氏　　名	関　宏鵬
学位の種類	博士（医学）
学位記番号	博士甲第７８７号
学位授与の要件	学位規則第４条第１項
学位授与年月日	平成２９年　９月１３日
学位論文題目	Mitochondrial ferritin protects SH-SY5Y cells against H2O2-induced oxidative stress and modulates α-synuclein expression （ミトコンドリアフェリチンは過酸化水素により誘導される酸化ストレスからSH-SY5Y細胞を保護し、α-シヌクレインの発現を調節する）
審査委員	主査　教授　漆谷　真
	副査　教授　等　誠司
	副査　教授　勝山　裕

別紙様式３（課程博士・論文博士共用）

論　文　内　容　要　旨

※整理番号	794	（ふりがな） 氏　　名	かん　ほんぼん 関　宏鵬
学位論文題目	Mitochondrial ferritin protects SH-SY5Y cells against H_2O_2-induced oxidative stress and modulates α-synuclein expression （ミトコンドリアフェリチンは過酸化水素により誘導される酸化ストレスからSH-SY5Y 細胞を保護し、α-シヌクレインの発現を調節する）		

Objective:

Mitochondrial ferritin (FtMt) is a type of ferritin that sequesters iron. Previous studies have shown that FtMt is expressed by dopaminergic neurons in the substantia nigra and that it may be involved in the pathology of Parkinson's disease. However, the functional roles of FtMt in dopaminergic neurons remain unclear. In this study, we investigated the function of FtMt in α-synuclein regulation and its antioxidant roles in dopaminergic cells using human dopaminergic neuroblastoma cells, SH-SY5Y.

Methods:

1. **Cell culture and transfection** SH-SY5Y cells were grown in DMEM/F-12 medium supplemented with 10% FBS. The growth conditions were maintained at 37 °C in a humidified environment containing 95% air and 5% CO_2. pEGFP-N1 as a control and pEGFP-N1/FtMt were transiently transfected into cells using FuGENE HD transfection reagent according to the manufacturer's instructions.
2. **Differentiation of SH-SY5Y cells** Cells were treated with 10 μmol/L RA in DMEM/F12 containing 2% FBS for 3 days, followed by replenishing with fresh medium containing 10 μmol/L RA in 0.5% FBS for another 3 days. Then, the cells were treated with DMEM/F12 containing 50 ng/ml of BDNF and 0.5% FBS for 3 days.
3. **Cell viability detection** Cell viability was investigated using MTT assay.
4. **Cell cytotoxicity detection** Cell cytotoxicity was investigated using LDH assay.
5. **RNA extraction and RT-PCR** Total RNA was purified from each sample using an RNeasy Plus Mini Kit according to the manufacturer's instructions. The cDNA was reverse-transcribed from 5 μg of total RNA using a Superscript III First-Strand Synthesis System with Oligo dT. Taqman Gene expression assays and Taqman Fast Advanced Master Mix were employed for RT-PCR. The data obtained were analyzed using Light Cycler software.
6. **RNA interference and transfection** FtMt siRNA or negative control siRNA (216 pmol each) was transfected into SH-SY5Y cells with METAFECTENE SI$^+$ according to the manufacturer's instructions.
7. **LIP measurement** Intracellular LIP was detected using a Calcein AM Assay Kit according to the manufacturer's instructions.
8. **Determination of iron in mitochondria** Mitochondria were isolated from SH-SY5Y cells using a Mitochondria Isolation Kit for Cultured Cells according to the manufacturer's instructions. Next, iron was determined in the mitochondria using an Iron Colorimetric Assay Kit according to the manufacturer's instructions.
9. **Protein extraction and Western blotting** Cells were washed twice with ice cold PBS before lysing with lysis buffer. The protein concentration was determined using BCA assay. Equal amounts of protein (10 μg) were denatured at 95 °C for 5 min, applied to the lanes in precast 15% polyacrylamide gels, electrophoresed, and transferred to a nitrocellulose

（備考）１．論文内容要旨は、研究の目的・方法・結果・考察・結論の順に記載し、２千字程度でタイプ等を用いて印字すること。
２．※印の欄には記入しないこと。

別紙様式３の２（課程博士・論文博士共用）

（続紙）

membranes. The membranes were blocked with 5% skim milk and incubated with the primary antibodies.

Results:

1. FtMt expression level was increased after SH-SY5Y cells were differentiated into dopaminergic neuron like cells.
2. FtMt suppresses α-synuclein expression without affecting α-synuclein mRNA levels.
3. FtMt overexpression abolishes the iron-induced upregulation of α-synuclein.
4. FtMt sequesters iron in mitochondria and decreases the intracellular LIP levels.
5. FtMt rescues SH-SY5Y cells from H_2O_2 -induced reactive oxygen species.
6. FtMt and α-synuclein are increased by H_2O_2 in SH-SY5Y cells.
7. FtMt partially rescues H_2O_2 -induced α-synuclein expression but not the mRNA level.

