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Mitofilin regulates cytochrome c release during apoptosis by controlling mitochondrial cristae remodeling

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ABSTRACT

Mitochondria amplify caspase-dependent apoptosis by releasing proapoptotic proteins, especially cytochrome c. This process is accompanied by mitochondrial cristae remodeling. Our studies demonstrated that mitofilin, a mitochondrial inner membrane protein, acted as a cristae controller to regulate cytochrome c release during apoptosis. Knockdown of mitofilin in HeLa cells with RNAi led to fragmentation of the mitochondrial network and disorganization of the cristae. Mitofilin-deficient cells showed cytochrome c redistribution between mitochondrial cristae and the intermembrane space (IMS) upon intrinsic apoptotic stimuli. *In vitro* cytochrome c release experiments further confirmed that, compared with the control group, tBid treatment led to an increase in cytochrome c release from mitofilin-deficient mitochondria. Furthermore, the cells with mitofilin knockdown were more prone to apoptosis by accelerating cytochrome c release upon the intrinsic apoptotic stimuli than controls. Moreover, mitofilin deficiency did not interfere with the activation of proapoptotic member Bax upon intrinsic apoptotic stimuli. Thus, mitofilin distinctly functions in cristae remodeling and controls cytochrome c release during apoptosis.

1. Introduction

Cell apoptosis plays essential roles in development and tissue homeostasis in all metazoan animals [1,2]. Mitochondria are important participants in amplifying apoptosis by releasing cytochrome c and other proapoptotic proteins into the cytoplasm and nucleus [3]. Cytochrome c in cytoplasm forms apoptosome with Apaf1 and procaspase 9, activates caspase activity, and subsequently promotes cell death [4]. Around 85% of total cytochrome c exists within the mitochondrial cristae, which is connected to the peripheral portion of the intermembrane space (IMS) by relatively narrow cristae junctions [5]. There are two steps required for facilitating cytochrome c complete release. The first step involves mitochondrial cristae remodeling at the early stage of apoptosis, which redistributes cytochrome c from mitochondrial cristae into IMS. The second step is the release of cytochrome c into the cytosol via the pores formed by Bax/Bak in the mitochondrial outer membrane [6].

Mitochondrial cristae remodeling is a complicated process, which is regulated by many inner membrane proteins such as OPA1 [7,8]. OPA1 is a GTPase that has been clearly proved to control mitochondrial cristae structure and morphology [9,10], and therefore prevents cytochrome c release from cristae into the IMS [11]. Another two mitochondrial proteins, Rhomboid PARL and prohibitin, regulate cytochrome c release during apoptosis via OPA1-dependent cristae remodeling [12,13].

Mitofilin is an inner membrane protein that has been defined as a mitochondria-shaping protein in controlling and maintaining mitochondrial cristae remodeling [14–16]. HeLa cells with RNAi-mediated knockdown of mitofilin exhibit disorganized mitochondrial inner membranes, which causes inner membranes to fail to form tubular or vesicular cristae [15]. However, whether mitofilin is involved in regulating cytochrome c release during apoptosis remains unclear.

In our studies, we found that knockdown of mitofilin caused disorganized cristae structures and loosed cristae junctions in the mitochondria. The stable cells with mitofilin deficiency were more prone to apoptosis upon several apoptotic stimuli, which was accompanied with cytochrome c redistribution and accelerated release, but irrelevant to the activation of proapoptotic member Bax in the mitochondrial outer membrane.

2. Materials and methods

2.1. Cell culture

HEK293T and HeLa cells were cultured in an atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen).

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2.2. RNA interference

Cell lines stably expressing GFP shRNA or mitofilin shRNA were established using a vector-based shRNA technique. ShRNA was designed and subcloned into retroviral vector pSIREN-RetroQ. Mitofilin shRNA targeted 5′ CCTTAGAGCCCGTTTCTAT 3′. Retroviruses were produced by co-transfecting HEK293T cells with one of the above retroviral vectors and packaging plasmids (pUMVC and pCMV-VSVG) using lipofectamine 2000 (Invitrogen). Viruses were collected at 48 h and 72 h after transfection. HeLa cells were transduced with the retrovirus containing mitofilin or GFP shRNA. Mitofilin knockdown efficiency was determined by RT-PCR and western blotting analysis.

