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Down-regulation of Mortalin Exacerbates Aβ-mediated Mitochondrial Fragmentation and Dysfunction*

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Background: Mitochondrial dysfunction is associated with neuronal disorders, and mitochondrial dynamics are altered in neurodegenerative diseases.
Results: Inhibition of mortalin potentiates amyloid-β-mediated mitochondrial dysfunction and cytotoxicity.
Conclusion: Inhibition of mortalin could lead to mitochondrial dysfunction through mitochondrial fragmentation.
Significance: Activation of mortalin may antagonize the progression of Aβ-mediated neuronal injury in which mitochondrial dysfunction has a key role.

Mitochondrial dynamics greatly influence the biogenesis and morphology of mitochondria. Mitochondria are particularly important in neurons, which have a high demand for energy. Therefore, mitochondrial dysfunction is strongly associated with neurodegenerative diseases. Until now various post-translational modifications for mitochondrial dynamic proteins and several regulatory proteins have explained complex mitochondrial dynamics. However, the precise mechanism that coordinates these complex processes remains unclear. To further understand the regulatory machinery of mitochondrial dynamics, we screened a mitochondrial siRNA library and identified mortalin as a potential regulatory protein. Both genetic and chemical inhibition of mortalin strongly induced mitochondrial fragmentation and synergistically increased Aβ-mediated cytotoxicity as well as mitochondrial dysfunction. Importantly we determined that the expression of mortalin in Alzheimer disease (AD) patients and in the triple transgenic-AD mouse model was considerably decreased. In contrast, overexpression of mortalin significantly suppressed Aβ-mediated mitochondrial fragmentation and cell death. Taken together, our results suggest that down-regulation of mortalin may potentiate Aβ-mediated mitochondrial fragmentation and dysfunction in AD.

Mitochondria, essential organelles for both life and death, are highly dynamic. They continuously undergo balanced fission and fusion processes, which are termed mitochondrial dynamics. Mitochondrial dynamics greatly affect the mitochondrial functions such as biogenesis as well as their morphology (1, 2). Imbalanced mitochondrial dynamics are directly linked to many human diseases including cancer, diabetes, and neurodegenerative diseases (3–5). Neurons are particularly dependent on mitochondrial function because of their higher metabolic activity and complex morphology (6). Mitochondria are pivotal for synaptic plasticity and the primary producers of reactive oxygen species (ROS), which contribute to mitochondrial dysfunction. Thus, disruptions of mitochondrial function and mitochondrial dynamics are prominent early events in neurodegenerative diseases such as Alzheimer disease (AD),[2] Parkinson disease (PD), Huntington disease, and amyotrophic lateral sclerosis (1, 6). Both the treatment of amyloid-β (Aβ) and the overexpression of either APP (Aβ precursor protein) or APPsw mutant efficiently induce mitochondrial fragmentation and synaptic injury in neuronal cells (7–9).

Mitochondrial fission and fusion processes are regulated by evolutionarily conserved molecular machinery. The large GTPase proteins, MFN1/2 (mitofusin-1/-2) and Opa1 (optic atrophy type 1) assists in the mitochondrial fusion process. Another GTPase protein, Drp1 (dynamin-related protein 1) promotes mitochondrial fission by interacting with mitochondrial outer membrane proteins such as Fis1 and mitochondrial fission factor. Loss-of-function mutations of MFN2 and Opa1 are directly linked to neurodegenerative diseases such as Charcot-Marie-Tooth subtype 2A and autosomal dominant optic atrophy (10, 11). Additionally, a mutation of Drp1 identified in an infant with lethal abnormal brain development also emphasizes the importance of mitochondrial dynamics in neurons (12). As a regulatory mechanism, various post-translational modifications such as phosphorylation, nitrosylation, sumoylation, ubiquitination, or GlcNAcylation of Drp1, proteolytic cleavage of Opa1, and ubiquitination or phosphorylation of

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[2] The abbreviations used are: AD, Alzheimer disease; PD, Parkinson disease; MEF, mouse embryo fibroblast; DIV, days *in vitro*; ROS, reactive oxygen species; DCFA-DA, 2′,7′-dichlorofluorescein diacetate; EGFP, enhanced green fluorescent protein; DCF, dichlorofluorescein; Drp1, dynamin-related protein 1.

MFN1/2 have explained the intricate mechanisms of mitochondrial dynamics in different signals (9, 13–27). Moreover, several other regulatory proteins have been identified. Knockdown of mitochondrial fission factor, GDAP1 (ganglioside-induced differentiation-associated protein-1), MiD49/51 (mitochondrial dynamics protein 49/−51), endophilin B1, or MTP18 (mitochondrial protein 18 kDa) resulted in elongated mitochondria, suggesting that these proteins are involved in the mitochondrial fragmentation processes (28–31). On the other hand, inhibition of prohibitin-2, SLP2 (stomatin-like protein 2), and mitofusion-binding protein promotes mitochondrial fragmentation, indicating that these proteins regulate the mitochondrial fusion process (32–34). Nonetheless, the precise mechanism that coordinates these complex processes of mitochondrial dynamics still remains unclear.

To further understand the regulatory machinery of mitochondrial dynamics, we established a cell-based functional screening system that identified mortalin as a potential regulatory molecule from siRNA library screening. The suppression of mortalin highly induced mitochondrial fragmentation and the suppression synergistically increased Aβ-mediated mitochondrial dysfunctions and cell death. However, up-regulation of mortalin remarkably reduced Aβ-mediated mitochondrial fragmentation and cytotoxicity. Importantly, we found that the expression level of mortalin was reduced in AD patients and in the AD-model mice. Taken together, our results suggest that down-regulation of mortalin exacerbates Aβ-mediated mitochondrial fragmentation and dysfunction in AD.