Discussion:

In this study, we first demonstrate that FtMt can repress the expression of α-synuclein. This result indicates that FtMt plays an important role in maintaining α-synuclein levels. Interestingly, these alterations in α-synuclein expression occurred at the protein levels but not at the mRNA levels, thereby suggesting that FtMt regulates α-synuclein expression in a posttranscriptional stage. We investigated the effect of iron on α-synuclein and found that it could increase α-synuclein protein expression but not alter its mRNA levels, suggesting that iron regulates α-synuclein at a posttranscriptional stage. Furthermore, we transfected an empty vector and FtMt plasmids into cells, before treatment with $FeCl_2$. Consequently, we found that $FeCl_2$ abolished the inhibitory effects of FtMt overexpression on α-synuclein expression. In addition, DFO suppressed α-synuclein expression in the same manner as FtMt. These results suggest that FtMt affects α-synuclein expression via iron regulation. We determined the iron levels in mitochondria after FtMt overexpression and knockdown, where the results showed that the iron levels in mitochondria were elevated after FtMt overexpression as well as exposure to $FeCl_2$. By contrast, the iron levels in mitochondria were decreased after FtMt knockdown. These results suggest that FtMt leads to iron transfer into mitochondria from LIP in the cytoplasm. We investigated the effects of FtMt on α-synuclein under H_2O_2-induced oxidative stress condition, which showed that FtMt overexpression could partially inhibit the enhanced α-synuclein expression induced by H_2O_2, whereas it was not reversed at the protein level. In addition, FtMt failed to prevent the increase in the α-synuclein mRNA level induced by H_2O_2. These results suggest that FtMt inhibits α-synuclein expression at a posttranscriptional stage under H_2O_2-induced oxidative stress conditions.

Conclusions:

In this study, we found FtMt expression was increased in differentiated SH-SY5Y cells and investigated the effects of FtMt on α-synuclein under physiological and H_2O_2-induced oxidative stress conditions in undifferentiated SH-SY5Y cells. Our results showed that FtMt leads to iron transfer into mitochondria from LIP in the cytoplasm. According to the mechanism of IRP/IRE system clarified by previous reports of other groups, reduction of iron levels in intracellular LIP caused enhancement of binding IRP to IRE, leading to inhibition of mRNA translation into protein. Consequently, α-synuclein was decreased by FtMt. Under pathological conditions, H_2O_2 increased the expression of α-synuclein through increasing mRNA levels. However, this phenomenon can be partially inhibited by FtMt via inhibiting α-synuclein mRNA translation. Furthermore, FtMt overexpression rescued SH-SY5Y cells from H_2O_2-induced oxidative stress. Overall, these results suggest that FtMt has neuroprotective effects against H_2O_2-induced oxidative stress as well as maintaining α-synuclein expression levels at the posttranscriptional level via iron regulation.

別紙様式８（課程・論文博士共用）

学位論文審査の結果の要旨

整理番号	７９４	氏　名	Hongpeng Guan
論文審査委員			

（学位論文審査の結果の要旨）（明朝体１１ポイント、６００字以内で作成のこと。）

中脳黒質ドパミン細胞に発現するミトコンドリアフェリチン（FtMt）が、パーキンソン病病態に如何に関与しているのかを調べるため、ヒト中脳不死化細胞 SH-SY5Y に対する酸化ストレスモデルを用いて、FtMt やαシヌクレインの発現量への影響と細胞死への影響について検討した。その結果、以下の点を明らかにした。

1．SH-SY5Y 細胞において、FtMt は α シヌクレインの発現を翻訳レベルで抑制した。一方、鉄イオンは α シヌクレイン発現を翻訳レベルで増加させたが、FtMt はこの増加を抑制した。
2．同細胞への FtMt の過剰発現は、細胞内の不安定鉄プール（LIP）のレベルを減少させ、ミトコンドリアの鉄濃度を上昇させた。
3．同細胞への FtMt の過剰発現は、過酸化水素を用いた酸化ストレスから細胞死を部分的に抑制し、α シヌクレインの発現レベルを低下させた。

本論文は、ミトコンドリアフェリチンがミトコンドリアへの鉄移行による、細胞質の鉄イオンプール量の低下を介してαシヌクレインの発現調節をしていることを初めて証明した研究成果である。さらにパーキンソン病における酸化ストレスや鉄代謝異常の病態への関与についても、αシヌクレイン毒性の観点から、細胞生物学的な実験により明らかにしており、パーキンソン病の病態解明と新規治療法の開発に貢献するものと考える。最終試験として論文内容に関連した試問を受け合格したので、博士（医学）の学位論文に値するものと認められた。

（総字数 585 字）

（平成２９年８月２９日　）

別紙様式９（課程博士用）