Therapeutics

ORIGINAL RESEARCH COMMUNICATION

Mitochondrial Ferritin Attenuates β-Amyloid-Induced Neurotoxicity: Reduction in Oxidative Damage Through the Erk/P38 Mitogen-Activated Protein Kinase Pathways

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Abstract

Aims: Mitochondrial ferritin (MtFt), which was recently discovered, plays an important role in preventing neuronal damage in 6-hydroxydopamine-induced Parkinsonism by maintaining mitochondrial iron homeostasis. Disruption of iron regulation also plays a key role in the etiology of Alzheimer's disease (AD). To explore the potential neuroprotective roles of MtFt, rats and cells were treated with $A\beta_{25-35}$ to establish an AD model. *Results:* We report that knockdown of MtFt expression significantly enhanced $A\beta_{25-35}$-induced neurotoxicity as shown by dysregulation of iron homeostasis, enhanced oxidative stress, and increased cell apoptosis. Opposite results were obtained when MtFt was overexpressed in SH-SY5Y cells prior to treatment with $A\beta_{25-35}$. Further, MtFt inhibited $A\beta_{25-35}$-induced P38 mitogen-activated protein kinase and activated extracellular signal-regulated kinase (Erk) signaling. *Innovation:* MtFt attenuated $A\beta_{25-35}$-induced neurotoxicity and reduced oxidative damage through Erk/P38 kinase signaling. *Conclusion:* Our results show a protective role of MtFt in AD and suggest that regulation of MtFt expression in neuronal cells may provide a new neuroprotective strategy for AD. *Antioxid. Redox Signal.* 18, 158–169.

Introduction

ALZHEIMER'S DISEASE (AD) is a common neurodegenerative disorder. The brains of AD patients are characterized by extracellular plaques of amyloid-β (Aβ), neurofibrillary tangles of tau protein, and loss of memory (24). Aβ plays an important role in the pathophysiological mechanisms of AD (26), as it accumulates to abnormally high levels in the brains of AD patients and has been shown to directly induce neuronal cell death (25). Synthetic analogs of naturally occurring Aβ are often used to model different aspects of AD in animal studies (29). $A\beta_{25-35}$ is a C-terminal peptide of $A\beta_{1-42}$ located in its hydrophobic functional domain (38), and its neurotoxic potency resembles the neurotoxic properties of the parent substance. A single administration of $A\beta_{25-35}$ into the lateral ventricles of mice or rats induces impairment of memory and delayed neurodegeneration in the hippocampus (32). These data confirmed that $A\beta_{25-35}$ is a useful tool for investigation of AD-related mechanisms in animal models (28).

Mitochondrial ferritin (MtFt) was identified as a ferritin heavy-chain-like (H-ferritin) protein in 2001 (15). Both human

Innovation

Previous studies have indicated that mitochondrial ferritin (MtFt) has a role in protecting mitochondria from oxidative damage by sequestering iron in a form that is not easily accessible to Fenton reactions. The underlying neuroprotection mechanism of MtFt in Alzheimer's disease (AD) has not been defined. Here, we reported that MtFt expression increased in the rat brain with aging and that experimentally lowering MtFt levels significantly enhanced $A\beta_{25-35}$-induced neuronal cell neurotoxicity and influenced extracellular signal-regulated kinase/P38 kinase signaling. Appropriate regulation of MtFt expression may prevent the damage to neuronal cells induced by neurotoxins and pathologic conditions such as those observed in AD patients.

and mouse MtFt precursors are imported into the mitochondria and processed into an ∼22-kDa mature protein in the

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mitochondrial matrix (22). In the matrix, these proteins assemble into homopolymeric ferritin shells with ferroxidase activity (14). Both H-ferritin and L-ferritin can sequester and store iron in a safe yet accessible form (1). Excess free iron can generate reactive oxygen species (ROS) during mitochondrial electron transport. Under these conditions, ferritins exert cellular protective roles against iron-mediated free radical damage (8). MtFt has been shown to dramatically modulate cellular iron metabolism (15), and our previous studies have shown that MtFt exerts a neuroprotective effect against 6-hydroxydopamine (6-OHDA)–induced dopaminergic cell damage (27).

In this study, we show that the endogenous expression of MtFt in the hippocampus of rats is age related. Other reports have shown that excessive activation of the mitogen-activated protein kinase (MAPK) pathway is one of the most important signals for neuronal cell death (5, 7). Inhibition of the MAPK pathway could be beneficial in the treatment of neurodegenerative diseases, including AD (23). A recent study confirmed that MtFt expression was significantly increased in the cerebral cortex of AD patients (34). However, the neuroprotective roles and the underlying mechanisms of MtFt action in AD and AD-like syndromes have not been studied. To explore these mechanisms, the $A\beta_{25-35}$ peptide was injected into the hippocampus of rats to produce an AD-like animal model. As in many studies (40), $A\beta$-treated SH-SY5Y cells were used as an AD cellular model. For *in vitro* studies, the mouse MtFt gene was stably transfected into SH-SY5Y cells to investigate the role of MtFt in a cell model of AD induced by $A\beta_{25-35}$. We demonstrated that MtFt could dramatically affect neuronal cell iron metabolism and prevent the neuronal cell damage induced by $A\beta_{25-35}$. We further demonstrate that the MAPK pathway plays an important role in this process.

Results

The expression of ferritins in the hippocampus at different ages in the rat

Ferritin is the major iron storage protein in almost all mammalian cells. We detected three forms of ferritin—H-ferritin, L-ferritin, and MtFt—in the rat hippocampus. As shown in Figure 1A and B, MtFt expression in the hippocampus was low at week 1 and increased with age (at 12, 44, and 88 weeks). L-ferritin expression peaked at week 44. H-ferritin expression increased from week 1 to week 12, reached its maximal value at week 44, and remained at this high level through week 88 (Fig. 1A, B).

The effect of MtFt short interfering RNA on the expression of MtFt and the release of cytochrome C in rats

To determine whether MtFt has protective effects against $A\beta_{25-35}$ neurotoxicity, rats were treated with MtFt short interfering RNA (siRNA). This siRNA specifically knocked down MtFt expression (Fig. 2Ai, Aii) without affecting the ferritin H or L chains (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars). MtFt siRNA treatment eliminated the increase in MtFt expression induced by the injection of $A\beta_{25-35}$.
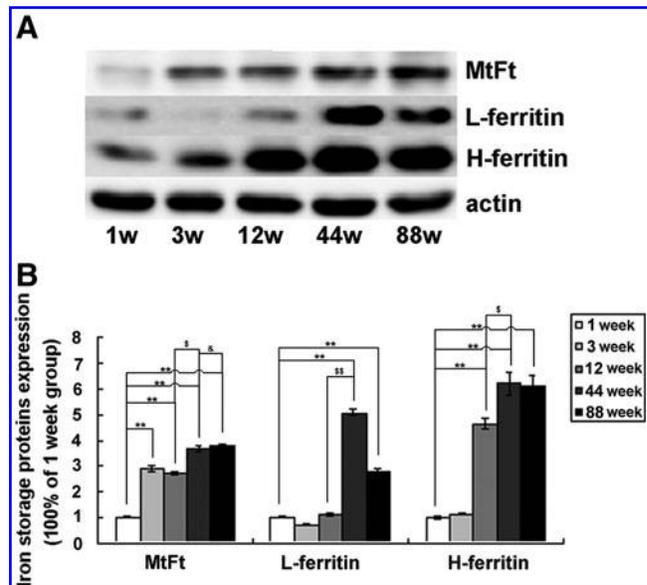

FIG. 1. Endogenous expression of MtFt, L-ferritin, and H-ferritin in the hippocampus of rats of different ages. Rats were studied at 1 (1w), 3 (3w), 12 (12w), 44 (44w), and 88 (88w) weeks of age. Hippocampal extracts were analyzed by western blotting using anti-MtFt, anti-L-ferritin, anti-H-ferritin, and anti-β-actin antibodies **(A)**. Relative band intensities of MtFt, L-ferritin, and H-ferritin are expressed as the ratio of each to β-actin **(B)**. Data are shown as the mean ± SD. **p < 0.01 *versus* 1-week-old rats; $p < 0.05, $$p < 0.01 *versus* 12-week-old rats; &p < 0.05 *versus* 44-week-old rats. MtFt, mitochondrial ferritin; SD, standard deviation.