EXPERIMENTAL PROCEDURES

Cell Culture and Measurement of Mitochondrial Length—SK-N-MC and SH-SY5Y neuroblastoma cells were obtained from the American Type Culture Collection (ATCC). Drp1-deficient mouse embryo fibroblast (MEF) cells were generously provided by Dr. Katsuyoshi Mihara (Kyushu University, Japan) (35). All cells were cultured at 37 °C in a 5% CO_2 incubator and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen). To generate stable cell lines (SK/mito-YFP, SY5Y/GFP, and SY5Y/GFP-MOT), SK-N-MC were transfected with pmito-YFP and SH-SY5Y were transfected with either pEGFP or pEGFP-mortalin using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Stable transfectants were selected by growth in selection medium containing G418 (1 mg/ml) for 10 days. After single cell dropping, the stable clones were selected under a fluorescence microscope and confirmed by Western blot analysis. Mean mitochondrial length in SK/mito-YFP cells was determined by measuring at least 20 individual mitochondria from images of multiple cells obtained by fluorescence microscopy.

Primary Cortical Neuron Culture—Cortices of 14-embryonic day old ICT mouse were dissected and triturated in Hanks' balanced salt solution containing glucose (5 mg/ml) and sucrose (7 mg/ml). Four hemispheres were plated onto a culture plate coated with poly-L-lysine (100 μg/ml) (Sigma) and laminin (1 μg/ml) (Sigma). Neurons were grown in DMEM (Invitrogen) containing 5% FBS (Hyclone, South Logan, UT), 5% horse serum (HS) (Invitrogen), glutamine (2 mM), and penicillin/streptomycin (Lonza, Basel, Switzerland). To kill proliferating glial cells and make pure neuronal culture, we treated the culture with arabinofuranosyl cytidine (10 μM) at 3 days *in vitro* (DIV). All experiments were performed at DIV 7.

Reagents—A YFP-fused MitoTracker plasmid (pmito-YFP) was provided by Dr. Yoon, GS (Ajou University, Korea) (36). Mortalin expression plasmid (pEGFP-mortalin) and MKT077 were kindly provided from Dr. Renu Wadhwa (National Institute of Advanced Industrial Science and Technology, Japan) (37, 38). Amyloid-$\beta_{(1-42)}$ (Aβ) was purchased from American Peptide Co. (Sunnyvale, CA). A MitoTracker® probe and Hoechst 33342 dye were purchased from Invitrogen. The validated siRNA targeting for mortalin (number 1, 5′-GACUAUCGCUCCAUGCCAA-3′ and number 2, 5′-AAACGCAAGUGGAAAUUAA-3′) and negative scrambled siRNA (5′-CCUACGCCACCAAUUUCGU-3′) were purchased from Dharmacon (Thermo Scientific) and previously validated Drp1 siRNA (5′-GAGGUUAUUGAACGACUCA-3′) and Opa1 siRNA (5′-CUGGAAAGACUAGUGUGUU-3′) were synthesized from Bioneer (Daejeon, Korea) (39).

Cell-based Functional Screening with siRNA Library—For the siRNA screening, we listed mitochondrial proteins based on a mitochondrial protein database (MitoProteom database). Among ~850 genes, we collected around 500 target genes by exclusion of unknown genes. Using the collected genes, we synthesized a custom siRNA library/mitochondrial siRNA library using the Dharmacon siGENOME SMART pool system (Dhamacon, Thermo Scientific). SK/mito-YFP (SK-N-MC stably expressing mito-YFP) cells were seeded in 96-well plates at ~1500 cells/well. After 24 h, each siRNA was transiently transfected into the cells with a final concentration ~50 pmol. After 3 and 7 days, mitochondrial morphology was observed under a fluorescence microscope to screen mitochondria dynamics regulator. siRNAs for OPA1 and Drp1 were used as positive controls for each experiment. The screening experiment was repeated two times with consistent results.

Western Blotting—For Western blotting, all lysates were prepared with protein sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromphenol blue) (Bio-Rad). Then the samples separated by SDS-PAGE were transferred to PVDF membrane (Bio-Rad). After blocking with 4% skim milk in TBST (25 mM Tris, 3 mM 140 mM NaCl, 0.05% Tween 20), the membranes were incubated overnight with specific primary antibodies at 4 °C. Anti-Drp1 antibody was from BD Biosciences (San Jose, CA); anti-mortalin antibody was from BD Biosciences; anti-Actin antibody was from Millipore (Temecula, CA). For protein detection, the membranes were incubated with HRP-conjugated secondary antibodies (Pierce).

ROS Measurement—Intracellular ROS levels were assayed using a fluorescent dye, dichloro-dihydro-fluorescein diacetate (DCFH-DA) (Invitrogen), which is converted to highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of oxidant. Briefly, cells plated in 96-well plate were transfected with siRNA. After 3 or 7 days from transfection, the cells were further treated with Aβ for 24 h. Then, the cells were incubated with DCFH-DA (20 μM) in serum-free medium for 30 min and measured the fluorescence (excitation/emission wavelength

358/485) (Victor X3, PerkinElmer Life Sciences). Relative ROS ratio was presented as the change in fluorescence of drug-treated samples compared with that of control samples.

Measurement of Mitochondria Membrane Potential and ATP Level—Mitochondrial membrane potential was examined with a unique fluorescent cationic dye, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, BD Biosciences) that detects loss of signal of mitochondrial membrane potential. The fluorescence intensity was measured using plate reader (PerkinElmer Life Sciences) at excitation and emission wavelengths of 485 and 535 nm, respectively, for the monomeric form as well as 535 and 590 nm for J-aggregate forms, respectively. Images were obtained using IX71 (Olympus, Tokyo, Japan) fluorescence microscopes and the cellular total ATP level was detected with an ATP bioluminescence detection kit (Promega, Madison, WI) according to the manufacturer's protocol.

Cell Proliferation Analysis—For the cell proliferation assay, cells seeded in 96-well plates were transfected with mortalin siRNA. After transfection, the cell proliferation rate was measured daily using a Cell Counting Kit-8 (CCK8) solution reagent (10 μM) (Dojindo Laboratories, Kumamoto, Japan) for 2 h. The absorbance was measured with spectrophotometer (Victor-X3, PerkinElmer Life Sciences).

