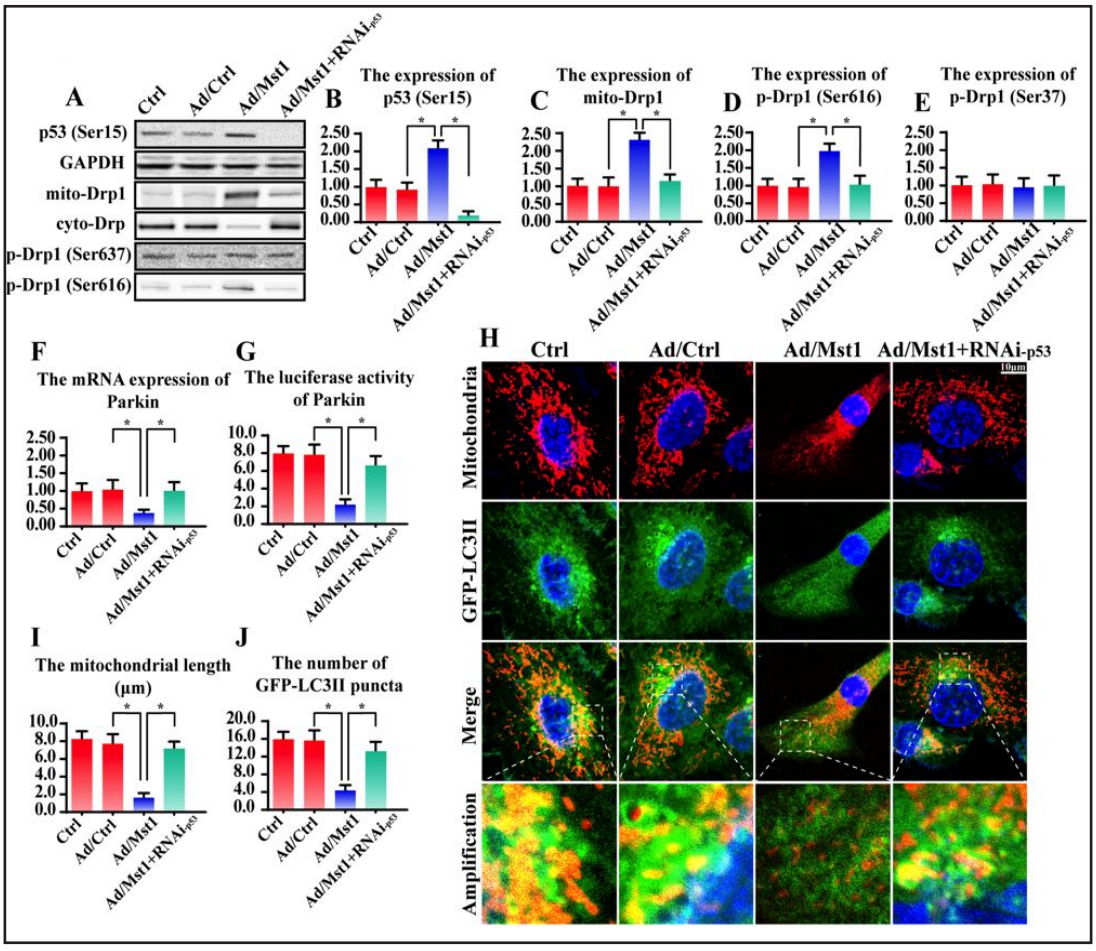


**Fig. 6.** Mst1 regulates Drp1-related mitochondrial fission and Parkin-required mitophagy via the p53 pathway. A-B. Mst1 overexpression was associated with p53 activation. A-E. Western blot assays were used to detect Drp1 migration. The loss of p53 reduced the expression of mito-Drp1 but increased the content of cyto-Drp1. In addition, Mst1 induced Drp1 activation via phosphorylation at Ser616, which was inhibited by p53. F-G. The qPCR experiment and transcriptional activity of Parkin was conducted with p53 knockdown or not. The loss of p53 reversed the Parkin transcription. H. The immunofluorescence of the mitochondria and LC3II. In the control cells, LC3II accumulated and interacted with the mitochondria, indicative of mitophagy. However, in the Ad/Mst1-treated cells, LC3II aggregation to the mitochondria was inhibited, which was accompanied with more mitochondrial debris, suggestive of mitophagy arrest and fission activation. This configuration change was rescued by p53 knockdown. I. Mitochondrial length was determined to reflect the mitochondrial fission. J. The number of LC3II puncta was counted to quantify mitophagy activity. *P<0.05.

mitochondria and lysosome at the same time. As shown in Fig. 4G-H, in the control cells, spindle mitochondria were partly tagged by lysosomes. However, after the overexpression of Mst1, the mitochondria divided into several fragmentations, which were separated from the lysosomes, indicative of mitophagy inhibition. Considering the inhibitory role of Mst1 on Parkin expression, we conducted a Parkin rescue experiment using an adenovirus-based overexpression (Ad/Parkin) of Parkin. The transfection efficiency is shown in Fig. 4A-B. As expected, re-introduction of Parkin promoted the lysosome interaction with the mitochondrial debris, which also sustained the network of the mitochondria. These data indicated that Mst1 controlled mitophagy via Parkin.