$A\beta$ accumulation in mitochondria is associated with mitochondrial dysfunctions (12) that include changes in membrane potential, which leads to the release of cytochrome C from the mitochondrial intermembrane space into the cytoplasm. This release of cytochrome C is a sign of mitochondrial-dependent apoptosis (33). Figure 2Bi and Bii shows that $A\beta_{25-35}$ increases the release of cytochrome C. Knockdown of MtFt induced more cytochrome C release into the cytoplasm, which exacerbated the toxicity of $A\beta_{25-35}$. These results indicate that $A\beta_{25-35}$ can induce mitochondrial dysfunction and that MtFt can play a protective role in the process.

The effect of MtFt on $A\beta_{25-35}$-induced neuronal cell apoptosis, malonyl dialdehyde levels, protein carbonyls, and hydroxynonenal–histidine in the hippocampus of rats

$A\beta_{25-35}$ induced prominent cell apoptosis (Fig. 3Ai, Aii). Knockdown of MtFt exacerbated the neuronal cell apoptosis induced by $A\beta_{25-35}$ in the dentate gyrus of the hippocampus (~62%). The predominant cells affected were neurons (Supplementary Fig. S2). Our results also show that the concentrations of malonyl dialdehyde (MDA), protein carbonyls, and hydroxynonenal–histidine (HNE–His) increased in the hippocampus of the control group and control siRNA group after injection of $A\beta_{25-35}$, but this increase was significantly greater when MtFt was downregulated (Fig. 3B–D). This finding is consistent with a previous report that lipid peroxidation increases after treatment with $A\beta$ (13).
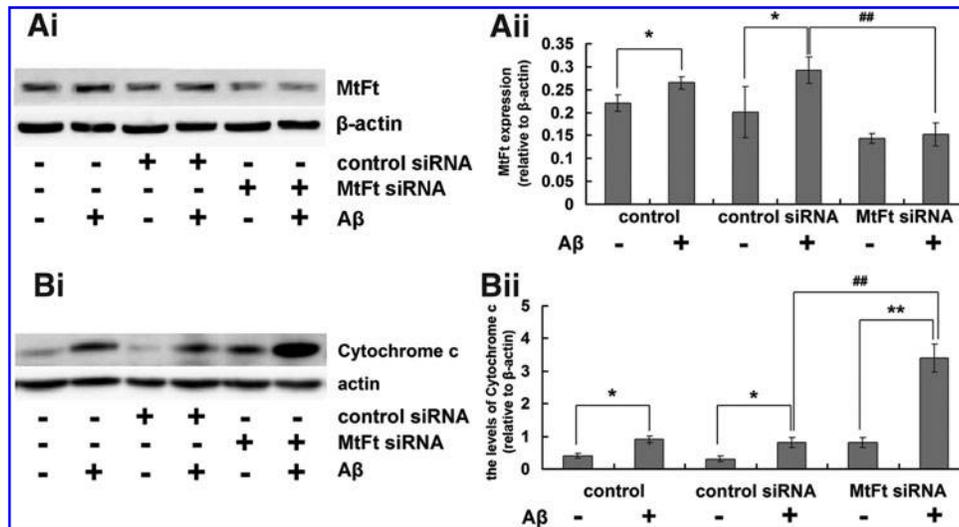
FIG. 2.   Effect of siRNA administration on expression of MtFt and the release of cytochrome C. siRNA administration decreased the expression of MtFt in the hippocampus **(A)** and increased the release of cytochrome C from mitochondria **(B)**. Rats were divided randomly into six groups, and 0.9% saline (groups I and II), control siRNA (groups III and IV), or MtFt siRNA (groups V and VI) was injected into the hippocampus. Groups II, IV, and VI were then injected with 10 μg of Aβ_{25-35} into the hippocampus. The expression of MtFt and the release of cytochrome C into the cytoplasm were assessed by western blotting **(Ai, Bi)**. Relative band intensities are shown in **(Aii)** and **(Bii)**. Data are expressed as the mean ± SD, $n = 3$; $*p < 0.05$ *versus* control group; $**p < 0.01$ *versus* control group; $^{\#\#}p < 0.01$ *versus* control siRNA- and Aβ_{25-35}-treated group. Aβ, amyloid-β; siRNA, short interfering RNA.

The effect of MtFt on the Bcl-2/Bax ratio, caspase-3 activation, and extracellular signal-regulated kinase/ P38 MAPK cell signaling in rats

The ratio of Bcl-2/Bax has been widely used to monitor the degree of apoptosis (27). Injection of MtFt siRNA significantly decreased the ratio of Bcl-2 to Bax (Fig. 4Ai, Aii) and increased the levels of cleaved caspase-3 (Fig. 4Bi, Bii) after Aβ_{25-35} treatment in rats. These results indicate that decreased expression of MtFt can prominently influence the Bcl-2/Bax ratio and lead to caspase-3 activation and a resulting increase in apoptosis after Aβ_{25-35} treatment in rats. The activation of MAPK is implicated in oxidative-stress-induced cell death. As shown in Figure 4Ci and Cii, Aβ_{25-35} significantly induced the activation of P-P38 in the hippocampus. After treatment with MtFt siRNA, P-P38 levels were remarkably enhanced, and the level of P-extracellular signal-regulated kinase (Erk) was less than that of the control and control siRNA groups after treatment with Aβ_{25-35} in hippocampus (Fig. 4Di, Dii).

The effect of MtFt on Aβ_{25-35}-induced neuronal cell toxicity in vitro

Cells stably transfected with MtFt showed increased expression of MtFt mRNA and protein (Fig. 5A, B). The endogenous expression of human MtFt was not changed by MtFt transfection (Fig. 5C). MtFt was slightly increased by Aβ_{25-35} treatment in MtFt-overexpressing cells (Fig. 5D). The ratios of apoptotic control (pcDNA3.1 transfected) cells increased (to ~15%), but MtFt-overexpressing cells showed no significant difference (Fig. 5Ei, Eii) following Aβ_{25-35} treatment. These findings suggest that MtFt has a neuroprotective role against Aβ_{25-35}-induced cell damage. We further examined the effect of MtFt on cell viability using the MTT method. Treatment with Aβ_{25-35} significantly decreased the viability

of SH-SY5Y and pcDNA3.1-transfected SH-SY5Y cells (Fig. 5F) by ~65% in each case; however, in the MtFt-transfected cells, viability decreased only slightly, and this change in viability was significantly different than that in the pcDNA3.1-transfected group. A similar protective role of MtFt on Aβ_{1-42}-induced neurotoxicity was also detected (Supplementary Fig. S3).

The effect of MtFt on Aβ_{25-35}-induced ROS generation in SH-SY5Y cells

6-OHDA can significantly increase ROS production in SH-SY5Y and pcDNA3.1 cells, resulting in cell apoptosis (27). ROS production increased more than twofold in untransfected and pcDNA3.1-transfected SH-SY5Y cells after Aβ_{25-35} treatment; however, ROS production did not change in MtFt-overexpressing cells (Fig. 6A). Thus, overexpression of MtFt can largely prevent Aβ_{25-35}-induced ROS generation in cells.