Apoptotic Cell Death Analysis—Apoptotic cell death was determined using an Annexin V-FITC/PI Apoptosis Detection Kit (BD Pharmingen) according to the manufacturer's protocol. Briefly, cells transfected with mortalin siRNA or treated with MKT077 were exposed to Aβ for 24 h. Then, the cells were stained with Annexin V-FITC and propdium iodide (BD Pharmingen). After staining, the cell death ratio was analyzed using a flow cytometer (BD Pharmingen).

Mouse and Human AD Samples—Triple transgenic AD mice (3xTgAD mice; swAPP, PS1-M146V, Tau-P301L) (40) that had been backcrossed to C57BL/6 mice for 8 generations were maintained in our animal facility under pathogen-free conditions. 3-, 6-, and 12-month-old male mice were euthanized, and brains were removed for analysis ($n = 5$). Inferior parietal lobule and cerebellum specimens from the brains of 5 patients with AD and 5 control subjects that had been enrolled in the University of Kentucky Alzheimer's Disease Center Autopsy Program were used for this study. All patients with AD met both clinical diagnostic criteria and neuropathological diagnostic criteria of AD (41, 42). The control subjects had no history or neuropathological signs of a brain disorder. At autopsy, tissue specimens were rapidly removed and frozen, and were stored at −80 °C.

Statistical Analysis—Data were obtained from least three independent experiments, and presented as mean ± S.E. Statistical evaluation of the results was performed with one-way analysis of variance. Data were considered significant at a value of $p < 0.05$.

RESULTS

Down-regulation of Mortalin Induces Drp1-dependent Mitochondrial Fragmentation and Dysfunction—It has been previously reported that there are ~850 proteins in mitochondria (43). For cell-based functional screening, we generated a mitochondrial siRNA library as described under "Experimental Procedures." In addition, we also established a cell-based functional screening system using SK-N-MC cells that stably expressed the YFP-fused mitochondria tracker, mito-YFP (SK/mito-YFP). Initially to identify novel genetic modulators of mitochondrial dynamics, we screened the siRNA library. Both Drp1 siRNA and Opa1 siRNA were used as positive controls in each experiment. Based on the screening results, we identified mortalin as a potent regulator of mitochondrial dynamics (Table 1).

TABLE 1
List of mitochondrial dynamics modulator

Gene name (siRNA)	Mitochondrial fragmentation	Mitochondrial elongation
Drp1	+++	
Mortalin	+++	
PHB1	++	
PHB2	+++	
ATP5I	+++	
STOML2	++	
TXN	++	
VDAC1	++	
Opa1		+++
LETM1		+++
VCP		+

To confirm the screening results, SK/mito-YFP cells were transiently transfected with a scrambled or specific siRNA against mortalin. The results showed that down-regulation of mortalin induced pronounced fragmentation of mitochondria (Fig. 1, *A–D*). Because Drp1 is a key regulator in mitochondrial fission, we next examined the effects of Drp1 on mortalin-mediated mitochondrial fragmentation. Mortalin siRNA was co-transfected with either scrambled or Drp1 siRNA in SK/mito-YFP cells. The knockdown of Drp1 was confirmed by Western blot analysis. The results showed that reduced Drp1 expression completely suppressed mitochondrial fragmentation induced by mortalin knockdown (Fig. 1*E*). Mitochondrial dynamics regulate mitochondrial functions as well as their morphology. Excessive mitochondrial fragmentation induces mitochondrial dysfunctions. Therefore, we addressed the effect of mortalin knockdown on mitochondrial function. Because the electrochemical gradient of mitochondrial membrane is essential for ATP synthesis, we detected both mitochondrial membrane potential and total cellular ATP levels following mortalin knockdown. The suppression of mortalin expression markedly induced mitochondrial membrane depolarization but reduced the cellular ATP levels, suggesting that knockdown of mortalin disrupts the mitochondrial membrane potential in neuroblastoma cells (Fig. 2, *A* and *B*). In addition, the loss of mitochondrial membrane potential was associated with excessive ROS generation. Thus, we further elucidated mitochondrial dysfunction in mortalin down-regulated cells. The suppression of mortalin expression by their siRNAs resulted in enhanced ROS production in neuroblastoma cells, whereas ROS inhibitors significantly suppressed ROS production, which was induced by down-regulation of mortalin (Fig. 2, *C* and *D*). Moreover, we investigated the effect of mortalin knockdown on proliferation. The down-regulation of mortalin suppressed cell proliferation in neuroblastoma cells, respectively (Fig. 2*E*). Taken together, our data suggest that down-regulation of reduced mortalin