2.3. Bcl-2-overexpressing cell line generation and culture

To generate a HeLa cell line stably overexpressing Bcl-2 and the control cell line containing only the plasmid vector, we packaged retrovirus with either pBabe-Bcl-2 or pBabe, infected them into HeLa cells and selected with puromycin for 5 days; thus, HeLa cells stably overexpressing Bcl-2 or the vector control were obtained, cultured and determined by Western blotting analysis. The details of the virus packaging were described as above.

2.4. Western blotting and antibodies

For Western blotting analysis, cell lysate was prepared, separated by SDS–PAGE and transferred to PVDF membrane. The following antibodies were used for immunodetection: rabbit polyclonal antibody against mitofilin (Novus, CO); rabbit polyclonal antibodies against Bcl-2 and Bax (Santa Cruz Biotechnology, CA); mouse monoclonal antibody against β-actin and rabbit polyclonal antibody against mtHSP60 (Sigma–Aldrich, Sr. Louis, MO).

2.5. Transmission electron microscopy

HeLa cells were fixed with a solution of 2.5% paraformaldehyde, 0.5% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4). The cells were then dehydrated with ethanol and immersed in a 1:1 mixture of propylene oxide and Epon. Samples were finally embedded in Epon by polymerization. Thin sections were imaged on a JEM-1010 electron microscope (Kabushiki Kaisha) at the Core Instrument Facility (Institute of Basic Medical Sciences, Chinese Academy of Medical Science).

2.6. Immunofluorescence

Cells grown on coverslips were fixed and permeabilized with 0.05% Triton X-100 in PBS for 15 min. Cells were incubated with a mouse monoclonal antibody against cytochrome c (BD Biosciences Pharmingen, clone 6H2.B4) overnight. Cells were then incubated with FITC-labeled secondary antibody (Molecular Probes), and later stained with MitoTrack Red (Molecular Probes). Images were acquired using Leica TCM-SP2 microscopy with a laser scanning confocal imaging system. The localization index was calculated as described before [17]. For the mitochondrial morphology analysis, cells were stained with MitoTrack Red only and images were obtained by Leica TCM-SP2 microscopy.

2.7. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay

HeLa cells were seeded in a 96-well plate at a density of 3×10^5 cells ml^{-1}. Cell proliferation was determined by incorporation of MTT as described previously [18].

2.8. Apoptosis induction and detection

Cells were grown to 80–90% confluence before treatments. Inducers of apoptosis included 1 mM H_2O_2, 1 μM staurosporine (STS), 2 μM etoposide and 50 ng/ml of tumor necrosis factor (TNF)-α. Apoptosis was induced by H_2O_2, STS, etoposide or TNF-α. After treatment, the cells were stained with annexin V-FITC (BD Biosciences Pharmingen) and analyzed by flow cytometry (BD Biosciences).

2.9. Isolation of mitochondria and in vitro assays for cytochrome c release

Mitochondria were isolated as described before [5]. Isolated mitochondria (30 μg) were incubated in the presence of caspase-8-cleaved recombinant human Bid (tBid; R&D Systems) or digitonin (Sigma–Aldrich). Cytochrome c release was determined with a colorimetric enzyme-linked immunosorbent assay (Quantikine DCTC0; R&D Systems) according to the manufacturer's instructions.

3. Results

3.1. Mitofilin deficiency caused disruption of the cristae structures in HeLa cells

Mitofilin controls mitochondrial inner membrane morphology and cristae structure [15]. To further explore the roles of mitofilin in the mitochondria, we established a mitofilin-deficient HeLa cell line using RNA interference technology (RNAi). Western blotting and realtime PCR (RT-PCR) were performed to detect the knockdown efficiency. Partial reduction (~70%) of mitofilin was observed after the first round of mitofilin shRNA infection, and ~95% mitofilin was silenced in the cells after a second round of infection (Fig. 1A and B). The latter caused disorganized mitochondrial cristae morphologies with onion-like structures (data not shown), which was consistent with previous research [15]. In addition, more than half of the cells with 95% mitofilin reduction became apoptotic by the fifth day after infection. Cells infected with mitofilin shRNA only once were capable of being cultured further with stable knockdown efficiency. Thus, cells infected with mitofilin shRNA only once (shmitofilin cells) were used to elucidate mitofilin functions in the subsequent experiments, and the cells infected with GFP shRNA only once were used as controls (shGFP cells).