MtFt expression attenuates the Aβ_{25-35}-induced reduction in mitochondrial membrane potential and prevents cytochrome C release

Measuring the mitochondrial membrane potential (MMP) in living cells is commonly used to assess the metabolic activity and functional integrity of mitochondria (41). To examine the underlying protective mechanism of MtFt in mitochondria, MMPs were measured. Aβ_{25-35} significantly decreased the MMP in SH-SY5Y and control cells but not in MtFt-overexpressing cells (Fig. 6B). These results indicate that MtFt expression is able to prevent the loss of membrane integrity and protects against the mitochondrial damage induced by Aβ_{25-35}.

Release of cytochrome C into the cytoplasm is characteristic of apoptosis, and it can activate a series of apoptosis cascades
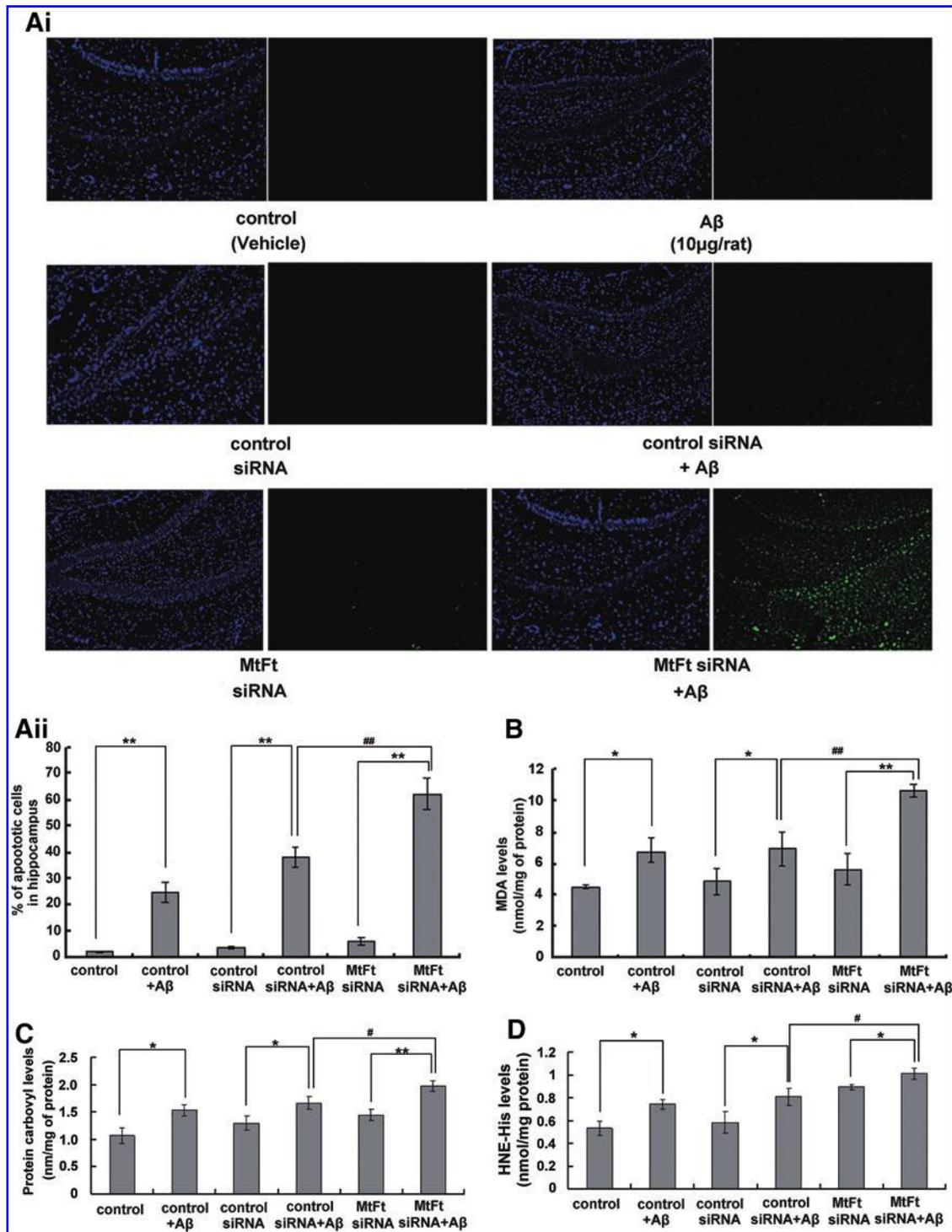
FIG. 3.   The effect of MtFt expression on Aβ$_{25-35}$-induced cell apoptosis and oxidative damage. Apoptotic cell death was determined by DAPI and TUNEL staining as described in the "Materials and Methods" section **(Ai)**. DAPI-stained TUNEL-positive cells were counted in three separate locations and are expressed as a percentage of total cells. Values are presented as the mean±SD from three rats **(Aii)**. The levels of MDA **(B)**, protein carbonyls **(C)**, and lipid peroxidation **(D)** after treatment with MtFt siRNA and Aβ$_{25-35}$ in the rat hippocampus are shown. *$p < 0.05$ *versus* control group; **$p < 0.01$ *versus* control group; #$p < 0.05$ *versus* control siRNA- and Aβ$_{25-35}$-treated group; ##$p < 0.01$. (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars.) DAPI, 4',6-diamidino-2-phenylindole; MDA, malonyl dialdehyde; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay.
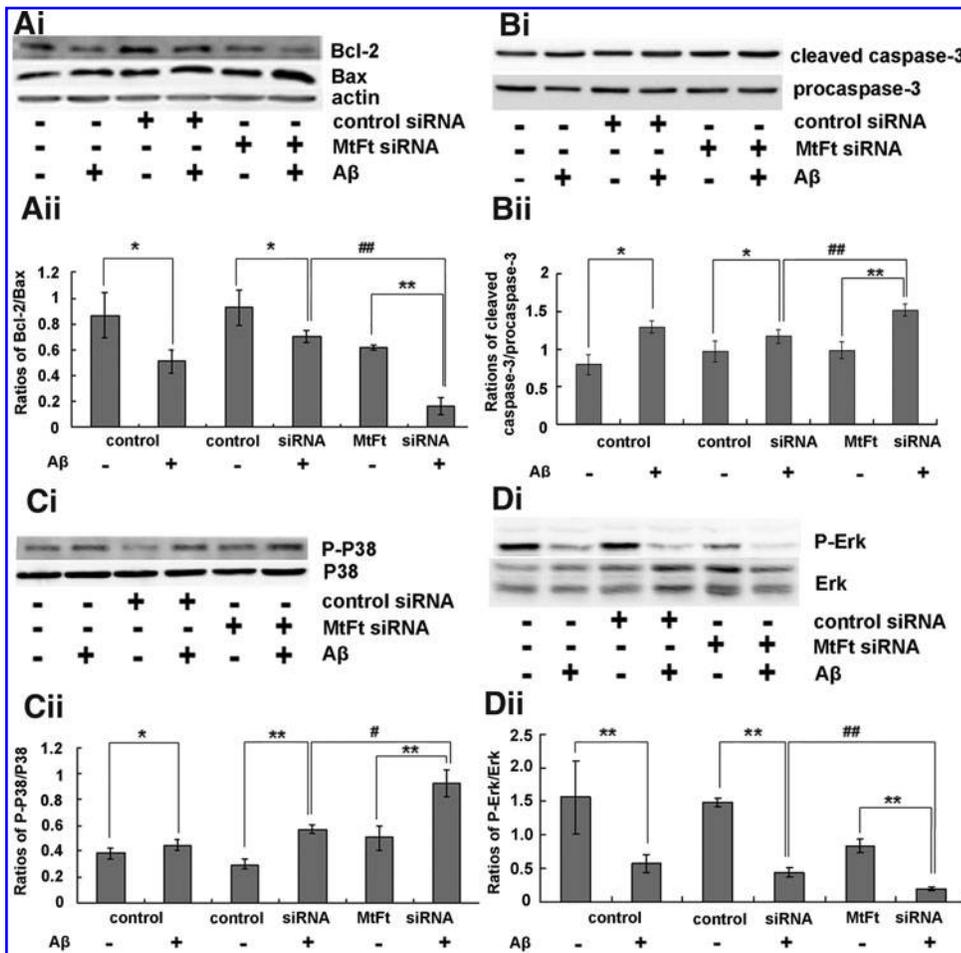
FIG. 4. The effect of MtFt expression on Aβ$_{25-35}$-induced activation of apoptosis-related proteins and MAPK signaling. Treatment with Aβ$_{25-35}$ decreased the ratio of Bcl-2 to Bax. After knockdown of MtFt, this ratio was significantly further decreased ($p < 0.01$) (Ai, Aii). The reduction in MtFt expression also significantly increased the content of cleaved caspase-3 (Bi, Bii). MAPK cell signaling components were detected by western blotting as described in the "Materials and Methods" section (Ci, Di), and relative band intensity is shown in (Cii) and (Dii). Data are expressed as the mean ± SD, $n = 3$; *$p < 0.05$ versus control group; **$p < 0.01$ versus control group; ##$p < 0.01$ versus control siRNA- and Aβ$_{25-35}$-treated group. MAPK, mitogen-activated protein kinase.