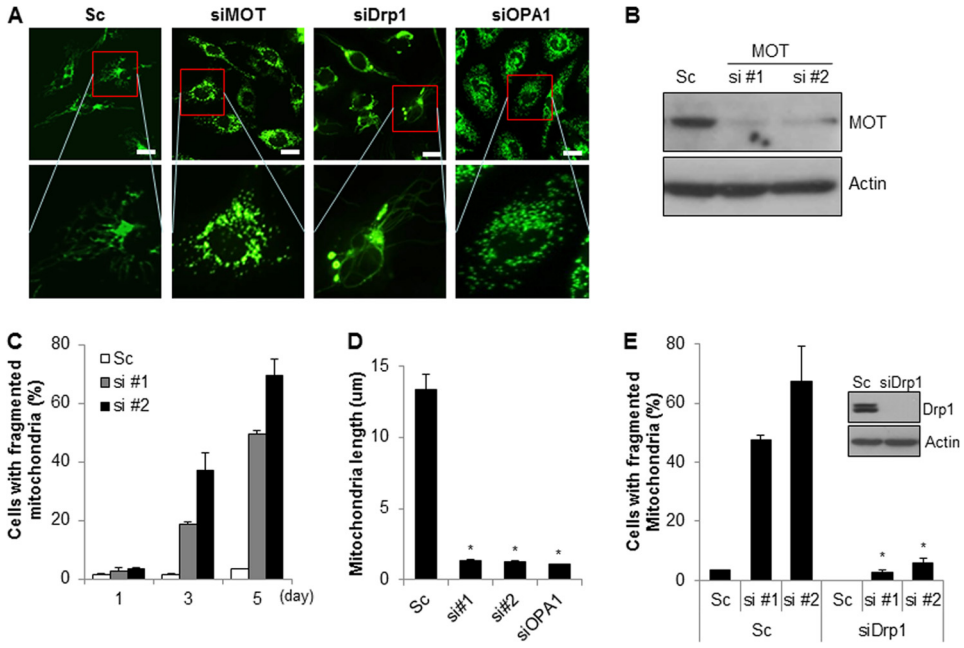

FIGURE 1. **Down-regulation of mortalin induces Drp1-dependent mitochondrial fragmentation and dysfunction.** *A*, SK-N-MC cells stably expressing mito-YFP (SK/mito-YFP) were transfected with either a control scrambled siRNA (*Sc*) or a specific siRNA against mortalin (*siMOT*), and then mitochondrial morphology was observed under a fluorescence microscope. Both Drp1 siRNA (*siDrp1*) and Opa1 siRNA (*siOpa1*) were used as controls. Representative fluorescence pictures of mitochondrial morphology by siRNAs are shown (*bar size*, 20 μm). *B* and *C*, SK/mito-YFP cells were transfected with either control scrambled siRNA (*Sc*) or specific siRNA against mortalin (*si#1* and *si#2*). After 5 days, mortalin expression was analyzed with Western blotting with anti-mortalin antibody (*B*). The mitochondrial morphology was observed under a fluorescence microscope (*C*). *D*, SK/mito-YFP were transfected with either a control scrambled siRNA (*Sc*) or a specific siRNA against mortalin (*siMOT*), and then the mitochondrial length was measured. *E*, Drp1 siRNA was co-transfected with mortalin siRNA (*si#1* and *si#2*) or Sc in SK/mito-YFP cells. After 7 days, mitochondrial fragmentation was measured under a fluorescence microscopy. Reduced expression of Drp1 by siRNA was confirmed by Western blotting. Data are represented as the mean ± S.E. ($n > 3$; *, $p < 0.01$).

induces mitochondrial fragmentation and mitochondrial dysfunction.

MKT077, a Mortalin Chemical Inhibitor Induces Both Mitochondrial Fragmentation and Mitochondrial Dysfunction—With the use of a chemical inhibitor, we investigated the effect of mortalin inhibition on mitochondrial fragmentation and dysfunction. MKT077 is a rhodacyanine dye analog that directly inhibits the function of mortalin (44). To investigate the effect of MKT077 on mitochondrial dynamics, SK/mito-YFP cells were incubated with MKT077 and the mitochondria were monitored (Fig. 3*A*). Similar to the knockdown experiments, treatment with MKT077 strongly increased mitochondrial fragmentation in a dose-dependent manner (Fig. 3*B*). Additionally, mitochondrial fragmentation induced by MKT077 was significantly blocked both in Drp1 down-regulated SK-N-MC cells and in Drp1-deficient MEF cells compared with that of the control cells (Fig. 3, *C* and *D*). These results suggest that MKT077 also induces Drp1-dependent mitochondrial fragmentation. We further investigated the effect of MKT077 on mitochondrial dysfunction. Wild type (WT) and Drp1-deficient MEF cells were exposed to MKT077, and mitochondrial dysfunction was investigated. Although MKT077 treatment enhanced mitochondrial membrane depolarization and ROS production in WT MEF cells, they were efficiently suppressed in Drp1-deficient MEF cells (Fig. 3, *E* and *F*). We further confirmed the effect of mortalin inhibition in primary cultured neurons. Consistently, treatment of MKT077 strongly induced mitochondrial fragmentation in mouse primary cultured neurons (Fig. 4, *A* and *B*). Moreover, treatment of MKT significantly increased mitochondrial membrane depolarization as well as ROS production in mouse primary cultured neurons (Fig. 4, *C–F*). These data suggest that MKT077 promotes mitochondria dysfunction via Drp1 activity.

Down-regulation of Mortalin Potentiates Aβ-mediated Mitochondria Dysfunction and Cytotoxicity—Aβ promotes mitochondrial dysfunction, which contributes to Alzheimer disease pathology. Moreover, it was recently reported that mitochondrial import of mortalin is influenced by Aβ (45). Thus, we examined the role of mortalin knockdown on Aβ-mediated mitochondria dysfunction. SK-N-MC cells transfected with either scrambled siRNA or mortalin siRNA were incubated with Aβ. Then, both the total cellular ATP level and ROS production were measured. Our results showed that the cellular ATP level decreased appreciably by down-regulation of mortlain compared with Aβ-treated control cells (Fig. 5*A*). Consistently, mortalin knockdown also significantly increased ROS generation compared with that of control cells following Aβ treatment (Fig. 5*B*).

Because mitochondrial dysfunction is highly associated with cytotoxicity, we next investigated the effect of mortalin knockdown on Aβ-induced cytotoxicity. SH-SY5Y cells transfected with mortalin siRNA were exposed to Aβ, and the cell death rate was determined by flow cytometry. The results suggested that down-regulation of mortalin more enhanced the Aβ-induced cell death than that of control cells (Fig. 5*C*). We further investigated the effect of mortalin inhibition on Aβ-induced cell death and mitochondrial dysfunction using a chemical