We observed the cristae structure in the shmitofilin and shGFP cells using transmission electron microscopy. Abnormal cristae vesicles with reduced diameter were predominant in the mitofilin-deficient cells, while classic long tubular cylinders were shown in the control cells (Fig. 1C and D). The mitochondria with mitofilin deficiency also presented completely unstructured cristae, as well as abnormally increased space between the membranes (Fig. 1C). Our results confirmed that mitofilin had an important role in controlling cristae morphology and structure.

To examine the morphology of cellular mitochondria, we stained mitochondria with MitoTrack Red. Fragmentation of mitochondria was obvious in the shmitofilin cells but not in the shGFP control cells (Fig. 1E and F).

3.2. Mitofilin deficiency reduced cell proliferation and increased cell sensitivity to apoptotic stimuli

Cell proliferation was assessed by MTT assay at indicated time points in the stable cell lines. As shown in Fig. 2A, the shmitofilin cells grew significantly slowly compared with the shGFP cells.

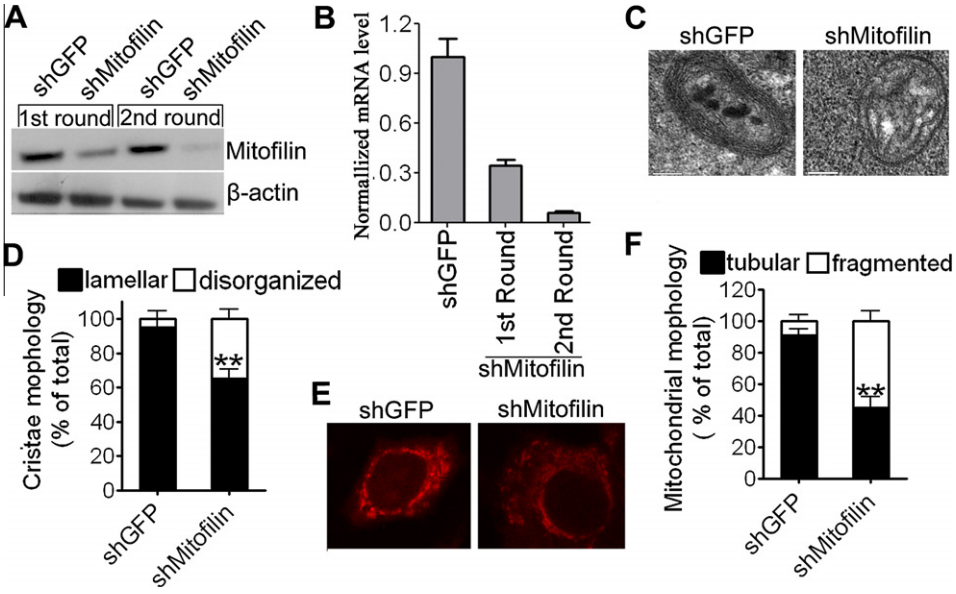

Fig. 1. Morphogenesis in the mitofilin-deficient cells. (A) HeLa cells were collected at 72 h after one or two-round of mitofilin or GFP shRNA infection. The cell lysate was separated on SDS–PAGE and analyzed by Western blotting with antibodies against mitofilin and β-actin. β-Actin was used as a loading control. (B) Real-time PCR analysis of the expression level of mitofilin mRNA in the shRNA-infected HeLa cells. (C) Defective mitochondrial ultrastructure in the shmitofilin cells. Representative transmission electron micrographs of mitochondria in the shGFP and shmitofilin cells were shown. The bars represent 0.2 μm. (D) Quantification of cristae morphology described in (C). Approximately 50% of cristae with disorganized morphology were present in the shmitofilin cells. $^{**}P < 0.01$. (E) Fragmentation of mitochondria in the mitofilin-deficient HeLa cells was detected by MitoTrack Red staining. (F) Quantification of mitochondrial morphology depicted in (E). Cells containing tubular (white bars) or fragmented (black bars) mitochondria were classified. $^{**}P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Cells were then treated with several