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Ferritin is associated with neural differentiation of bone marrow-derived mesenchymal stem cells under extremely low-frequency electromagnetic field

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Abstract

Extremely low-frequency electromagnetic field (ELFEF) is a well-known mechanical stimulation that induces neural differentiation. It is potentially an effective treatment for neurodegenerative diseases. In a previous study, ferritin light chain was upregulated in ELFEF-exposed human bone marrow-derived mesenchymal stem cells (BM-MSCs). Ferritin light chain is a component of ferritin, a highly conserved iron-binding protein. In this study, to identify molecules associated with ferritin during neural differentiation of BM-MSCs, we performed reverse transcription polymerase chain reaction (RT-PCR), western blotting, and ATP analysis. Our data indicated that ELFEF triggers the upregulation of ferritin light chain (FLC) and ferritin heavy chain (FHC) in BM-MSCs. The elevated levels of FLC and FHC correlated positively with the differentiation of BM-MSCs into neural cells. Moreover ELFEF induced the activation of iron regulatory protein-1 (IRP-1) and cofilin, which are downstream targets of ferritin. These results suggest that ELFEF induces neural differentiation through activation of a ferritin-regulated mechanism.

Key words: Extremely low-frequency electromagnetic field (ELFEF), bone marrow-derived mesenchymal stem cells (BM-MSCs), neural differentiation, ferritin, iron regulatory protein-1 (IRP-1), cofilin.

Introduction

Extremely low-frequency electromagnetic field (ELFEF) affects a number of biological functions, including cell fate determination, gene expression, and cell differentiation (1-4). It can control the binding of neurotransmitters, antibodies, and hormones to binding sites on the cell surface. ELFEF exposure also influences the biophysical properties of cell membranes, including the permeability to calcium ions, thereby enhancing communication potential across cell membranes (5-6).

Mesenchymal stem cells (MSCs), which are multipotent, can differentiate into multiple cell types such as adipocytes, chondrocytes, and osteoblasts (7). This makes MSCs a promising target for cell therapy in tissue engineering and regenerative medicine (8-10). Ongoing studies indicate that specific experimental conditions like ELFEF can induce differentiation of MSCs (11).

In our previous studies, exposure to 50 Hz, 1 mT of ELFEF for 12 days induced neural differentiation of BM-MSCs. Exposed BM-MSCs became morphologically more similar to neuron-like cells, and these cells increased the expression of specific neural markers. When compared to control cells, eight proteins were identified specifically in ELFEF cells using two dimensional gel electrophoresis (2-DE). One of these proteins, the ferritin light chain, a subunit of ferritin, was upregulated in the ELFEF-exposed cells.

Ferritin is an intracellular protein that stores iron in a redox inactive form, and releases it in response to an intracellular pool of labile iron. Ferritin can be used to regulate metabolic processes in response to the changing iron requirements of the cell (12-15). Ferritin is an important element for controlling iron ion homeostasis. In conditions of iron demand, degraded-ferritin chains and iron, stored in ferritin, can be liberated (16). When the content of cellular iron is low, ferritin synthesis also decreases; conversely, when the content of cellular iron is high, ferritin synthesis increases. Thus, the level of ferritin in the cytoplasm is regulated by the translation of FHC and FLC in response to intracellular chelatable iron. This process is mediated by interactions between RNA binding proteins and a region of the FHC and FLC mRNAs, termed the iron responsive element (IRE). RNA binding proteins, including iron regulatory protein-1 (IRP-1), bind to the IRE and inhibit mRNA translation. When iron is abundant, IRP-1 exists as a cytosolic aconitase; conversely, when iron is scarce, IRP-1 can bind the IRE, acting as a repressor of ferritin translation (17-18). In humans, cofilin is prevalent in the central nervous system (CNS) and is concentrated in the cell body and in growth cones of axonal and dendritic processes. Cofilin, an actin-binding protein, binds to both actin monomers and polymers, and promotes the disassembly of actin filaments. During reorganization of actin filaments, cofilin levels increase significantly (19).