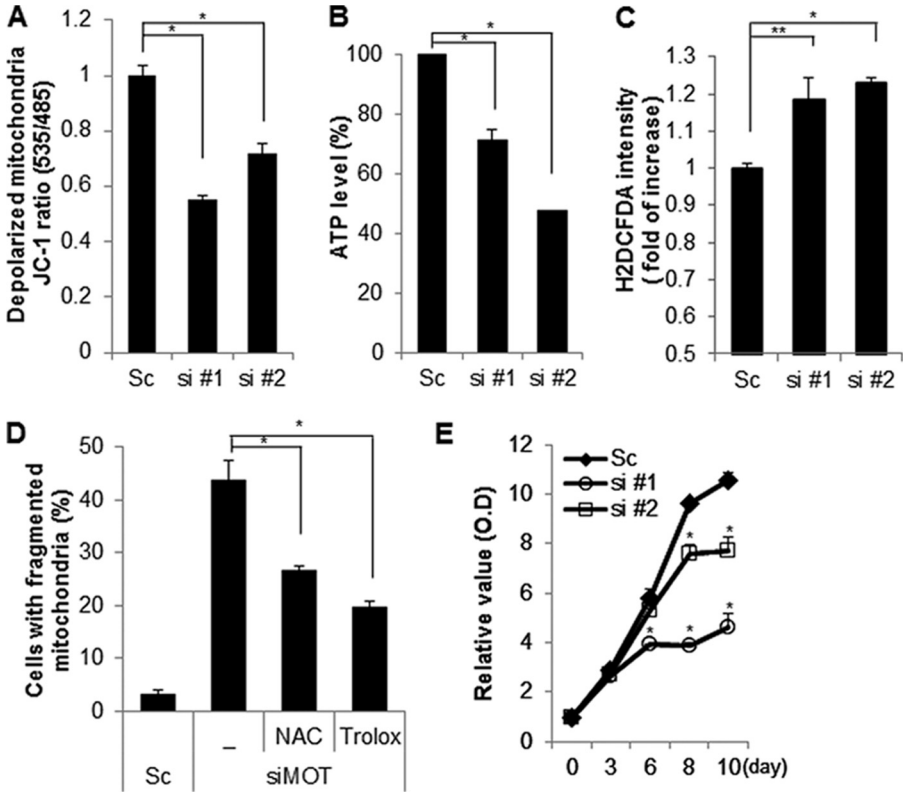

FIGURE 2. **Down-regulation of mortalin induces mitochondrial dysfunction.** *A–C*, SK-N-MC cells were transfected with a control scrambled (*Sc*) or mortalin siRNA (*si #1* and *si #2*). After 7 days, the alteration of mitochondrial membrane potential was monitored by the MitoProbe JC-1 assay (*A*). The cellular total ATP level was examined by an ATP bioluminescence detection assay (*B*). The intracellular ROS level was measured by a DCFH-DA fluorescence ROS detection assay (*C*). *D,* SK-N-MC cells were transfected with Sc or mortalin siRNA (*siMOT*) and additionally incubated with ROS inhibitors (*NAC* and *Trolox*). After 7 days, mitochondrial fragmentation was measured under a fluorescence microscopy. *E,* SK-N-MC cells were transfected with a Sc or mortalin siRNA (*si #1* and *si #2*). The cell proliferation rate was determined daily using a cell proliferation assay kit. Data are represented as the mean ± S.E. ($n > 3$; *, $p < 0.01$; **, $p < 0.05$).

inhibitor (MKT077). SK-N-MC cells were exposed to MKT077 in the presence or absence of Aβ, and then the cellular ATP and ROS levels as well as cytotoxicity were examined. Similar to genetic inhibition, MKT077 treatment synergistically affected the Aβ-mediated reduction of ATP levels, induction of ROS generation, and cell death (Fig. 5, *D–F*). Collectively, these data suggest that mortalin inhibition exacerbates Aβ-induced mitochondrial dysfunction in neuroblastoma cells.

Over-expression of Mortalin Prevents Aβ-mediated Cytotoxicity in Neuroblastoma Cells—Our previous results imply that the inhibition of mortalin is involved in mitochondrial dysfunction, which is associated with cytotoxicity. To demonstrate the effect of mortalin over-expression on mitochondrial morphology and cell death, we established a cell line (SH-SY5Y/mortalin) stably expressing mortain (Fig. 6*A*). Then we examined the effect of mortalin on Aβ-mediated cell death as well as mitochondrial fragmentation. Consistent with mortalin knockdown experiments, both Aβ-mediated mitochondrial fragmentation and cell death were significantly suppressed in mortalin over-expressing cells compared with that of control cells (Fig. 6, *B* and *C*). These results further suggest that over-expression of mortalin prevents Aβ-induced cytotoxicity in neuroblastoma cells.

Mortalin Is Down-regulated in Brain Samples from 3xTg-AD Mice and Human Alzheimer Patients—In recent reports, mortalin has been implicated in neurodegenerative diseases, such as Parkinson disease (46, 47). However, the implication of mortalin expression with AD has not been elucidated. Therefore, we investigated the expressional regulation of mortalin by employing a triple transgenic mouse model of AD (3xTg-AD) harboring three mutant genes: Aβ precursor protein (*APPSwe*), presenilin-1 (*PS1M146V*), and *tauP301L* (40). The endogenous expression of mortalin gradually increased during normal brain development in mice. In contrast, mortalin expression was not increased in the brain of a 3xTg-AD mouse by 12 months (Fig. 7*A*). Importantly, the expression of mortalin in brain tissues from human AD patients was considerably decreased compared with that of age-matched normal controls (Fig. 7*B*). Taken together, these results suggest that mortalin is down-regulated in human AD patients as well as in the AD mice models.