apoptotic stimuli (H_2O_2, STS and etoposide) individually. Cell apoptosis was measured by annexin V staining. As shown in Fig. 2B and C, after treatment with either H_2O_2 or STS at different time points, there was a significantly higher proportion of apoptotic populations in the shmitofilin cells compared with the shGFP cells. Similarly, more apoptotic cells were observed in the shmitofilin cells than in the shGFP cells after treatment with etoposide for 6 h (Fig. 2D). To clarify whether the mitofilin-deficient cells were also sensitive to extrinsic apoptotic stimuli, shmitofilin cells and shGFP cells were treated with TNFα, a cytokine related to the death receptor pathway that bypasses the mitochondrial amplificatory loop [19]. There was no difference in apoptosis rates between these two cell lines (Fig. 2E), thus, the cells with mitofilin deficiency were specifically sensitive to intrinsic apoptotic stimuli.

3.3. Mitofilin deficiency accelerated the release of cytochrome c from mitochondria

Recently, several researchers have proven that the reorganization of mitochondrial cristae is required for cytochrome c redistribution between cristae and IMS, which results in complete

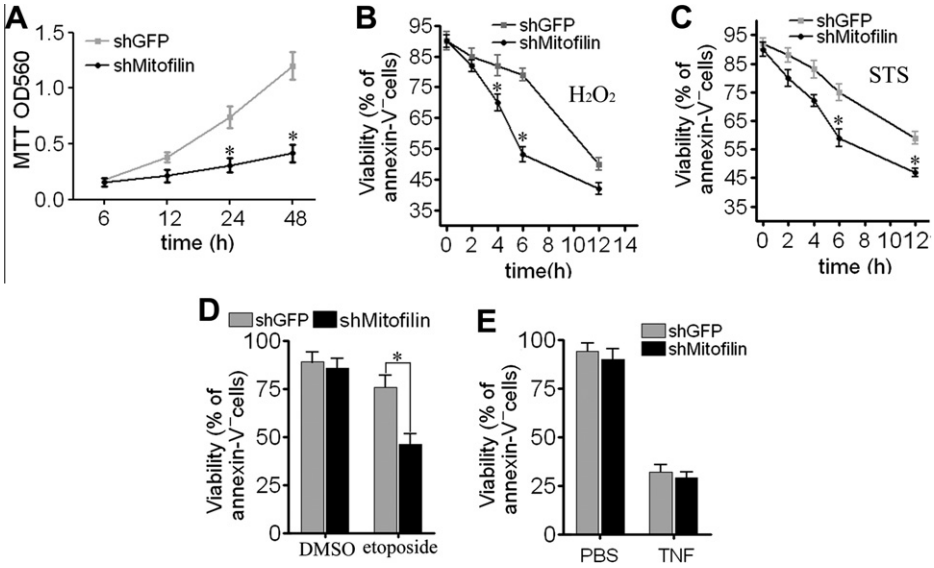

Fig. 2. Mitofilin deficiency caused cells sensitive to the intrinsic apoptotic stimuli. (A) Decreased growth in the shmitofilin cells compared with the control shGFP cells by MTT. Data represent means ± SEM of three independent experiments. $^{*}P < 0.05$. (B and C) Measurement of the survived cells at different time points upon apoptotic treatment by H_2O_2 (B) and STS (C). Data represent means ± SEM of three independent experiments. $^{*}P < 0.05$. (D) Measurement of the surviving cells treated with etoposide for 6 h. Data represent means ± SEM of three independent experiments. $^{*}P < 0.05$. (E) Measurement of the surviving cells treated with TNFα for 6 h. Data represent means ± SEM of three independent experiments.

cytochrome c release from the mitochondria during apoptosis [5,9,11,13]. Mitofilin is responsible for maintaining the mitochondrial cristae structure. Lack of mitofilin might trigger cristae remodeling and cristae junction widening, thus causing a complete release of cytochrome c upon exposure to death signals.