leading to cell death. Figure 6Ci and Cii shows that Aβ$_{25-35}$ significantly increased the release of cytochrome C in SH-SY5Y and control cells but not in MtFt-overexpressing cells. These results further emphasize that MtFt expression strongly prevents the apoptosis of SH-SY5Y cells associated with Aβ$_{25-35}$ treatment.

The effect of MtFt on the Bcl-2/Bax ratio and caspase-3 activation in cells

In vivo, we found that the ratio of Bcl-2 to Bax decreased and that cleaved caspase-3 levels increased significantly after knocking down MtFt in the Aβ$_{25-35}$-treated rat hippocampus. In contrast, the ratio of Bcl-2 to Bax did not change noticeably in MtFt-overexpressing cells when cells were treated with Aβ$_{25-35}$. However, in SH-SY5Y and pcDNA3.1 cells, the Bcl-2/Bax ratio decreased significantly compared with the control group (Fig. 7Ai, Aii). Aβ$_{25-35}$ treatment dramatically activated caspase-3 in SH-SY5Y and pcDNA3.1 cells but not in MtFt cells (Fig. 7Bi, Bii). These observations indicate that MtFt can maintain the normal ratio of Bcl-2 to Bax and prevent caspase-3 activation to protect the cells against Aβ$_{25-35}$ toxicity.

MtFt represses Aβ$_{25-35}$-induced activation of Erk/P38/ MAPK in cultured cells

The activation of MAPK is implicated in oxidative-stress-induced cell death. MtFt overexpression blocked the activation of P-P38 and P-Erk (Fig. 7C, D). These results indicate

that Erk/P38/MAPK signaling may be involved in MtFt-mediated protection against Aβ$_{25-35}$ toxicity in cultured cells.

MtFt contributes to the oxidative damage and signaling associated with endogenous amyloid precursor protein

To determine whether MtFt can block endogenously derived amyloid precursor protein (APP)–induced oxidative damage, we transiently transfected plasmids that expressed APP into MtFt cells (Fig. 8A). MMP and cell viability analyses indicated that MtFt expression reduced the damage induced by APP (Fig. 8B, C). The P-P38/P38 and P-Erk/Erk ratios also changed significantly after APP transfection into MtFt cells (Fig. 8D, E). These results indicate that MtFt has a role not only in ameliorating Aβ$_{25-35}$ damage but also in reducing the toxicity associated with APP.

MtFt decreases labile iron pool levels by regulating iron redistribution in Aβ$_{25-35}$-treated cells

Iron is an essential cofactor for many proteins, particularly those involved in oxidative metabolism; however, excess free iron contributes to the enhanced generation of ROS and oxidative stress (39). To clarify the mechanisms by which MtFt depresses ROS levels in Aβ$_{25-35}$-treated cells, we measured the levels of the labile iron pool (LIP). MtFt overexpression slightly decreased LIP levels but dramatically inhibited the elevation of LIP levels in cells treated with Aβ$_{25-35}$ (Fig. 9A).
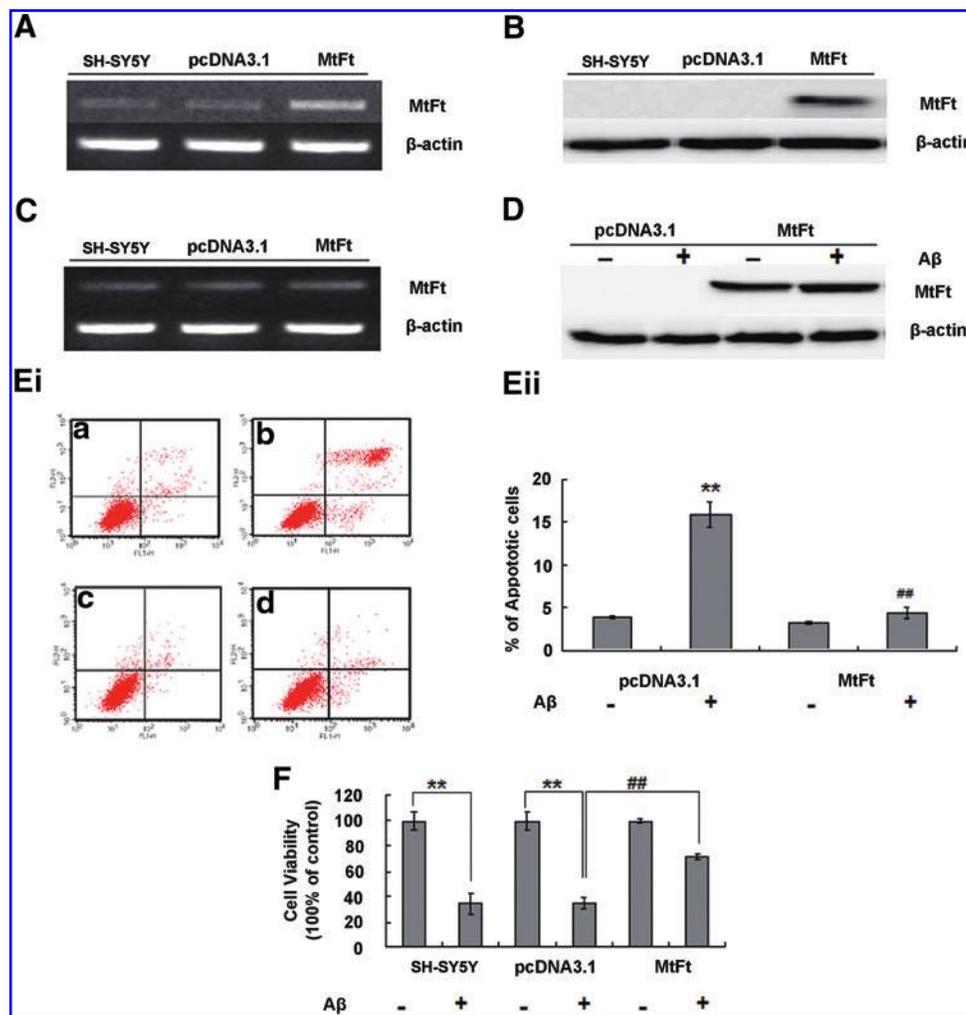
FIG. 5. **The overexpression of mouse MtFt in SH-SY5Y cells and its effects on cell viability and apoptosis following treatment with Aβ$_{25-35}$.** Overexpression of MtFt in the stably transfected SH-SY5Y cells was detected by RT-PCR **(A)** and by western blotting using an anti-HA antibody **(B)**. Endogenous human MtFt in SH-SY5Y cells was also detected with RT-PCR. No differences between the three types of cells were observed **(C)**. After treatment with Aβ$_{25-35}$, the expression of MtFt increased in the MtFt cells **(D)**. Wild-type SH-SY5Y cells (SH-SY5Y), empty vector transfectants (pcDNA3.1), and MtFt transfectants (MtFt) were treated with 25 μM Aβ$_{25-35}$ for 24 h **(Ei, Eii)**. Cell apoptosis was measured by flow cytometry. Data are expressed as the mean ± SD, $n=3$; **$p<0.01$ compared with the control cells; ##$p<0.01$ *versus* the Aβ$_{25-35}$-treated control cells. **(F)** Cell viability was measured with the MTT assay. Data are expressed as the percentage of the viable cells in the untreated control group ± SD, $n=8$; **$p<0.01$ *versus* the control cells; ##$p<0.01$ *versus* the Aβ$_{25-35}$-treated cells (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars.) RT-PCR, reverse transcription-PCR.