DISCUSSION

Mitochondrial dysfunction is associated with neuropathies, and mitochondrial dynamics are altered in neurodegenerative diseases (1, 48). In this study, we synthesized a siRNA library that consisted of mitochondrial proteins and screened the library to identify novel regulators of mitochondrial dynamics. From the screening, we found several already known mitochondrial dynamics modulators such as prohibitin-2, LETM-1 (leucine zipper-EF hand containing transmembrane protein-1), VCP (valosin-containing protein), VDAC, and some mitochondrial ATPase subunits as well as mortalin (Table 1) (33, 49–51). In this article, we demonstrated that the inhibition of mortalin

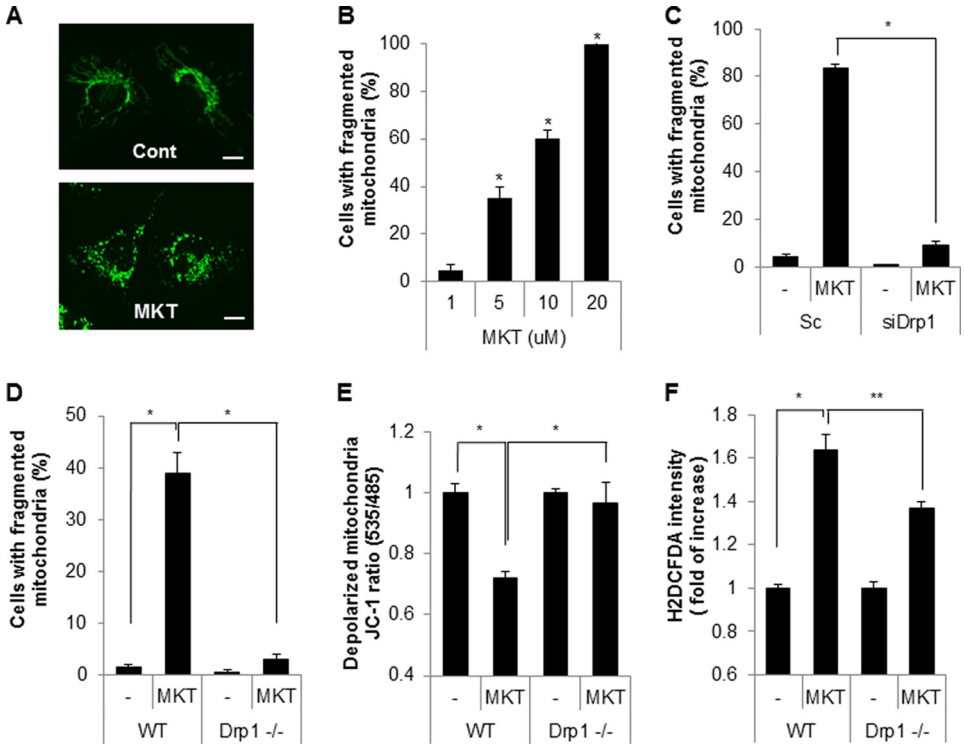

FIGURE 3. **MKT077, a mortalin chemical inhibitor induces Drp1-mediated mitochondrial fragmentation and mitochondrial dysfunction.** *A*, SK/mito-YFP cells were treated with MKT077 (*MKT*, 10 μM) for 4 h and then imaged using a fluorescence microscope. *B*, SK/mito-YFP cells were incubated with increasing concentrations of MKT, and the fragmented mitochondria were observed after 4 h. *C*, SK/mito-YFP cells transfected with either scrambled siRNA (*Sc*) or siRNA against Drp1 (*siDrp1*) were exposed to MKT (10 μM) for 6 h. *D*, mitochondria in wild type MEF (WT) and Drp1-deficient MEF (Drp1$^{-/-}$) treated with MKT077 (10 μM) were labeled with a fluorescence MitoTracker (100 nM). Then, cells with fragmented mitochondria were observed by fluorescence microscopy. *E*, depolarized mitochondrial membrane was analyzed by MitoProbe JC-1 dye in WT and Drp1$^{-/-}$ MEF cells after MKT077 (10 μM) treatment. *F*, WT and Drp1$^{-/-}$ MEF cells treated with MKT077 (10 μM) were stained with DCF-DA, a ROS labeling dye and the intracellular ROS level was assessed by flow cytometric analysis. Data are represented as the mean ± S.E. ($n > 3$; *, $p < 0.01$ or **, $p < 0.05$).

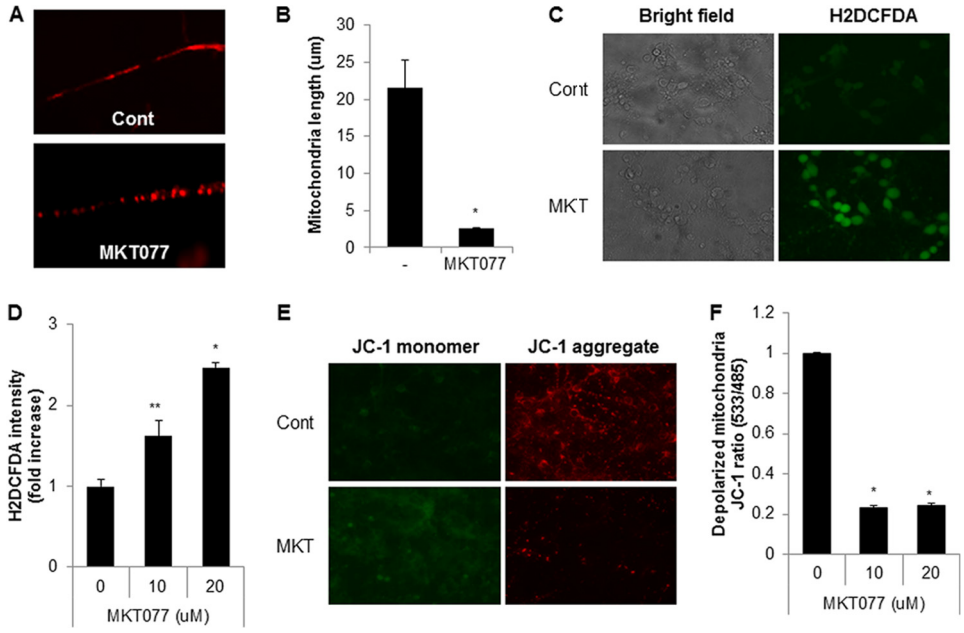

FIGURE 4. **MKT077 also induces mitochondrial fragmentation and mitochondrial dysfunction in primary cultured neuronal cells.** *A* and *B*, pure cortical neuronal cultures (*DIV 7*) were treated with MKT077 (10 μM) for 4 h and stained with a MitoTracker probe. Mitochondrial morphology by MKT077 (*A*) and mitochondrial length was measured (*B*) by fluorescence. *C* and *D*, pure cortical neuronal cultures (DIV 7) were treated with MKT077 (10 or 20 μM) for 4 h and stained with CM-H_2DCFDA (2 μM) for 20 min. The cellular ROS level was observed (*C*), and measured with a fluorescence microplate reader (*D*). *E* and *F*, MKT077-treated cells were stained with JC-1 staining solution and mitochondria dysfunction was measured by a fluorescence microplate reader (*F*), and images were observed under fluorescence microscope (*E*). Data are represented as the mean ± S.E. ($n > 3$; *, $p < 0.02$; **, $p < 0.05$).