To test this hypothesis, we measured cytochrome c translocation by immunofluorescence after treating the shmitofilin and shGFP cells with H_2O_2. After 2 h, complete cytochrome c release was observed in the shmitofilin cells, whereas there was no change in the control cells (Fig. 3A). We then statistically analyzed the cytochrome c localization at different time points after H_2O_2 treatment. Compared with the control cells, cytochrome c was released faster from the mitochondrion with mitofilin deficiency (Fig. 3B).

An *in vitro* cytochrome c release experiment was carried out using mitochondria isolated from the shmitofilin and shGFP cells. Mitochondria were incubated with tBid, a BH3-only member of the Bcl-2 family that induces intrinsic apoptosis with complete cytochrome c release [5]. After incubating with increasing doses of tBid for 20 min, a significant difference was observed between the shGFP and shmitofilin cells with both 0.1 nM and 0.01 nM tBid treatments (Fig. 3C). Mitochondria from the mitofilin-deficient cells exhibited complete cytochrome c release with 0.1 nM tBid. In the control cells, 10-fold tBid was needed to achieve the similar level of cytochrome c release (Fig. 3C). Next, mitochondria were incubated with 10 nM tBid at indicated time points. Release of cytochrome c from the mitofilin-deficient mitochondria was faster than that from the control mitochondria at all the time points measured (Fig. 3D). These results suggest that mitofilin deficiency facilitated cytochrome c release from mitochondria in response to intrinsic apoptotic stimuli.

Digitonin can be used to selectively permeabilize the mitochondrial outer membrane, releasing the IMS pool of cytochrome c [20]. We treated the mitochondria either with 40 pmol digitonin alone or with 40 pmol digitonin combined with 1 nM tBid. In the group treated with digitonin only, there was no difference found between the mitofilin-deficient mitochondrion and the controls. However, in the group treated with both digitonin and tBid, ~80% cytochrome c was released from the mitofilin-deficient mitochondria 2 min after treatment, compared to only ~60% from control mitochondria at the same time point (Fig. 3E). These results suggest that mitofilin stabilized the pool of cytochrome c shuttling between the cristae and IMS.

3.4. Mitofilin deficiency accelerated STS-induced intrinsic apoptotic pathway without interfering with the activation of Bax

The final release of cytochrome c from mitochondrial IMS to cytoplasm requires the activation and oligomerization of the multidomain proapoptotic members Bax and Bak. This process can be inhibited by anti-apoptotic protein Bcl-2 [21,22]. To test whether mitofilin deficiency would cause the activation and translocation of Bax to the mitochondrial outer membrane upon detection of intrinsic apoptotic stimuli, we isolated mitochondria from the shmitofilin and shGFP cells after STS treatment, and performed Western blotting analysis. As shown in Fig. 4A, similar levels of Bax were activated in both stable cell lines upon STS treatment, which indicated that mitofilin deficiency had no detectable effect on Bax activation. Furthermore, we investigated whether Bcl-2 could rescue intrinsic apoptotic events by suppressing Bax activation. To this end, the Bcl-2-overexpressing cell line was infected with either mitofilin shRNA or control GFP shRNA virus. Some of the cells were collected and the overexpression level of Bcl-2 and knockdown efficiency of mitofilin was observed by Western blotting (Fig. 4B). The remaining cells were then treated with STS for 6 h to induce apoptosis. Mitochondria were isolated from the treated cells and lysed for Western blotting analysis. Bax activation was significantly inhibited in both the mitofilin-deficient and control cells when Bcl-2 was overexpressed (Fig. 4C). These treated