Recent studies indicated that ferritin binds to a selective region of the brain, which results in the complete progression of neural differentiation (14). However, the role of ferritin in neural differentiation is not well studied. Furthermore, few studies have addressed the mechanism of neural differentiation in response to ferritin. Thus, this study investigates the relation between ELFEF-stimulated neural differentiation and ferritin.

Materials and methods

Cell culture

BM-MSCs were purchased from Lonza (PT-2501, Lonza Walkersville Inc., MD, USA). BM-MSCs were thawed and maintained using non-hematopoietic expansion medium (NH Expansion Medium, Miltenyi Biotec

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Table 1. Primers for RT-PCR.

Name	Forward : 5'→3'	Reverse : 5'→ 3'	Expected size
β-tubulin III	GAGCGGATCAGCGTCTACTA	TCCTCACACTTTTGACGCTG	252
GAPDH	AAGGTCGGAGTCAACGGATTTGGT	TACTGGTGTCAGGTACGGTAGTGA	534
MAP2	TGCCATCTTGGTGCCGA	GGACCTCCACCATTACAGTTG	460
Nestin	TGGCTCAGAGGAAGAGTCTGA	AGTGTCGTACATTTACCCCCT	169
Neurofilament-M	AAGCCACTCAGACCAGAATA	GACCTATATCTTTAGCGACG	366
Oct 3/4	GTATTCAGCCAAACGACCATC	GCTTTCTCTTTCGCTTGGTC	183

GmbH, Bergisch Gladbach, Germany) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco BRL, Gaithersburg, MD, USA) in a humidified incubator at 37°C and 5% CO_2.

ELFEF stimulation

BM-MSCs were cultured either in the presence of ELFEF at 50 Hz, 1 mT for 12 days or without ELFEF (control group), according to the methods described by Hyun-Jung Kim *et al*. BM-MSCs at early passages (between 3 and 6 passages) were used in all experiments. When BM-MSCs were exposed to ELFEF, the medium was replaced with Dulbecco's Modified Eagle Medium Low Glucose (DMEM-LG, Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Lonza Walkersville Inc., MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Gaithersburg, MD, USA), and the cells were incubated in a humidified incubator at 37°C and 5% CO_2.

Proliferation assay

After being cultured under ELFEF conditions, cells were trypsinized and seeded at a density of 4,000 cells/well in 96-well microplates and allowed to attach for 24 hours. Then, CCK-8 solution (Dojindo laboratory, Kumamoto, Japan) was added to each well and the cells were incubated for 2 hours at 37°C. Cell proliferation was assessed by measuring absorbance at 450 nm using a microplate reader.

Cell viability assay

Cells were seeded in 96-well microplates and allowed to attach for 24 hours. Deferoxamine (DFO) was treated at concentrations of 20, 50, 80, 100, 120 and 150 μM for 24 and 48 hours, respectively. Then, CCK-8 solution was added to each well and the cells were incubated for 2 hours at 37°C. Cell cytotoxicity was assessed by measuring absorbance at 450 nm using a microplate reader.

Western blotting

After being cultured under ELFEF conditions, the cells were immediately lysed in RIPA buffer (Sigma, Missouri, USA) supplemented with Xpert Protease Inhibitor Cocktail Solution (GenDEPOT, Barker, TX, USA). The primary antibodies used for western blot analysis were ferritin light chain, ferritin heavy chain, iron regulatory protein 1 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), cofilin, and GAPDH (Abcam, Cambridge, UK). Bands from western blotting were scanned using a biomolecular imaging system (Fujifilm, Tokyo, Japan).

RT-PCR

cDNAs were synthesized using the Superscript III First Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's protocols. Primers for PCR were purchased from Cosmogenetech (Seoul, Korea) (Table 1). All PCR techniques were conducted in 22~32 cycles. The PCR products were separated via electrophoresis on a 1.5% agarose gel, and then the product bands were detected using the Molecular Imager® GelDoc™ XR (BioRad, Hercules, CA, USA).