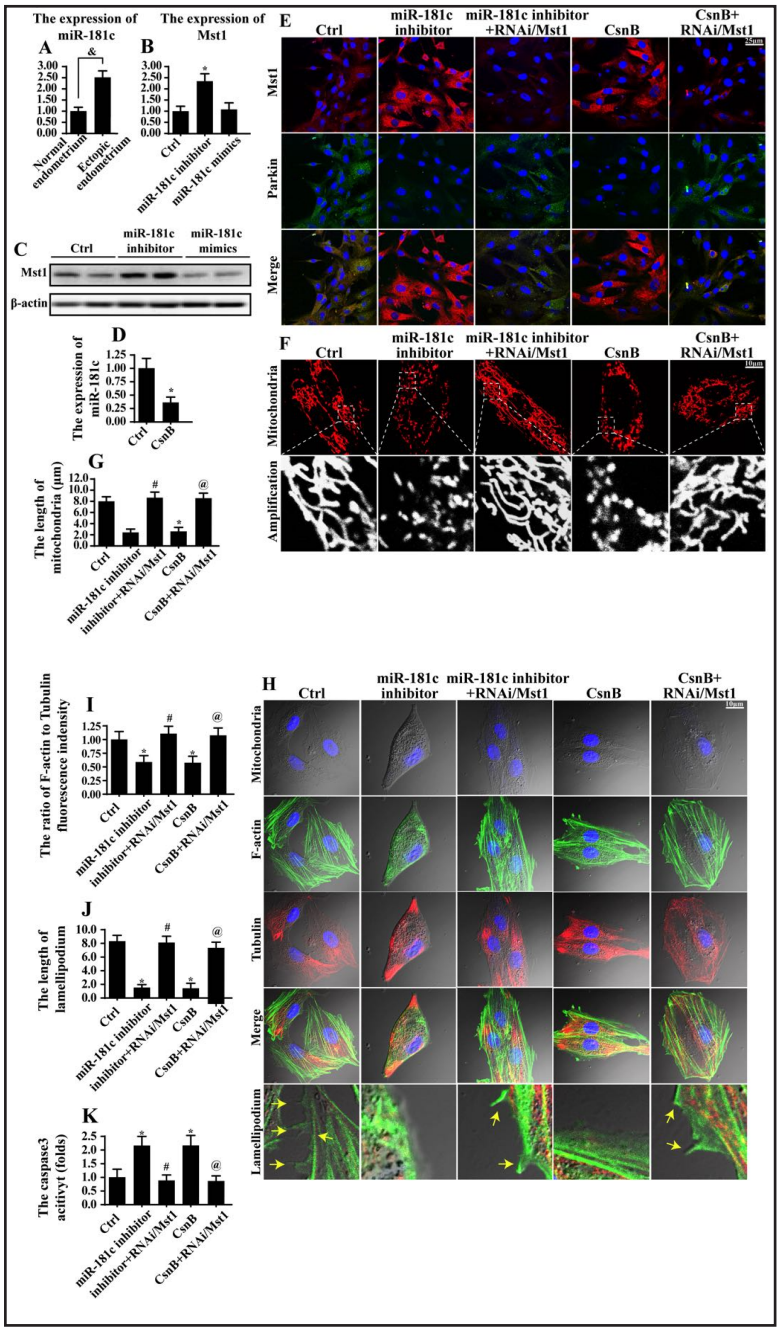


**Fig. 7.** NR4A/miR-181c is the upstream regulator of Mst1 expression, contributing to mitochondrial homeostasis, cellular survival and migration. A. The miR-181c expression was increased in the ectopic endometrium when compared to the normal endometrium. &P<0.05. B-C. The inhibition of miR-181c via its inhibitor restored the expression of Mst1. In contrast, the miR-181c mimics slightly reduced the expression of Mst1. However, no significant difference was observed between the control group and the miR-181c mimics group in the ESCs. D. The miR-181c expression in the ESCs was regulated via NR4A. The activation of NR4A via Cytosporone B (CsnB) reduced the expression of miR-181c. E. The immunofluorescence of Mst1 and Parkin in the ESCs. The inhibition of miR-181c or the activation of NR4A via CsnB reversed the expression of Mst1 but reduced the Parkin content. These effects were cancelled by Mst1 siRNA. F-G. The mitochondrial fragmentations were observed, and the mitochondrial length was determined to reflect the role of NR4A/miR-181c in fission. H. The immunofluorescence of F-actin and tubulin. In the amplified panel, the F-actin-based lamellipodium outside of the cell was measured. I. The F-actin expression was normalized to tubulin, which is another element of the cytoskeleton, but it has no role in cellular migration. The inhibition of miR-181c or the activation of NR4A via CsnB reduced F-actin expression. J. The length of the lamellipodium was determined. K. The caspase 3 activity assay was conducted to explore the role of NR4A/miR-181c in cellular apoptosis. *P<0.05 vs control group, #P<0.05 vs miR-181c inhibitor group, @P<0.05 vs CsnB group.

*Mitophagy inhibition mediates energy disorder, oxidative stress and calcium overload in ESCs*

Mitophagy arrest results in mitochondrial dis-homeostasis. Energy production is the central role of the mitochondria, in our study, we found that Mst1-mediated mitophagy

inhibition caused a significant drop in ATP production, which was reversed by Parkin overexpression (Fig. 5A). In addition to the energy disorder, mitophagy suppression was also associated with cellular oxidative stress as evidenced by higher ROS production (Fig. 5B-C). This effect was concomitant with a decline in the contents of anti-oxidant factors, such as SOD and GSH (Fig. 5D-F). In contrast, the lipid peroxide MDA increased under Mst1 overexpression, whereas regaining Parkin reversed the redox balance. These data indicated that Mst1-mediated mitophagy suppression contributed to cellular injury, which might be involved in ESC apoptosis.