To clarify the change of LIP in MtFt protection, iron-related proteins were also measured. The iron uptake proteins TfR1 and DMT1 (+IRE) decreased and the iron release protein FPN1 increased in pcDNA3.1 cells after Aβ$_{25-35}$ treatment (Fig. 9B, C). These changes in protein expression are consistent with increased levels and regulation of cytoplasmic iron *via* the IRE–IRP system. Consistent with this idea, H-ferritin levels also increased (Fig. 9B). Interestingly, DMT1 (−IRE) expression also significantly increased (Fig. 9C), which may explain why iron levels increased in Aβ$_{25-35}$-treated cells. Overexpression of MtFt increased TfR1 and decreased H-ferritin levels (Fig. 9Bi, Bii), suggesting a reduction in cytoplasmic iron levels, which is in accord with our previous studies (27). After treatment with Aβ$_{25-35}$, TfR1, DMT1 (+IRE) or DMT1 (−IRE), FPN1, and ferritin did not change significantly in the MtFt cells (Fig. 9B, C). These results may

explain why LIP increases in the SH-SY5Y and pcDNA3.1 cells but not in the MtFt cells; MtFt protein acts to redistribute iron from the cytoplasm to the mitochondria, thereby decreasing LIP after treatment with Aβ$_{25-35}$. The increase in DMT1 (−IRE) levels was restrained by MtFt overexpression, which decreased the iron uptake induced by Aβ$_{25-35}$ treatment.

Discussion

Iron is absolutely required by virtually every cell, and cells of the central nervous system, which have high levels of metabolic activity and oxygen consumption, have particularly high iron requirements. However, excess iron can catalyze the generation of ROS (27); therefore, iron is considered an important factor contributing to neurotoxicity in several
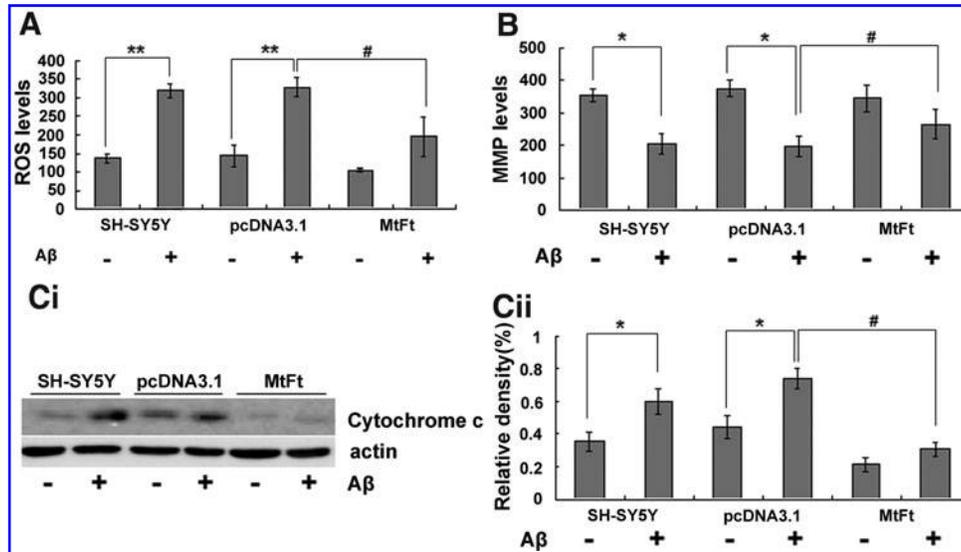
FIG. 6.   Effect of MtFt expression on ROS, MMP, and the release of cytochrome C. MtFt expression attenuates $A\beta_{25-35}$-induced accumulation of ROS **(A)**, MMP **(B)**, and the release of cytochrome C **(Ci, Cii)**. Cells were treated with 25 μM $A\beta_{25-35}$ for 24 h, and ROS levels were detected by measuring 2,7-dichlorofluorescein diacetate fluorescence. MMP levels were detected *via* the fluorescence of rhodamine 123, and the release of cytochrome C into the cytoplasm was detected by western blotting. Data are expressed as the mean ± SD, $n = 3$; *$p < 0.05$ *versus* the control cells; **$p < 0.01$ *versus* the control cells; #$p < 0.05$ *versus* the $A\beta_{25-35}$-treated control cells. MMP, mitochondrial membrane potential; ROS, reactive oxygen species.
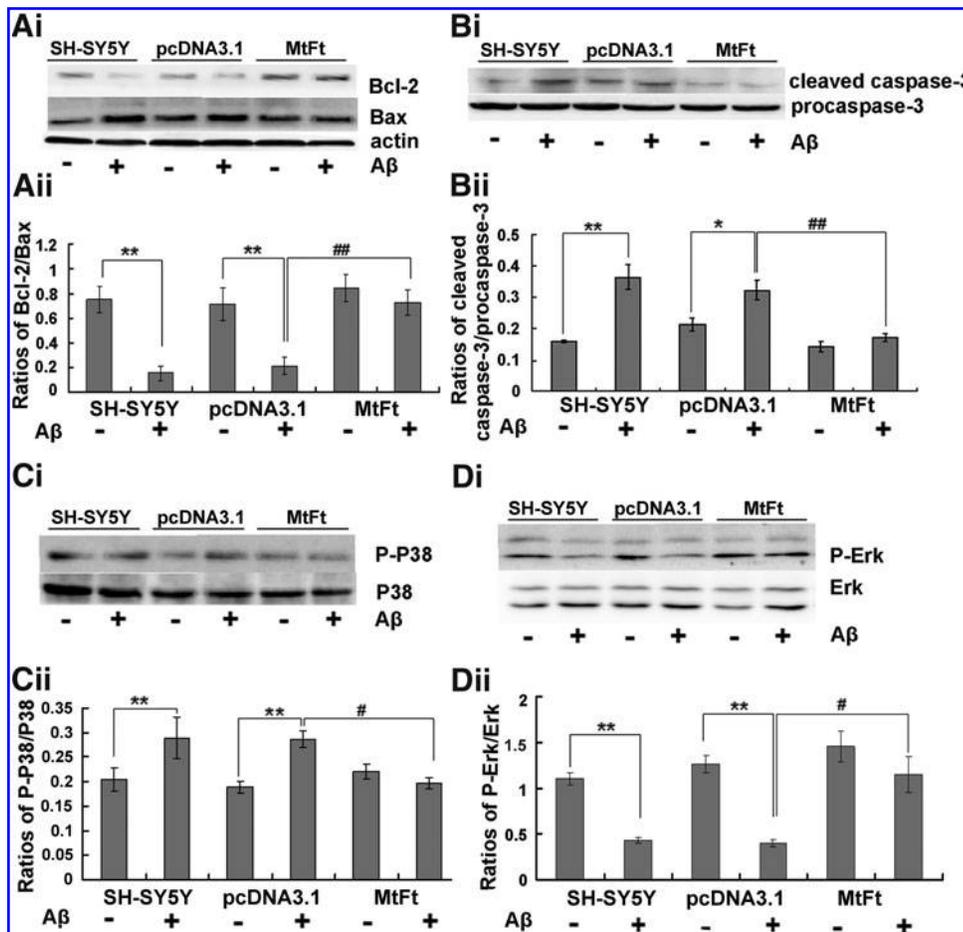