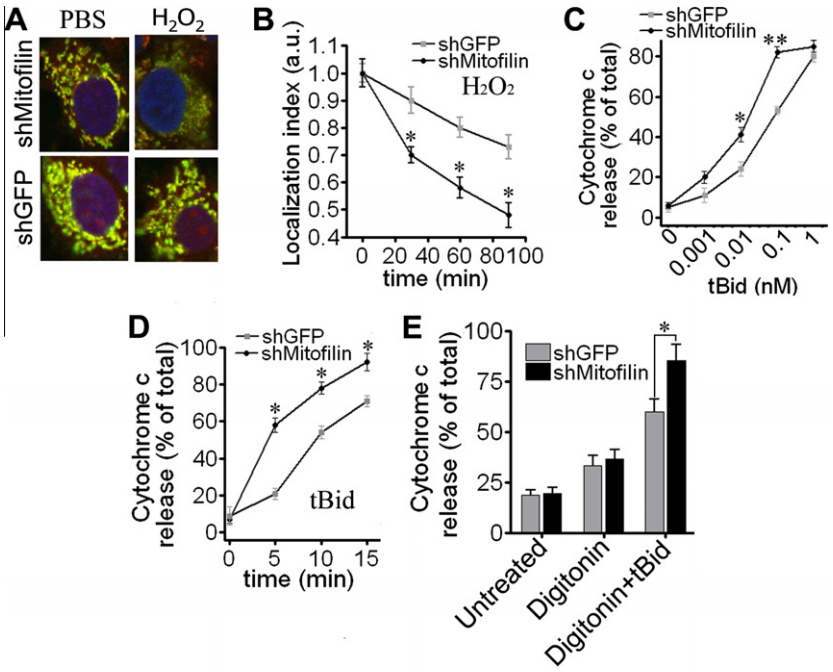

Fig. 3. Mitofilin deficiency accelerated cytochrome c release from mitochondria. (A) Immunofluorescence showed representative images of subcellular cytochrome c distribution after H_2O_2 treatment for 2 h. (B) Localization index of cytochrome c. Experiments were performed as in (A), except that cells were fixed at indicated time points. Data represent means ± SEM of five independent experiments. $^*P < 0.05$. (C and D) Mitochondria isolated from both the shmitofilin and shGFP cells were treated with tBid at indicated doses (C) or at indicated time points (D). After centrifugation, the amount of cytochrome c in the supernatant was determined by a specific ELISA. Data represent means ± SEM of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$. (E) Effects of tBid on the digitonin-releasable cytochrome c pool in the mitochondria isolated from both the shmitofilin and shGFP cells. $^*P < 0.05$.