As for the role of mitophagy in ESC mobilization, we focused on the balance of cytoplasmic calcium ($[Ca^{2+}]c$) and mitochondrial calcium ($[Ca^{2+}]m$). Cellular migration depends on a moderate $[Ca^{2+}]c$ concentration. A $[Ca^{2+}]c$ overload hinders cellular contraction and mobilization. Thus, we first detected the synchronous change in $[Ca^{2+}]m$ and $[Ca^{2+}]c$ fluorescence in Ad/Mst1-treated ESCs. As shown in Fig. 5G, Ad/Mst1 elevated the fluorescence intensity of $[Ca^{2+}]m$ and $[Ca^{2+}]c$ compared to the control group. However, the recovery of Parkin reduced $[Ca^{2+}]m$ and $[Ca^{2+}]c$ fluorescence. Furthermore, the calcium map of $[Ca^{2+}]c$ was also in agreement with the above findings that Mst1 amplified the calcium signal in a mitophagy-dependent manner (Fig. 5H-I). At last, we observed a change in F-actin, which is a necessary element for cell migration. As shown in Fig. 5J-K, Ad/Mst1 reduced the F-actin expression, which was reversed by Parkin overexpression, suggesting that mitophagy maintained the F-actin homeostasis.

*Mst1 regulates Drp1-related fission and Parkin-required mitophagy via p53*

To explain the mechanism by which Mst1 manipulates fission and mitophagy, we focused on p53. The Mst1-Hippo pathway is the activator of p53, which regulates fission and mitophagy. In the current study, we found that Mst1 overexpression elevated the expression of phosphorylated p53 at Ser15 (Fig. 6A-B), which was indicative of p53 activation. This change was accompanied with an increase in mito-Drp1 and a drop in cyto-Drp1 (Fig. 6A-C). However, with the knockdown of p53 via siRNA, the expression of mito-Drp1 was reduced and cyto-Drp1 was increased (Fig. 6A-C). These data indicated that Mst1 regulated Drp1 via p53. Furthermore, Drp1 translocation to the mitochondria was determined via a post-transcriptional modification. Drp1 has two key phosphorylation sites, including Ser616 and Ser637. Drp1 is activated by phosphorylation at Ser616 but is inactivated by phosphorylation at Ser637. The balance of these two phosphorylation sites determines the activity of Drp1. We found that Ad/Mst1 primarily induced the phosphorylation of Drp1 at Ser616. (Fig. 6A, D-E). However, this effect was nullified by p53 silence. Notably, no change was observed in p-Drp1$^{Ser637}$ regardless of Mst1 overexpression or p53 knockdown (Fig. 6A, D-E). These data indicated that p53 controlled Drp1 activity via a post-transcriptional modification.

To further explain whether p53 controlled Parkin expression, we focused Parkin gene transcription. We found that Parkin mRNA expression decreased in response to Mst1 overexpression (Fig. 6F) but was increased with the knockdown of p53. These data indicated that p53 was the transcriptional repressor of Parkin. Furthermore, we conducted a luciferase assay of Parkin with Mst1 overexpression and p53 knockdown. As shown in Fig. 6G, Mst1 repressed the transcriptional activity of Parkin, which was enhanced by p53 silencing.

To determinee whether p53 was also involved in fission and mitophagy, we used co-immunofluorescence to stain the mitochondria and LC3II. As shown in Fig. 6H-J, in the control group, the spindle mitochondria are partly marked by dotted or aggregated LC3II, indicative of mitochondrial fission. However, Mst1 overexpression induced mitochondrial mass division into several fragmentations. In addition, Ad/Mst1 also inhibited LC3II accumulation, which is indicative of autophagy flux arrest. However, the knockdown of p53 maintained the mitochondrial network and reversed the LC3II accumulation. These data confirmed that Mst1 controlled fission and mitophagy via p53. At the molecular level, p53 regulated Drp1 and Parkin at the post-transcriptional and mRNA levels, respectively.

*NR4A represses Mst1 expression via miR-181c*

Lastly, we investigated the upstream signaling for Mst1 downregulation. A recent study suggests that mi-R181c [45] is the transcriptional repressor of Mst1 in pancreatic cancer. In the present study, we found that miR-181c was also increased in the ectopic endometrium compared to the normal endometrium (Fig. 7A). Upon inhibition of miR-181c via its inhibitor, Mst1 expression was reversed (Fig. 7B-C). Notably, the miR-181c mimics slightly limited the expression of Mst1 in the ESCs (Fig. 7B-C). However, no significant difference was observed between the miR-181c mimics and the control group. These data indicated that in the normal ESCs, Mst1 was strongly inhibited by miR-181c, and thus, the application of miR-181c mimics could not further reduce the expression of Mst1.

Furthermore, we wanted to understand the regulatory signaling for the miR-181c upregulation. A recent study identified that endometriosis progression was associated with impaired endometrial decidualization, which is regulated by the orphan nuclear receptor subfamily 4 group A (NR4A). The decreased expression of NR4A disrupted endometrial decidualization, representing a novel mechanism to explain subfertility, adenomyosis and endometriosis. In the present study, we found that the NR4A agonist cytosporone B (CsnB) reduced the expression of miR-181c (Fig. 7D), suggesting that NR4A plays a role in inhibiting the expression of miR-181c. Through immunofluorescence, we found that the activation of NR4A increased the expression of Mst1 (Fig. 7E), similar to the results in the miR-181c inhibition, suggesting that NR4A is the upstream activator of Mst1 via repressing miR-181c.

Next, to determine whether NR4A/miR-181c is involved in mitophagy and fission, we co-stained Parkin. As shown in Fig. 7E, the inhibition of miR-181c or the activation of NR4A via CsnB reduced the expression of Parkin. However, these effects were inhibited by the knockdown of Mst1 via siRNA, suggesting that NR4A/miR-181c regulation of Parkin required Mst1. Similarly, the miR-181c inhibitor or the NR4A agonist induced more mitochondrial fragmentations, and these effects were nullified by Mst1 siRNA (Fig. 7F-G).