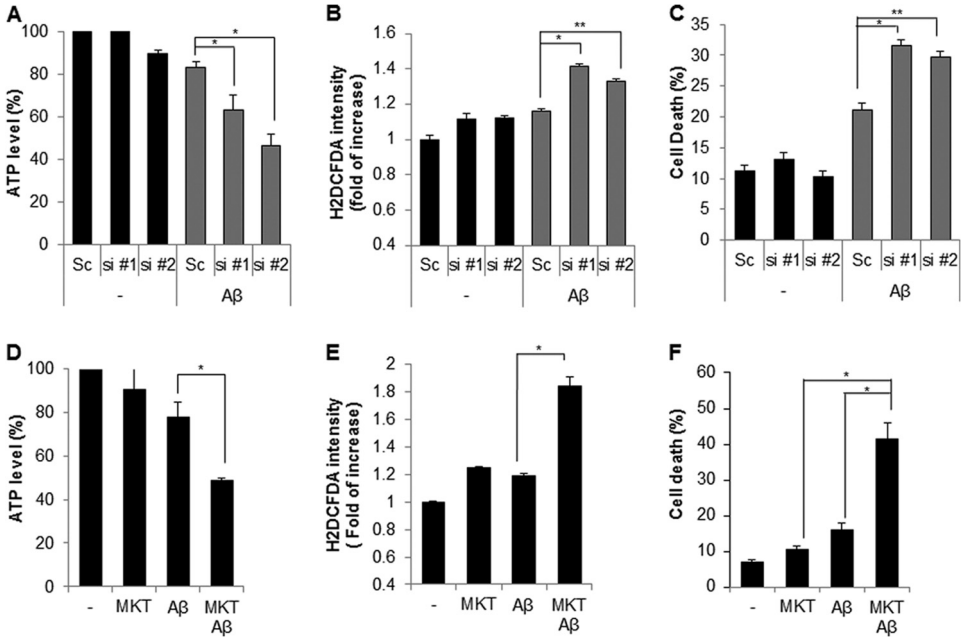

FIGURE 5. **Inhibition of mortalin potentiates Aβ-mediated mitochondria dysfunction and cell death.** *A* and *B*, SK-N-MC cells were transiently transfected with scrambled (*Sc*) or mortalin siRNA (*si #1* and *si #2*). After 3 days the cells were further treated with Aβ (10 μM) or not. The cellular ATP and ROS levels were determined using an ATP bioluminescence detection assay (*A*) or a ROS detection assay (*B*), respectively. *C*, SH-SY5Y cells transiently transfected with scrambled or mortalin siRNA (*si #1* and *si #2*) were treated with Aβ (10 μM) then, apoptotic cell death was measured by a flow cytometric analysis with Annexin V staining. *D-F*, SK-N-MC cells were treated with MKT (10 μM) in the presence or absence of Aβ (10 μM) for 20 h. Then the total ATP cellular level, ROS production, and cell death ratio were also determined. Data are represented by the mean ± S.E. ($n > 3$). Data are considered significant at p values of (*) <0.02 or (**) <0.05.

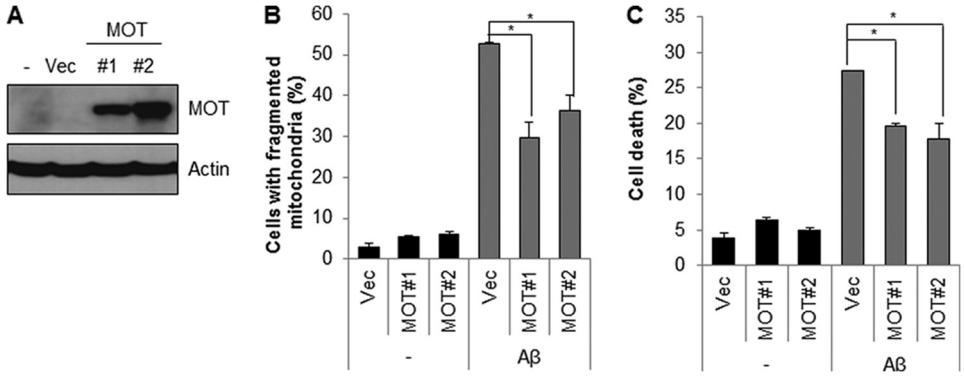

FIGURE 6. **Ectopic expression of mortalin protects Aβ-induced mitochondrial fragmentation and cell death.** *A*, SH-SY5Y cells stably expressing mortalin were generated in SH-SY5Y cells (SY5Y/MOT). Overexpression was confirmed by Western blot analysis with anti-GFP antibody. *B*, SY5Y/MOT (*MOT #1* and *MOT #2*) cells treated with Aβ (10 μM) for 5 h were stained with MitoTracker dye (100 nM). Then, the cells with fragmented mitochondria were counted under a fluorescence microscope. *C*, SY5Y/MOT cells were incubated with Aβ (10 μM) for 24 h and stained with propidium iodide. The apoptotic cell death was measured. Data are represented as the mean ± S.E. ($n = 5$). Data were considered significant at a (*) $p < 0.02$ value.