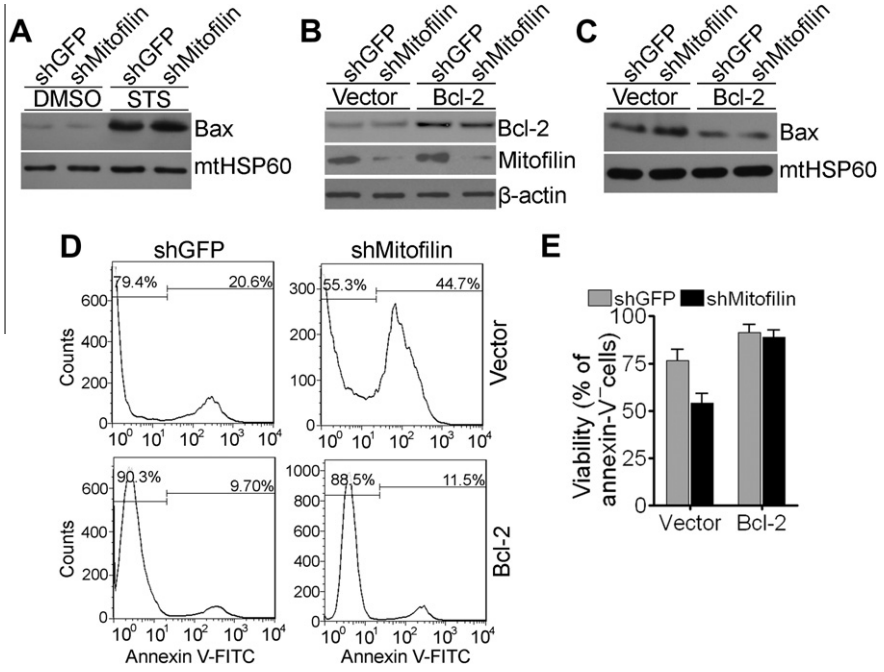

Fig. 4. Mitofilin deficiency accelerated STS induced cytochrome c release without interfering with the activation of Bax. (A) The shmitofilin and shGFP cells were treated with STS or DMSO (vehicle) for 6 h. Mitochondria were isolated, lysed and analyzed by Western blotting with antibodies against Bax and mtHSP60. mtHSP60 was used as a loading control. (B) Bcl-2-overexpressing cells infected with either the shGFP or shmitofilin virus were harvested and lysed for Western blotting analysis with antibodies against mitofilin, Bcl-2 and β-actin. β-Actin was used as a loading control. (C) Cells from (B) were treated with STS for 6 h, and then mitochondria were isolated and used for Western blotting analysis with antibodies against Bax and mtHSP60. mtHSP60 was used as a loading control. (D) Cells from (B) were treated with STS for 6 h and further processed by flow cytometry analysis of annexin V-FITC-labeled apoptotic cells. (E) Quantification of surviving cells after apoptotic stimuli as described in (D).

cells were also stained with anexin V-FITC antibody and analyzed by flow cytometry. Overexpression of Bcl-2 in the shmitofilin cells completely inhibited the apoptosis induced by STS treatment (Fig. 4D and E).

4. Discussion

We targeted mitofilin with specific shRNA to define its cellular activities. Our results indicated that mitofilin controlled mitochondrial cristae morphology as a mitochondrial inner membrane protein. The fragmented mitochondria with highly disorganized and swollen cristae in the mitofilin-deficient cells strikingly resembled the mitochondria that were found in OPA1-deficient and prohibitin-null cells [9,13]. The absence of mitofilin increased the susceptibility of cells to intrinsic apoptotic stimuli by accelerating cytochrome c release, without affecting the activation and translocation of Bax to the mitochondria.

Mitofilin oligomers involve in the formation and maintenance of the cristae junction. Mitochondrial cristae form onion-like structure with mitofilin deficiency [15]. Our studies provide detailed proofs that mitochondrial cristae structure largely depends on the intracellular level of mitofilin. Severely abnormal cristae morphology with onion-like structure was only found in the cells with 95% mitofilin loss (two-round mitofilin shRNA-infected cells). In shmitofilin cells (one-round mitofilin shRNA-infected cells), there were only typical vesicle-like cristae with increased spaces between the membranes. This specialized structure may allow cristae localized cytochrome c to flow freely into IMS, the results of which suggest that mitofilin is essential for the formation of normal tubular cristae as well as cristae junctions. Like OPA1, mitofilin forms oligomers and keeps the cristae junction closely, which may sequester cytochrome c in mitochondrial cristae. Disassembling OPA1 or mitofilin oligomers might widen the cristae junction, which facilitates cytochrome c release and the progression of the apoptotic program after stimulation. Our results are consistent with the new conception that the cytochrome c release during apoptosis could only require cristae junction remodeling but not gross cristae rearrangements in mitochondrial structure [23,24].

Permeabilization of the mitochondrial outer membrane is important for the final release of cytochrome c [25]. In this step, Bax/Bak is activated and forms oligomers in the outer membrane which allow cytochrome c to flow freely into cytosol [25]. Our studies show that mitofilin deficiency induces cytochrome c release and cell apoptosis under the intrinsic apoptotic stimuli, while the activation of Bax remains unaffected. Overexpression of anti-apoptotic factor Bcl-2 inhibits downstream apoptotic events by inhibiting the translocation of Bax to the mitochondria. These data suggest that mitofilin controls cytochrome c release by modulating the cristae remodeling, but does not affect the permeability of the mitochondrial outer membrane.

In conclusion, mitofilin, together with other proteins, forms a functionally complex network that maintains the mitochondrial cristae structure. Mitofilin acts as a gatekeeper to control cytochrome c release from cristae to IMS and further participates in the intrinsic apoptotic pathway.

Acknowledgments

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