Furthermore, we also found that the miR-181c inhibitor or the NR4A agonist induced F-action dysfunction and impaired lamellipodium formation (Fig. 7H-J), which were reversed by Mst1 siRNA. Similarly, the caspase 3 activity assay in Fig. 7K also suggested that NR4A/miR-181c influenced ESC apoptosis via Mst1.

## Discussion

Ample evidence suggests the involvement of Mst1 in the inhibition of hepatocellular carcinoma metastasis, pancreatic cancer relapse, gastric cancer survival, cardiac reperfusion injury and metabolic liver injury. However, little is known about the role of Mst1 in the protection of endometriosis. In the present study, we found the following: (1) Mst1 was significantly downregulated in the ectopic endometrium of endometriosis; (2) the overexpression of Mst1 was closely associated with the inability of ESCs to migrate and survive; (3) mechanistically, regaining Mst1 powerfully enhanced Drp1 post-transcriptional phosphorylation at Ser616 and repressed Parkin transcription activity via p53, leading to mitochondrial fission activation and mitophagy inhibition; (4) Drp1-related fission greatly induced ESC apoptosis and migration arrest in a HtrA2/Omi-dependent manner; (5) additionally, defective Parkin-required mitophagy dramatically evoked cellular oxidative stress, energy metabolism and calcium overload; (6) through excessive mitochondrial fission and aberrant mitophagy, Mst1 launched caspase 9-related mitochondrial apoptosis and abrogated F-actin/lamellipodium-dependent cellular migration; and (7) finally, we identified that NR4A/miR181c was the upstream signal for the Mst1 dysfunction in endometriosis. As far as we know, this study comprehensively described the important role of the NR4A-miR181c-Mst1 pathway in endometriosis apoptosis and migration via regulation of Drp1-related mitochondrial fission and Parkin-required mitophagy for the first time (Fig. 8).

Endometriosis, a benign gynecological disease defined as the presence of endometrial cells outside the uterus, seriously impacts the quality of life and reproductive ability of 6–10% of reproductive-aged women and is strongly associated with chronic pelvic pain and

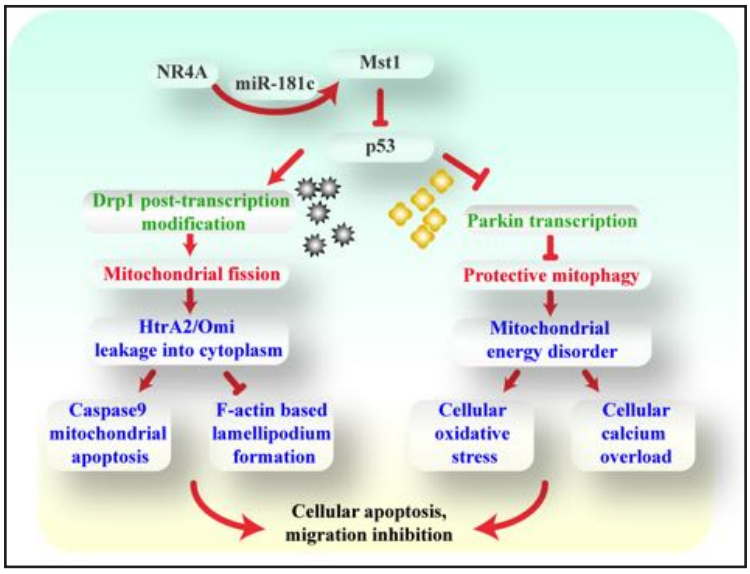


**Fig. 8.** In the ectopic endometrium, Mst1 is downregulated compared to the normal endometrium. The re-introduction of Mst1 reduced ESC migration and increased ESC apoptosis. Mechanistically, regaining Mst1 enhanced Drp1 post-transcriptional phosphorylation at Ser616 and repressed Parkin transcription activity via p53, leading to mitochondrial fission activation and mitophagy inhibition. On the one hand, Drp1-related fission greatly induced ESC apoptosis and migration arrest by releasing HtrA2/Omi into the cytoplasm. HtrA2/Omi not only activated the caspase 9-dependent mitochondrial apoptosis pathway but also induced F-actin degradation and lamellipodium collapse. On the other hand, defective Parkin-required mitophagy dramatically evoked cellular oxidative stress, energy metabolism and calcium overload, which impaired cellular viability and F-actin homeostasis. Through excessive mitochondrial fission and aberrant mitophagy, Mst1 induced ESC apoptosis and migration inhibition. Finally, the upstream regulatory signaling for Mst1 dysfunction contributed to NR4A/miR-181c. The activation of NR4A or the inhibition of miR-181c balanced Mst1 expression, leading to ESC mitochondrial damage, mobilization failure and apoptosis.

infertility. The endometrium of the uterus is lined by a columnar epithelium that is supported by a stromal cell foundation, which consists of a variety of cell types [46]. The endometrial epithelial cells and ESCs are the main two cell types, as they have the absolute predominance in quantitative terms. The ESCs actively produce numerous growth factors and cytokines that affect epithelial function. According to Sampson's hypothesis, ESCs migrate to and survive in ectopic locations, which is the key feature of endometriosis. Accordingly, the molecular mechanism of ESCs survival and mobilization should be considered, with a goal of discerning the rationale for therapeutically targeting endometriosis