strongly induced mitochondrial fragmentation and dysfunction. In addition, the expression of mortalin was reduced in brain tissues from the AD mice and AD patients. Mortalin is a mitochondrial chaperone protein and a member of the heat shock protein 70 (HSP70) family (52, 53). However, unlike most other HSP70 members, mortalin is not inducible by heat shock but is sensitive to oxidative stress, glucose deprivation, low-level of radiation, and some cytotoxins (54), suggesting that mortalin is a multifunctional protein in various stress conditions. Moreover, mortalin is a key regulatory protein for the import of mitochondrial ATPase components (55). In fact, HSP70 chaperone activity is important to protein quality control and function in mitochondria (56). According to this notion, our results indicated that mortalin has a crucial role in mitochondrial function. The mitochondrial fragmentation and dysfunction caused by mortalin inhibition were dependent on Drp1, suggesting that mitochondrial chaperon activity modulates function of the mitochondrial dynamics (Figs. 1–3). The immune precipitation assay, to examine the direct interaction between Drp1 and mortalin, suggested that mortalin was not directly interacted with Drp1 (data not shown). However, increased ROS by mortalin inhibition may be a key mediator in Drp1 activation in mortalin inhibition-induced mitochondrial fragmentation (Figs. 2*D*, 3*F*, and 4*C*).

Interestingly, recent proteomic analysis with post-mortem PD substantia nigra showed down-regulation of mortalin in PD brain, and mortalin is also deceased in the 6-hydroxydopamine-treated PD rat models (57, 58). Moreover, several PD-associated genetic variants of mortalin have been identified in PD patients (49). The PD-related mutants of mortalin increase

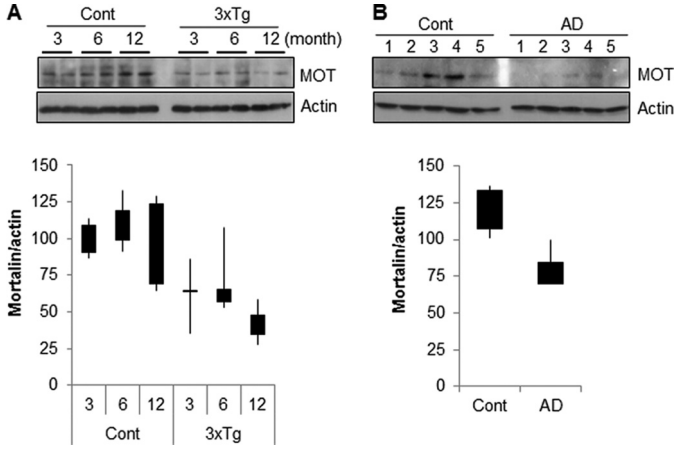

FIGURE 7. **Mortalin expression is down-regulated in Alzheimer disease patient and mice model.** *A*, whole brain extracts from wild type mice (3, 6, and 12 months) and age-matched triple transgenic mice were assessed by Western blotting with mortalin and actin antibodies (*upper panel*). The relative expression value was calculated using densitometer analysis (*lower panel*, $n = 5$). *B*, the expression level of mortalin was examined in brain tissues from normal and age-matched Alzheimer disease patients (*upper panel*) and the relative expression value was analyzed using densitometry (*lower panel*).

both mitochondrial fragmentation and mitochondrial oxidative stress, which eventually impair mitochondrial homeostasis. The mitochondrial dysfunction mediated by down-regulation of mortalin is recovered by over-expression of Parkin, implying that the dysfunction of mortalin is highly associated with PD (59). Moreover, we found that mitochondrial translocation of Parkin was slightly enhanced by treatment of MKT077 (data not shown). However, the roles of mortalin in AD are not well understood. In this study, we examined the expressional regulation of mortalin in AD patients. Our expression analysis revealed that mortalin is down-regulated in brain tissues from 3xTg-AD mouse and AD patients (Fig. 7). Recently, it was shown that ectopic expression of mortalin attenuates Aβ-mediated oxidative stress and neurotoxicity, whereas the suppression of mortalin promotes mitochondrial dysfunction and neuronal injury (60, 61). According to previous results (60, 61), we also observed that the knockdown (by siRNA) and inhibition (by MKT077) of mortalin potentiated Aβ-induced mitochondrial dysfunction and cell death (Fig. 5). Although, the underlying mechanism for mortalin down-regulation in AD and the role of mortalin in Aβ-related neuronal injury are needed to further elucidation in neurodegenerative diseases, our results suggest that down-regulation of mortalin potentiates Aβ-mediated mitochondrial fragmentation and dysfunction in AD. Mortalin expression was differentially regulated in the hippocampus of human APOE4-targeted replacement mice, and the oxidation of mortalin was increased in APOE knock-out mice (62, 63). Oxidized mortalin induced mitochondrial aggregation and resulted in cell death in yeast (64). Therefore, future investigation of the transcriptional and translational regulation of mortalin in AD is needed.

Unlike PD or AD, mortalin is abundantly expressed in many human cancers (65). The up-regulation of mortalin in primary cells reduces cellular senescence and apoptosis but increases the life span of worms (66, 67). Indeed, we observed that over-expression of mortalin suppressed Aβ-induced cell death in neuroblastoma cells (Fig. 5*C*). As a chaperon, mortalin negatively regulates p53, a tumor suppressor protein (65, 68). MKT077 inhibits the chaperone function of mortalin and disrupts the binding of mortalin and p53, resulting in p53 accumulation (44, 69). The p53 protein is up-regulated in the superior temporal gyrus of AD patients and that increases Tau phosphorylation (70, 71). Although the relationship between mortalin and p53 in AD remains unclear, previous results suggest that up-regulation of mortalin reduces neuronal damage in neurodegenerative diseases. Indeed, Wang *et al.* (72) recently concluded that activation of HSP70 reduces neurotoxicity mediated by aggregation of the polyglutamine (poly-Q) protein, which is associated with Huntington disease.

In conclusion, we have suggested that mortalin is down-regulated in AD that could lead to mitochondrial dysfunction through mitochondrial fragmentation. Thus, up-regulation or activation of mortalin through drug targeting may antagonize the progression of AD in which mitochondrial dysfunction plays a key role.

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