ATP analysis

The assay is based on the reaction of luciferase with ATP. Through this reaction, light is produced. This light was detected with the Luminoskan Ascent Luminometer (Thermo Electron Corporation, Waltham, MA, USA). After exposure to ELFEF for 12 days, the BM-MSCs were harvested and the ATP concentration was detected using the ATP Determination Kit (Invitrogen, Rockville, MD, USA). All procedures were performed according to the manufacturer's directions.

Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Student's *t*-test or one-way analysis of variance (ANOVA) was used to determine the statistical significance of the results and to compare the means of the groups using the SPSS statistical software version 12.0.

Results

ELFEF induces differentiation of BM-MSCs

After being exposed to ELFEF for one day or for 12 days, the morphology of the BM-MSCs was examined. While the un-exposed BM-MSCs had spindle-like, flattened, or fibroblast-like features, ELFEF-stimulated BM-MSCs had a narrow and elongated shape, with branch-like forms. ELFEF induced morphological changes that resembled neuron-like features (Figure 1 (A)).

To investigate the differences between the cell proliferative abilities of the two groups, a cell viability assay was performed after the cells were exposed 50 Hz, 1 mT ELFEF for 12 days. As shown in Figure 1 (B), there was a significant difference in the viability of cells with and without ELFEF exposure. This indicates that ELFEF positively affects the differentiation of BM-MSCs.

RT-PCR analysis was performed, using mRNA from the BM-MSCs, to assess the transcriptional levels of the following neural-specific markers: the proneural marker, nestin; the mature neural markers, β-tubulin III,

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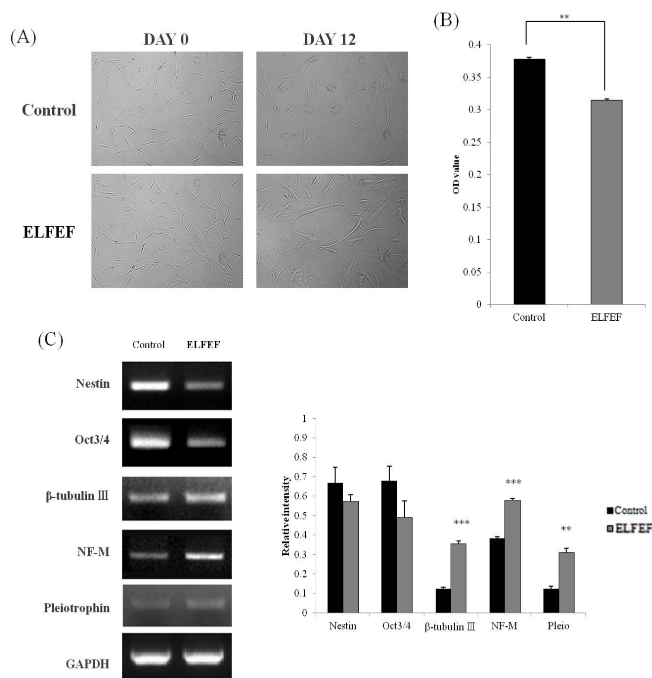

Figure 1. Changes in differentiation of BM-MSCs after ELFEF exposure. (A) Morphology of BM-MSCs after ELFEF exposure. Cells were cultured for 12 days with or without ELFEF stimulation of 50 Hz, 1 mT. Magnification: 100×. (B) ELFEF exposure affects proliferation of BM-MSCs. Proliferative capacity was assessed using the CCK-8 assay after ELFEF exposure. (C) RT-PCR was conducted to detect the gene expression of neural differentiation and stemness markers after ELFEF exposure. GAPDH served as a loading control. Error bars represent mean ± SEM. (*P < 0.05, **P < 0.01, ***P < 0.001).

neurofilament-M (NF-M), and pleiotrophin; and the stemness gene, oct 3/4. The mRNA levels of both nestin and oct 3/4 were downregulated in ELFEF-exposed cells. In contrast, β-tubulin III, NF-M, and pleiotrophin were upregulated in EFLEF-exposed cells. Thus, mRNA expression of both neural and stemness markers was different between ELFEF exposed and control cells (Figure 1 (C)). This result suggests that ELFEF exposure enhances neural differentiation in BM-MSCs.