In the present study, we found that Mst1 dysregulation was responsible for the excessive survival and migration of ESCs. Mst1, a critical regulator of the hippo pathway, is closely related to evolutionarily conserved serine/threonine protein kinases that play a pivotal role in cell proliferation, survival, morphology and motility and organ size [47]. Recent studies in conditional Mst1 ablation and transgenic Mst1/2 double-knockout mice exhibit abrogation of Yap phosphorylation with intense nuclear accumulation, leading to the development of hepatocellular carcinoma, pancreatic cancer, cardiac reperfusion injury and metabolic liver disease [48, 49]. However, our data introduced Mst1 into the etiology of endometriosis. We demonstrated that Mst1 was downregulated in the ectopic endometrium, which negatively correlated with ESC survival and migration. Once Mst1 activity was recovered in the ESCs, the ability of the ESCs to survive and mobilize was impaired. These data indicate that Mst1 acts as a negative regulator of endometriosis, dictating ESCs to excessive apoptosis and migration inhibition. Therefore, our findings unveil the neglected role of Mst1 in endometriosis, highlighting a novel therapeutic target of endometriosis, involving ESC apoptosis and migration. However, more clinical evidence is needed to support our notion.

One mechanism by which Mst1 impacts ESCs apoptosis and migration is through the activation of Drp1-related mitochondrial fission by p53[50]. p53 amplifies Drp1 activity via a post-transcriptional modification at Ser616. Excessive Drp1-related fission forces the mitochondria to liberate HtrA2/Omi into the cytoplasm where HtrA2/Omi activates

the caspase 9-related mitochondrial apoptosis pathway. Meanwhile, HtrA2/Omi abolishes ESC migration by minimizing the stability of F-actin, leading to lamellipodium collapse. Another critical regulatory mechanism of Mst1 in ESC apoptosis/migration is Parkin-required mitophagy. Mst1 blocks Parkin transcription and expression via p53, resulting in a mitophagy delay. The worn-out mitophagy fails to remove the damaged or fragmented mitochondria, evoking cellular oxidative stress, energy disorder and a calcium overload. Thus, through excessive fission and deficient mitophagy, Mst1 enhanced ESCs apoptosis and limited its migration.

The concept that fission or mitophagy modulates cell apoptosis is supported by a plethora of studies [8, 51, 52]. With respect to cell migration, several researchers reasoned that fission requires F-actin to form the potential contraction point at the surface of the mitochondria, which extensively consumes F-actin stress fibers, a necessary element for cell migration [53]. Moreover, oxidative stress and energy deletion, as a result of bad-structured mitophagy or fission, have the ability to induce the F-actin degradation via the regulation of cofilin, a depolymerization agent of F-actin [52]. However, in the present study, we identified that HtrA2/Omi was the pro-apoptotic signal for ESC apoptosis induced by fission. Meanwhile, HtrA2/Omi induced F-actin collapse, which impaired the formation of lamellipodium. HtrA2/Omi is a serine protease and chaperone protein, and it binds to and cleaves the inhibitor of apoptosis proteins (IAPs), leading to cellular apoptosis [54]. Additionally, previous studies have also reported that the F-actin cytoskeleton is regulated by p53 via the activation of HtrA2/Omi [55]. These findings are in agreement with our results. Importantly, our data established the links between Mst1, Drp1-related mitochondrial fission and Parkin-required mitophagy with respect to cell migration. To the best of our knowledge, this is the first study to explore the interactions of the Mst1-Hippo pathway and mitochondrial dynamics [56].

Finally, we found that Mst1 dysregulation was controlled by NR4A and miR181c. Nuclear receptors represent a family of transcription factors responsible for the regulation of many intracellular pathways, such as cancer, metabolic and proliferative diseases [57, 58]. They are termed orphan because their endogenous ligands have not yet been identified. The orphan nuclear receptor subfamily 4 group A (NR4A) is progressively decreased in adenomyosis, which impairs endometrial decidualization [59]. In the present study, we found that the activation of NR4A reversed Mst1 expression via miR-181c, suggesting that NR4A/miR-181c could be involved in the development of endometriosis. Ample evidence identifies miR-181c as the transcriptional repressor of Mst1[45]. Moreover, numerous studies have profiled the role of miR-181c in mitochondrial protection [60-62]. Furthermore, a high expression of miR-181c is considered a predictive marker of the recurrence in colorectal cancer, suggesting an inhibitory effect of miR-181c on cell survival and proliferation. These findings are in accordance with our results. However, the question of how NR4A regulates miR-181c expression remains unanswered. More studies are needed to explore the underlying mechanism.

## Conclusion

In summary, our report illustrates that Mst1 functions as a novel endometriosis suppressor involving ESC viability and mobility. Mechanically, Mst1 is triggered by NR4A/miR-181c and regulates Drp1-related mitochondrial fission and Parkin-required mitophagy via p53. Thus, strategies to regulate the balance of the Mst1-Hippo pathway, Drp1-related mitochondrial fission and Parkin-required mitophagy could be a therapeutic target with respect to endometriosis.

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## Disclosure Statement

The authors have declared that they have no conflicts of interest.