FIG. 7.   Effect of MtFt on apoptosis-related proteins and MAPK signaling. Overexpression of MtFt attenuates $A\beta_{25-35}$-induced activation of apoptosis-related proteins **(A, B)** and MAPK signaling **(C, D)**. Treatment with $A\beta_{25-35}$ decreased the ratio of Bcl-2 to Bax in SH-SY5Y and pcDNA3.1 cells; however, in MtFt cells, the decrease of this ratio was inhibited compared with the control cells **(Ai, Aii)**. The ratio increased significantly more in MtFt cells treated with $A\beta_{25-35}$ than in the pcDNA3.1 cells. The overexpression of MtFt also decreased the content of cleaved caspase-3 significantly **(Bi, Bii)**. The expression of MAPK signaling proteins was assessed by western blotting as described in the "Materials and Methods" section **(Ci, Di)**, and relative band intensities are shown in **(Cii)** and **(Dii)**. Data are expressed as the mean ± SD, $n = 3$; *$p < 0.05$ *versus* control cells; **$p < 0.01$ *versus* control cells; #$p < 0.05$ *versus* pcDNA3.1 cells treated with $A\beta_{25-35}$; ##$p < 0.01$ *versus* pcDNA3.1 cells treated with $A\beta_{25-35}$.
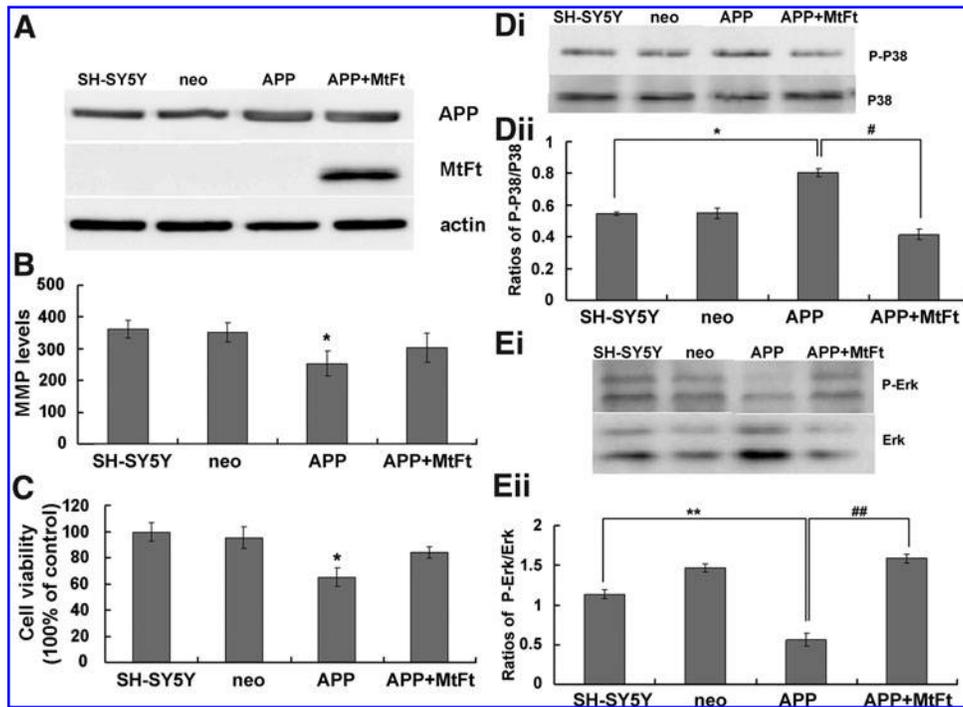
FIG. 8. MtFt expression blocked Aβ-induced oxidative damage and activation of the associated cell signaling pathways. The effectiveness of transient transfection of APP and MtFt in SH-SY5Y cells was assessed by western blotting using anti-APP and anti-HA antibodies **(A)**. Empty-vector-transfected cells (neo) and untransfected SH-SY5Y cells served as control groups. After treating the cells with 25 μM Aβ_{25-35} for 24 h, MMP levels were measured *via* the fluorescence of rhodamine 123 **(B)**, and the MTT assay was used to assess cell viability **(C)**. The expression of MAPK cell signaling components was assessed by western blotting as described in the "Materials and Methods" section **(Di, Ei)**, and relative band intensities are shown in **(Dii)** and **(Eii)**. Data are expressed as the mean±SD, $n=3$; *$p<0.05$ *versus* control group; **$p<0.01$ *versus* control group; [#]$p<0.05$; [##]$p<0.01$ *versus* control siRNA- and Aβ_{25-35}-treated group. APP, amyloid precursor protein.

FIG. 9. Overexpression of MtFt alters iron homeostasis in SH-SY5Y cells after treatment with Aβ$_{25-35}$. (A) The effect of Aβ_{25-35} treatment on the intracellular LIP. The cells were treated with 25 μM Aβ_{25-35} for 24 h, and the LIP levels were detected with the calcein-AM assay. The effects of MtFt on the expression of TfR1, ferritin, FPN1, DMT1 (−IRE), and DMT1 (+IRE) are shown in **(B, C)**. Total cell extracts were analyzed by western blotting using anti-TfR1 and anti-ferritin **(Bi)**, anti-FPN1, anti-DMT1 (−IRE), and anti-DMT1 (+IRE) antibodies **(Ci)**. Relative band intensities are shown in **(Bii)** and **(Cii)**. Data are expressed as the mean±SD, $n=3$; *$p<0.05$ *versus* control cells; **$p<0.01$ *versus* control cells; [##]$p<0.01$ *versus* pcDNA3.1 cells treated with Aβ_{25-35}. LIP, labile iron pool.
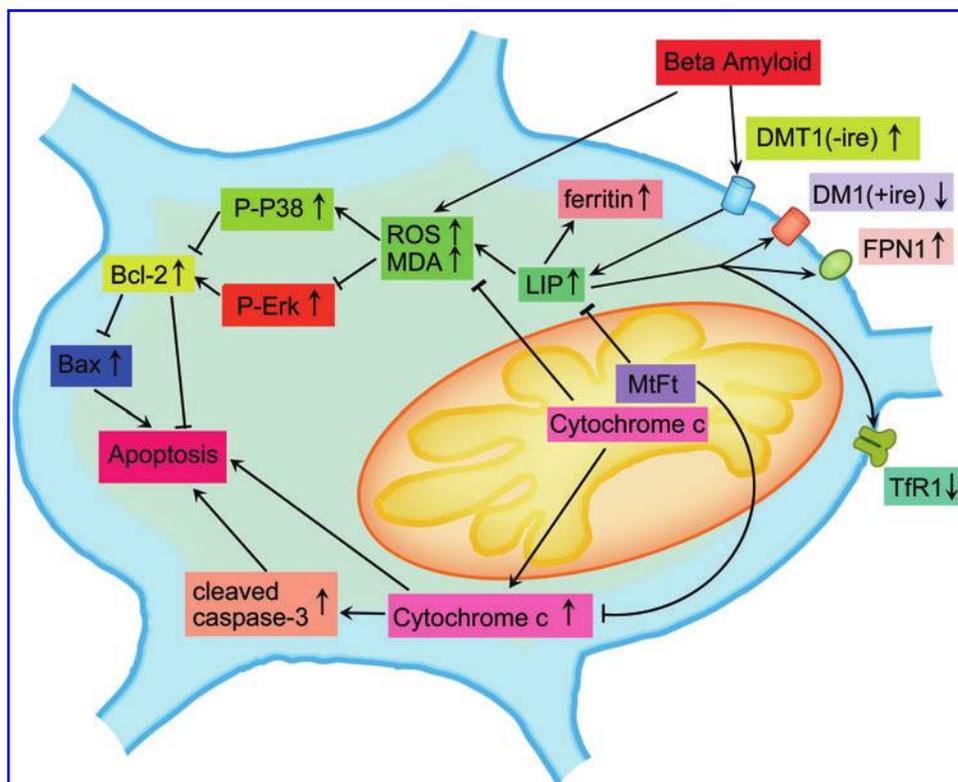

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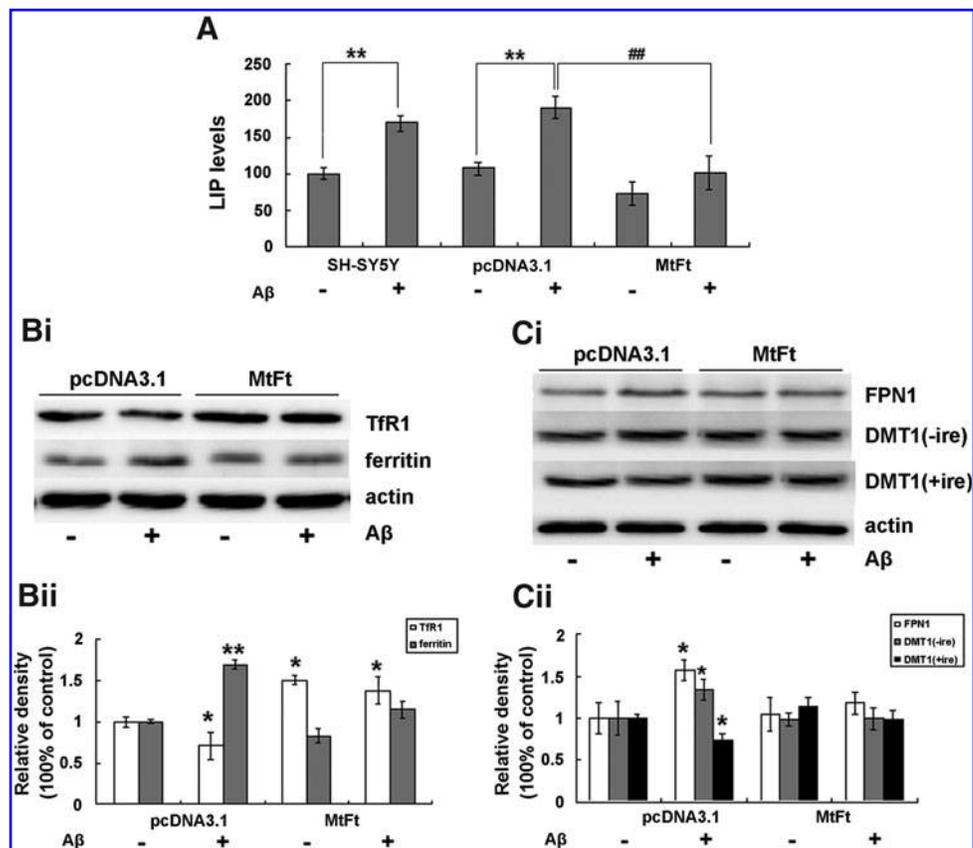
FIG. 10.   A schematic representation of the proposed neuroprotective mechanism of MtFt following treatment with Aβ$_{25-35}$. Aβ$_{25-35}$ changes the expression of iron-related proteins resulting in an increase in the intracellular LIP. This iron may donate electrons for the generation of ROS and lipid peroxidation, and these changes may signal the cell to begin the process of programmed cell death through the MAPK pathway. Alternatively, the overexpression of MtFt may withdraw iron from the cytoplasmic pool, decreasing LIP level. This in turn attenuates Aβ$_{25-35}$-induced neurotoxicity and reduces oxidative damage through Erk/P38 kinase signaling. The MtFt prevents the release of cytochrome C into the cytoplasm; perhaps, it is another pathway to decrease the cell apoptosis (To see this illustration in color the reader is referred to the web version of this article at www.liebertpub.com/ars.)

neurodegenerative disorders, including AD (17). Oxidative stress has been reported in the brains of AD patients. Aβ-associated free radicals can initiate lipid peroxidation, protein oxidation, ROS formation, and mitochondrial Ca^{2+} accumulation, eventually leading to the death of neurons. Other potential sources of oxidative stress in the pathogenesis of AD include the concentration of iron, the activation of microglial cells, and a decrease of complex IV activity. There is strong evidence to suggest that the MAPK pathway is strongly activated in AD (16).

MtFt, a recently discovered H-ferritin-like protein that is expressed only in mitochondria, is found in tissues with high levels of metabolic activity and oxygen consumption, including the testes, brain, heart, and kidneys. This tissue distribution pattern suggests that the major role of MtFt may be to protect mitochondria from iron-dependent oxidative damage. Recent research suggests that MtFt is involved in the pathology of AD and may play a neuroprotective role by reducing oxidative stress; however, the mechanism of this action has not been reported (35).

In the current study, we found that the expression of MtFt increased with age over the period from weeks 12 to 88 in the hippocampi of rats (Fig. 1). Cytochrome C and oxidative stress play a central role in apoptosis by signaling the cell to begin the process of programmed cell death (9). To confirm

the protective role of MtFt in Aβ-induced neurotoxicity, siR-NA was injected into the hippocampus of rats. We demonstrated that decreased expression of MtFt significantly increased the release of cytochrome C into the cytoplasm (Fig. 2B), thereby increasing the number of apoptotic cells (Fig. 3Aii) and decreasing the Bcl-2/Bax ratio (Fig. 4Aii). Thus, MtFt plays a clear neuroprotective role *in vivo*. After treatment with Aβ$_{25-35}$, MtFt knockdown aggravated apoptosis in the hippocampus and oxidative damage to the tissue, as evidenced by increased levels of MDA, protein carbonyls, and HNE–His (Fig. 3A–D). The activities of the mitochondrial complex enzymes I–III (A), II (B), and IV (C) were also significantly decreased (Supplementary Fig. S1). To verify that the increased apoptosis was related to the modulation of MtFt levels, we carried out further studies using SH-SY5Y cells that stably overexpressed MtFt. MtFt overexpression reduced cellular apoptosis in response to Aβ$_{25-35}$ and reduced the production of ROS, as predicted by the animal studies (Figs. 5 and 6). These observations demonstrate that MtFt has a protective effect in the brain, and the regulation of MtFt expression is a potential approach to diminish Aβ$_{25-35}$-induced oxidation and cell apoptosis.

It is well documented that developmental neuronal cell body degeneration requires the apoptotic effectors Bax and caspase-3 (36) and that Bcl-2 protects neurons against

oxidative stress and apoptosis (31). In our studies, we observed an increase in caspase-3 protein, a reduction in the Bcl-2/Bax protein ratio, and a loss of MMP following $A\beta_{25-35}$ treatment (6). MtFt gene knockdown decreased the Bcl-2/Bax ratio and enhanced caspase-3 activation (Fig. 4A, B). Opposite effects were observed when MtFt was overexpressed in SH-SY5Y cells that were treated with $A\beta_{25-35}$ (Fig. 7A, B).