## References

1 Lagana AS, La Rosa VL, Rapisarda AMC, Valenti G, Sapia F, Chiofalo B, Rossetti D, Ban Frangez H, Vrtacnik Bokal E, Vitale SG: Anxiety and depression in patients with endometriosis: impact and management challenges. Int J Womens Health 2017;9:323-330.
2 Nezhat C, Falik R, McKinney S, King LP: Pathophysiology and management of urinary tract endometriosis. Nat Rev Urol 2017;14:359-372.
3 Lessey BA, Kim JJ: Endometrial receptivity in the eutopic endometrium of women with endometriosis: it is affected, and let me show you why. Fertil Steril 2017;108:19-27.
4 Baker JM, Al-Nakkash L, Herbst-Kralovetz MM: Estrogen-gut microbiome axis: Physiological and clinical implications. Maturitas 2017;103:45-53.
5 Suliman HB, Piantadosi CA: Mitochondrial Quality Control as a Therapeutic Target. Pharmacol Rev 2016;68:20-48.
6 Xu S, Pi H, Zhang L, Zhang N, Li Y, Zhang H, Tang J, Li H, Feng M, Deng P, Guo P, Tian L, Xie J, He M, Lu Y, Zhong M, Zhang Y, Wang W, Reiter RJ, Yu Z, Zhou Z: Melatonin prevents abnormal mitochondrial dynamics resulting from the neurotoxicity of cadmium by blocking calcium-dependent translocation of Drp1 to the mitochondria. J Pineal Res 2016;60:291-302.
7 Zhou H, Zhang Y, Hu S, Shi C, Zhu P, Ma Q, Jin Q, Cao F, Tian F, Chen Y: Melatonin protects cardiac microvasculature against ischemia/reperfusion injury via suppression of mitochondrial fission-VDAC1-HK2-mPTP-mitophagy axis. J Pineal Res 2017;63:e12413.
8 Zhou H, Zhu P, Guo J, Hu N, Wang S, Li D, Hu S, Ren J, Cao F, Chen Y: Ripk3 induces mitochondrial apoptosis via inhibition of FUNDC1 mitophagy in cardiac IR injury. Redox Biol 2017;13:498-507.
9 Fuhrmann DC, Brune B: Mitochondrial composition and function under the control of hypoxia. Redox Biol 2017;12:208-215.
10 Smith MR, Vayalil PK, Zhou F, Benavides GA, Beggs RR, Golzarian H, Nijampatnam B, Oliver PG, Smith RA, Murphy MP, Velu SE, Landar A: Mitochondrial thiol modification by a targeted electrophile inhibits metabolism in breast adenocarcinoma cells by inhibiting enzyme activity and protein levels. Redox Biol 2016;8:136-148.
11 Quijano C, Trujillo M, Castro L, Trostchansky A: Interplay between oxidant species and energy metabolism. Redox Biol 2016;8:28-42.
12 Zhou W, Yu L, Fan J, Wan B, Jiang T, Yin J, Huang Y, Li Q, Yin G, Hu Z: Endogenous Parathyroid Hormone Promotes Fracture Healing by Increasing Expression of BMPR2 through cAMP/PKA/CREB Pathway in Mice. Cell Physiol Biochem 2017;42:551-563.
13 Yang HH, Chen Y, Gao CY, Cui ZT, Yao JM: Protective Effects of MicroRNA-126 on Human Cardiac Microvascular Endothelial Cells Against Hypoxia/Reoxygenation-Induced Injury and Inflammatory Response by Activating PI3K/Akt/eNOS Signaling Pathway. Cell Physiol Biochem 2017;42:506-518.
14 Harvey KF, Zhang X, Thomas DM: The Hippo pathway and human cancer. Nat Rev Cancer 2013;13:246-257.
15 Zhou D, Zhang Y, Wu H, Barry E, Yin Y, Lawrence E, Dawson D, Willis JE, Markowitz SD, Camargo FD, Avruch J: Mst1 and Mst2 protein kinases restrain intestinal stem cell proliferation and colonic tumorigenesis by inhibition of Yes-associated protein (Yap) overabundance. Proc Natl Acad Sci U S A 2011;108:E1312-1320.
16 Hu SY, Zhang Y, Zhu PJ, Zhou H, Chen YD: Liraglutide directly protects cardiomyocytes against reperfusion injury possibly via modulation of intracellular calcium homeostasis. J Geriatr Cardiol 2017;14:57-66.
17 Nakamura M, Zhai P, Del Re DP, Maejima Y, Sadoshima J: Mst1-mediated phosphorylation of Bcl-xL is required for myocardial reperfusion injury. JCI Insight 2016;1:e86217.
18 Zhang M, Zhang L, Hu J, Lin J, Wang T, Duan Y, Man W, Feng J, Sun L, Jia H, Li C, Zhang R, Wang H, Sun D: MST1 coordinately regulates autophagy and apoptosis in diabetic cardiomyopathy in mice. Diabetologia 2016;59:2435-2447.
19 Zhou H, Du W, Li Y, Shi C, Hu N, Ma S, Wang W, Ren J: Effects of melatonin on fatty liver disease: The role of NR4A1/DNA-PKcs/p53 pathway, mitochondrial fission, and mitophagy. J Pineal Res 2018;64:e12450.
20 Ho HY, Lin CW, Chien MH, Reiter RJ, Su SC, Hsieh YH, Yang SF: Melatonin suppresses TPA-induced metastasis by downregulating matrix metalloproteinase-9 expression through JNK/SP-1 signaling in nasopharyngeal carcinoma. J Pineal Res 2016;61:479-492.