There is a large amount of evidence that demonstrates that Aβ-induced neuronal injury triggers transcriptional and post-transcriptional processes that regulate neuronal fate, including the activation of MAPK pathways by neurotrophins and neurotransmitters and the induction or activation of pro- and antiapoptotic proteins (e.g., those of the Bcl-2 family) (37). Among the MAPK pathways, P38 is often implicated in cell death (4), and Erk has been widely associated with cell survival (19). Recent reports have shown that Erk may act as a physiological Bcl-2 kinase to prevent cell death in response to cytotoxic stimuli (10) and that activation of the P38 MAPK pathway leads to P53 phosphorylation (30), which, in turn, stimulates the expression of Bax in Aβ-induced cells (11). This suggests that Erk plays a neuroprotective role in cortical neurons and that P38 exerts toxic effects (37). Our study showed that MtFt siRNA treatment decreased P-Erk expression and increased P-P38 levels after the injection of $A\beta_{25-35}$. In SH-SY5Y cells, after the transfection of MtFt gene, the opposite results were obtained. In summary, MtFt may have direct neuroprotective effects against $A\beta_{25-35}$ toxicity via activation of the MAPK pathway in neurons.

An increasing number of recent studies have suggested that oxidative stress may be associated with AD neurodegeneration. Metals, such as iron, copper, and zinc, have been shown to be key factors in biochemical reactions that produce free radicals, which lead to peroxidation of cellular lipids and neuronal damage or death (3). Increased iron concentrations are found in specific brain areas of AD patients (20). In this study, we found that overexpression of MtFt decreased LIP levels and significantly reduced $A\beta_{25-35}$-induced ROS levels. The increase in LIP levels that occurred in response to $A\beta_{25-35}$ treatment decreased the expression of the iron import proteins TfR1 and DMT1 (+IRE) and increased the expression of the iron export protein FPN1. A schematic representation of the mechanisms suggested by our study is provided in Figure 10. We propose that $A\beta_{25-35}$ increases DMT1 (−IRE) expression, which in turn enhances cellular iron uptake, leading to an increase in LIP levels and enhanced ferritin levels. The excess LIP donates electrons for the generation of ROS and lipid peroxidation. These changes may cause the cell to begin the process of programmed cell death through the MAPK pathway (by increasing P38 phosphorylation and decreasing that of Erk). MtFt could lead to cellular iron redistribution, wherein large amounts of iron are translocated to the mitochondria and LIP levels are decreased (21). Our data suggest that MtFt can attenuate $A\beta_{25-35}$-induced neurotoxicity and reduce oxidative damage through Erk/P38 kinase signaling and that these effects are coordinately regulated by intracellular LIP levels.

The current work is the first to demonstrate that the expression of MtFt in the rat hippocampus is age related and that its neuroprotective effect on the toxicity induced by $A\beta_{25-35}$ may depend on the MAPK pathway. Appropriate regulation of MtFt expression may prevent the damage to neuronal cells induced by neurotoxins and pathologic conditions such as those observed in patients with AD.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium, fetal calf serum, and MTT were purchased from Invitrogen. $A\beta_{25-35}$, annexin V/PI, rhodamine 123, calcein-AM, and 2,7-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma. TRIzol reagent, Lipofectamine 2000, and rat MtFt siRNA were purchased from Invitrogen. Anti-HA, Bcl-2, Bax, and cytochrome C antibodies were purchased from Santa Cruz Biotechnology. Antibodies to Erk, phosphorylated Erk (P-Erk), P38, phosphorylated P38 (P-P38), and caspase-3 were purchased from Cell Signaling Technology. H-ferritin, L-ferritin, DMT1 (+IRE), DMT1 (−IRE), and β-actin antibodies were purchased from Alpha Diagnostic International. The anti-MtFt antibody was a gift from Professor Sonia Levi.

Animals

Adult male Sprague Dawley rats were obtained from the Experimental Animal Center of Hebei Medical University. Animals were housed in a temperature- and humidity-controlled environment on a 12-h light–dark cycle with free access to food and water. All experiments were approved by the Animal Care and Use Committee of the Hebei Science and Technical Bureau in the RPC.

siRNA generation and treatment

siRNA targeting rat MtFt was obtained commercially from Invitrogen. The sequences are UAAGCUUCUCUGCAUG CUCCCUCUC (siRNA1) and GAGAGGGAGCAUGCAGA GAAGCUUA (siRNA2). Transfection of siRNAs into the hippocampus of rats was carried out using Lipofectamine 2000, and the reagent was delivered as described in the following section.

Preparation of aggregated $A\beta_{25-35}$ and rat surgery

Aggregated $A\beta_{25-35}$ was solubilized in sterile isotonic saline solution [pH 7.4 (28)] to a final concentration of 2 μg/μl and then incubated at 37°C for 36 h. Five microliters of this solution was injected over 5 min into the rat hippocampus (18). The rats were divided randomly into six groups with six rats in each group. The control group was injected with 0.9% saline. The second group was injected with $A\beta_{25-35}$. The third group was injected with control siRNA. The fourth group was injected with $A\beta_{25-35}$ 24 h after the injection of control siRNA. The fifth group was injected with MtFt siRNA. The last group was injected with $A\beta_{25-35}$ 24 h after the injection of MtFt siRNA. The rats were then kept for 10 days under normal conditions. Three rats from each group were dissected for biochemical and molecular analysis of the hippocampus, and the brains of the other three rats were fixed in 50 ml of phosphate buffered solution containing 4% paraformaldehyde. Serial coronal sections of the brains (20 μm) were cut with a freezing microtome (13).

Cell culture and treatment

Plasmids expressing mouse MtFt (MtFt-pcDNA3.1) or the corresponding empty vector were transfected into SH-SY5Y cells as previously described (27). After SH-SY5Y cells (SH-SY5Y), MtFt-transfected cells (MtFt), and vector-transfected

cells (pcDNA3.1) had grown to ~70% confluency, 25 μM $A\beta_{25-35}$ was added. The cells were then incubated at 37°C for 24 h prior to analysis.

Western blot and reverse transcription-PCR analysis

Protein expression was assessed by western blotting as previously described (27). mRNA expression was assessed by reverse transcription-PCR using total RNA extracted with TRIzol reagent according to the literature (27). The primer sequences used are listed in Supplementary Table S1. All of the experiments were performed at least three times. The relative band intensities of the proteins are expressed as the ratio of each to β-actin.

Detection of apoptosis

The presence of apoptosis in the dentate gyrus of rat hippocampi was assessed by the terminal deoxynucleotidyl transferase-mediated FITC-dUDP nick-end labeling method (13), and apoptosis in SH-SY5Y cells was measured by flow cytometry using annexin V/PI staining as previously described (27).

Measurement of lipid peroxidation and protein carbonyl levels

MDA, a reliable marker of lipid peroxidation, was determined with thiobarbituric acid according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). The levels of protein carbonyls and HNE-His were determined as previously described (13).

Measurement of intracellular ROS and MMP

The level of intracellular ROS was quantified by measuring the fluorescence of DCFH-DA according to the methods of Bass *et al.* (2). Changes in MMP were assayed by measuring the retention of rhodamine 123 as previously described (27).

Measurement of the intracellular LIP

Intracellular LIP levels were measured according to the literature (27). For these studies, cells were treated with 25 μM $A\beta_{25-35}$.

Statistical analysis

All experiments were performed at least in triplicate. One-way analysis of variance was used to estimate overall significance and was followed by Tukey's *post hoc* test corrected for multiple comparisons. Data are presented as the mean ± standard deviation. A probability level of 95% ($p < 0.05$) was considered significant.

Author Disclosure Statement

The authors declare no competing financial interests.

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Abbreviations Used

6-OHDA = 6-hydroxydopamine
Aβ = amyloid-β
AD = Alzheimer's disease
APP = amyloid precursor protein
DAPI = 4′,6-diamidino-2-phenylindole
DCFH-DA = 2,7-dichlorofluorescein diacetate
Erk = extracellular signal-regulated kinase
HNE–His = hydroxynonenal–histidine
LIP = labile iron pool
MAPK = mitogen-activated protein kinase
MDA = malonyl dialdehyde
MMP = mitochondria membrane potential
MtFt = mitochondrial ferritin
PBS = phosphate buffered solution
ROS = reactive oxygen species
RT-PCR = reverse transcription-PCR
siRNA = short interfering RNA
TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling assay

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