21 Quintana C, Cabrera J, Perdomo J, Estevez F, Loro JF, Reiter RJ, Quintana J: Melatonin enhances hyperthermia-induced apoptotic cell death in human leukemia cells. J Pineal Res 2016;61:381-395.
22 Hambright WS, Fonseca RS, Chen L, Na R, Ran Q: Ablation of ferroptosis regulator glutathione peroxidase 4 in forebrain neurons promotes cognitive impairment and neurodegeneration. Redox Biol 2017;12:8-17.
23 Li M, Pi H, Yang Z, Reiter RJ, Xu S, Chen X, Chen C, Zhang L, Yang M, Li Y, Guo P, Li G, Tu M, Tian L, Xie J, He M, Lu Y, Zhong M, Zhang Y, Yu Z, Zhou Z: Melatonin antagonizes cadmium-induced neurotoxicity by activating the transcription factor EB-dependent autophagy-lysosome machinery in mouse neuroblastoma cells. J Pineal Res 2016;61:353-369.
24 Liu L, Li H, Cui Y, Li R, Meng F, Ye Z, Zhang X: Calcium Channel Opening Rather than the Release of ATP Causes the Apoptosis of Osteoblasts Induced by Overloaded Mechanical Stimulation. Cell Physiol Biochem 2017;42:441-454.
25 Carrasco-Pozo C, Tan KN, Reyes-Farias M, De La Jara N, Ngo ST, Garcia-Diaz DF, Llanos P, Cires MJ, Borges K: The deleterious effect of cholesterol and protection by quercetin on mitochondrial bioenergetics of pancreatic beta-cells, glycemic control and inflammation: *In vitro* and *in vivo* studies. Redox Biol 2016;9:229-243.
26 Lin C, Chao H, Li Z, Xu X, Liu Y, Hou L, Liu N, Ji J: Melatonin attenuates traumatic brain injury-induced inflammation: a possible role for mitophagy. J Pineal Res 2016;61:177-186.
27 Das N, Mandala A, Naaz S, Giri S, Jain M, Bandyopadhyay D, Reiter RJ, Roy SS: Melatonin protects against lipid-induced mitochondrial dysfunction in hepatocytes and inhibits stellate cell activation during hepatic fibrosis in mice. J Pineal Res 2017;62:e12404.
28 Li J, Chen L, Xiong Y, Zheng X, Xie Q, Zhou Q, Shi L, Wu C, Jiang J, Wang H: Knockdown of PD-L1 in Human Gastric Cancer Cells Inhibits Tumor Progression and Improves the Cytotoxic Sensitivity to CIK Therapy. Cell Physiol Biochem 2017;41:907-920.
29 Mailloux RJ, Craig Ayre D, Christian SL: Induction of mitochondrial reactive oxygen species production by GSH mediated S-glutathionylation of 2-oxoglutarate dehydrogenase. Redox Biol 2016;8:285-297.
30 Yu S, Wang X, Geng P, Tang X, Xiang L, Lu X, Li J, Ruan Z, Chen J, Xie G, Wang Z, Ou J, Peng Y, Luo X, Zhang X, Dong Y, Pang X, Miao H, Chen H, Liang H: Melatonin regulates PARP1 to control the senescence-associated secretory phenotype (SASP) in human fetal lung fibroblast cells. J Pineal Res 2017;63:e12405.
31 Kang JW, Hong JM, Lee SM: Melatonin enhances mitophagy and mitochondrial biogenesis in rats with carbon tetrachloride-induced liver fibrosis. J Pineal Res 2016;60:383-393.
32 Dong X, Fu J, Yin X, Qu C, Yang C, He H, Ni J: Induction of Apoptosis in HepaRG Cell Line by Aloe-Emodin through Generation of Reactive Oxygen Species and the Mitochondrial Pathway. Cell Physiol Biochem 2017;42:685-696.
33 Tallman KA, Kim HH, Korade Z, Genaro-Mattos TC, Wages PA, Liu W, Porter NA: Probes for protein adduction in cholesterol biosynthesis disorders: Alkynyl lanosterol as a viable sterol precursor. Redox Biol 2017;12:182-190.
34 Kalyanaraman B: Teaching the basics of cancer metabolism: Developing antitumor strategies by exploiting the differences between normal and cancer cell metabolism. Redox Biol 2017;12:833-842.
35 Zhou H, Hu S, Jin Q, Shi C, Zhang Y, Zhu P, Ma Q, Tian F, Chen Y: 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:e005328.
36 Zhou H, Li D, Zhu P, Hu S, Hu N, Ma S, Zhang Y, Han T, Ren J, Cao F, Chen Y: Melatonin suppresses platelet activation and function against cardiac ischemia/reperfusion injury via PPARgamma/FUNDC1/mitophagy pathways. J Pineal Res 2017;63:e12438.
37 Iggena D, Winter Y, Steiner B: Melatonin restores hippocampal neural precursor cell proliferation and prevents cognitive deficits induced by jet lag simulation in adult mice. J Pineal Res 2017;62:e12397.
38 Lee HJ, Jung YH, Choi GE, Ko SH, Lee SJ, Lee SH, Han HJ: BNIP3 induction by hypoxia stimulates FASN-dependent free fatty acid production enhancing therapeutic potential of umbilical cord blood-derived human mesenchymal stem cells. Redox Biol 2017;13:426-443.
39 Pariente R, Pariente JA, Rodriguez AB, Espino J: Melatonin sensitizes human cervical cancer HeLa cells to cisplatin-induced cytotoxicity and apoptosis: effects on oxidative stress and DNA fragmentation. J Pineal Res 2016;60:55-64.
40 Wang N, Liu H, Li X, Zhang Q, Chen M, Jin Y, Deng X: Activities of MSCs Derived from Transgenic Mice Seeded on ADM Scaffolds in Wound Healing and Assessment by Advanced Optical Techniques. Cell Physiol Biochem 2017;42:623-639.
41 Tan DX, Hardeland R, Back K, Manchester LC, Alatorre-Jimenez MA, Reiter RJ: On the significance of an alternate pathway of melatonin synthesis via 5-methoxytryptamine: comparisons across species. J Pineal

Res 2016;61:27-40.
42 Lin S, Hoffmann K, Gao C, Petrulionis M, Herr I, Schemmer P: Melatonin promotes sorafenib-induced apoptosis through synergistic activation of JNK/c-jun pathway in human hepatocellular carcinoma. J Pineal Res 2017;62:e12398.
43 Zhang Y, Zhou H, Wu W, Shi C, Hu S, Yin T, Ma Q, Han T, Zhang Y, Tian F, Chen Y: 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.
44 Jokinen R, Pirnes-Karhu S, Pietilainen KH, Pirinen E: Adipose tissue NAD(+)-homeostasis, sirtuins and poly(ADP-ribose) polymerases -important players in mitochondrial metabolism and metabolic health. Redox Biol 2017;12:246-263.
45 Zhu H, Jin Q, Li Y, Ma Q, Wang J, Li D, Zhou H, Chen Y: 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;23:101-113.
46 Zhou H, Yang J, Xin T, Zhang T, Hu S, Zhou S, Chen G, Chen Y: Exendin-4 enhances the migration of adipose-derived stem cells to neonatal rat ventricular cardiomyocyte-derived conditioned medium via the phosphoinositide 3-kinase/Akt-stromal cell-derived factor-1alpha/CXC chemokine receptor 4 pathway. Mol Med Rep 2015;11:4063-4072.
47 Yu T, Ji J, Guo YL: MST1 activation by curcumin mediates JNK activation, Foxo3a nuclear translocation and apoptosis in melanoma cells. Biochem Biophys Res Commun 2013;441:53-58.
48 Hubbard JM, Grothey A: Colorectal cancer in 2014: progress in defining first-line and maintenance therapies. Nat Rev Clin Oncol 2015;12:73-74.
49 Ahmed AA, Mohamed AD, Gener M, Li W, Taboada E: YAP and the Hippo pathway in pediatric cancer. Mol Cell Oncol 2017;4:e1295127.
50 Wang DB, Kinoshita C, Kinoshita Y, Morrison RS: p53 and mitochondrial function in neurons. Biochim Biophys Acta 2014;1842:1186-1197.
51 Lee K, Back K: Overexpression of rice serotonin N-acetyltransferase 1 in transgenic rice plants confers resistance to cadmium and senescence and increases grain yield. J Pineal Res 2017;62:e12392.
52 Shi C, Cai Y, Li Y, Li Y, Hu N, Ma S, Hu S, Zhu P, Wang W, Zhou H: Yap promotes hepatocellular carcinoma metastasis and mobilization via governing cofilin/F-actin/lamellipodium axis by regulation of JNK/Bnip3/SERCA/CaMKII pathways. Redox Biol 2018;14:59-71.
53 Jin Q, Li R, Hu N, Xin T, Zhu P, Hu S, Ma S, Zhu H, Ren J, Zhou H: 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.
54 Zhou H, Li D, Shi C, Xin T, Yang J, Zhou Y, Hu S, Tian F, Wang J, Chen Y: Effects of Exendin-4 on bone marrow mesenchymal stem cell proliferation, migration and apoptosis *in vitro*. Sci Rep 2015;5:12898.
55 Yamauchi S, Hou YY, Guo AK, Hirata H, Nakajima W, Yip AK, Yu CH, Harada I, Chiam KH, Sawada Y, Tanaka N, Kawauchi K: p53-mediated activation of the mitochondrial protease HtrA2/Omi prevents cell invasion. J Cell Biol 2014;204:1191-1207.
56 Gao Y, Xiao X, Zhang C, Yu W, Guo W, Zhang Z, Li Z, Feng X, Hao J, Zhang K, Xiao B, Chen M, Huang W, Xiong S, Wu X, Deng W: Melatonin synergizes the chemotherapeutic effect of 5-fluorouracil in colon cancer by suppressing PI3K/AKT and NF-kappaB/iNOS signaling pathways. J Pineal Res 2017;62:e12380
57 Kolluri SK, Zhu X, Zhou X, Lin B, Chen Y, Sun K, Tian X, Town J, Cao X, Lin F, Zhai D, Kitada S, Luciano F, O'Donnell E, Cao Y, He F, Lin J, Reed JC, Satterthwait AC, Zhang XK: A short Nur77-derived peptide converts Bcl-2 from a protector to a killer. Cancer Cell 2008;14:285-298.
58 Pawlak A, Strzadala L, Kalas W: Non-genomic effects of the NR4A1/Nur77/TR3/NGFIB orphan nuclear receptor. Steroids 2015;95:1-6.
59 Jiang Y, Jiang R, Cheng X, Zhang Q, Hu Y, Zhang H, Cao Y, Zhang M, Wang J, Ding L, Diao Z, Sun H, Yan G: Decreased expression of NR4A nuclear receptors in adenomyosis impairs endometrial decidualization. Mol Hum Reprod 2016;22:655-668.
60 Jung KA, Lee S, Kwak MK: NFE2L2/NRF2 activity is linked to mitochondria and AMP-activated protein kinase signaling in cancers through miR-181c/mitochondria-encoded cytochrome c oxidase regulation. Antioxid Redox Signal 2017;27:945-961.
61 Zhou H, Wang S, Zhu P, Hu S, Chen Y, Ren J: Empagliflozin rescues diabetic myocardial microvascular injury via AMPK-mediated inhibition of mitochondrial fission. Redox Biol 2017;15:335-346.
62 Zhou H, Yang J, Xin T, Li D, Guo J, Hu S, Zhou S, Zhang T, Zhang Y, Han T, Chen Y: Exendin-4 protects adipose-derived mesenchymal stem cells from apoptosis induced by hydrogen peroxide through the PI3K/Akt-Sfrp2 pathways. Free Radic Biol Med 2014;77:363-375.