ELFEF exposure reduces ferritin synthesis

ELFEF exposure induces mild stress on BM-MSCs. This stimulus influences the ferritin synthesis. We conducted western blotting to verify the expression levels of both FLC and FHC in BM-MSCs after ELFEF exposure (Figure 2). In the ELFEF cells, protein levels of both FLC and FHC were elevated compared to those in control cells.

Deferoxamine (DFO)-mediated ferritin degradation suggests that ferritin is required for the differentiation of BM-MSCs

Ferritin is degraded in lysosomes by DFO which is a known specific chelator of iron and iron release is dependent on ferritin degradation (16). In our study, a cell viability assay was performed to determine the IC50 of DFO required to induce ferritin degradation in BM-MSCs. For ferritin degradation, DFO was administered at concentrations of 20, 50, 80, 100, 120 and 150 µM for 24 and 48 hours, respectively. DFO treatment for 48 hours was more effective than treatment for 24 hours (Figure 3 (A)). BM-MSCs were treated with DFO

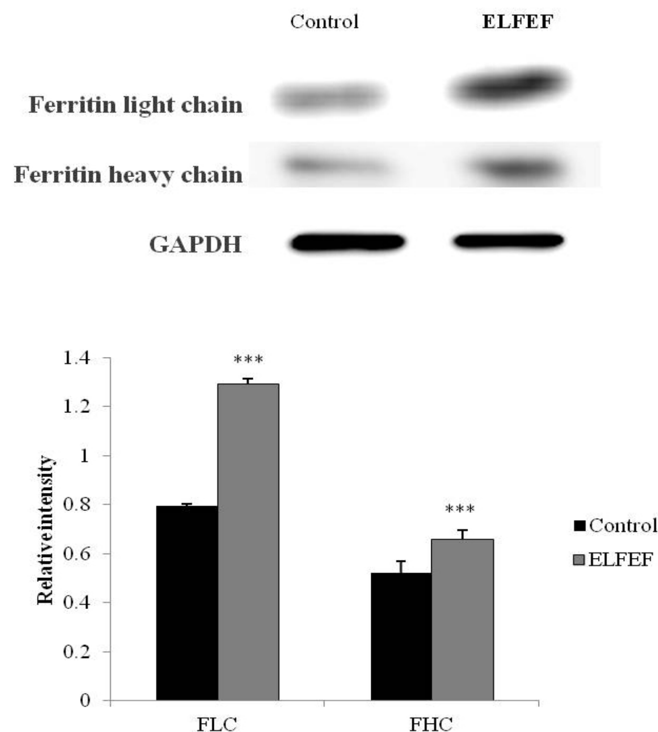

Figure 2. Confirmation of cellular ferritin expression in BM-MSCs after ELFEF exposure. Western blot analysis showing upregulation of FLC and FHC compared with GAPDH loading control. Error bars represent mean ± SEM. (*P < 0.05, **P < 0.01, ***P < 0.001).

100 µM for 48 hours before the exposure to ELFEF. To evaluate the effect of DFO, the expression levels of FLC and FHC were measured using western blot (Figure 3 (B)). Protein levels of FLC and FHC were upregulated in ELFEF-exposed cells; however, in DFO-treated, ELFEF-exposed cells, protein levels of FLC and FHC were significantly downregulated. These results suggest that DFO effectively induces ferritin degradation even with subsequent exposure to ELFEF.

After treatment of BM-MSCs with DFO, RT-PCR analysis was conducted to examine the effects of ferritin degradation on ELFEF-induced neural differentiation. The transcriptional levels of neural-specific and stemness markers were different in DFO-treated and untreated cells (Figure 3 (C)). When compared to expression in ELFEF-exposed cells and control cells, the expression of nestin and oct 3/4 in DFO-treated, ELFEF-exposed cells increased; however, the expression of the mature neural markers, β-tubulin III, NF-M, and pleiotrophin decreased in DFO-treated, ELFEF- exposed cells. This result suggests that ferritin plays an important role in ELFEF-induced neural differentiation and in maintaining of the characteristics of BM-MSCs.

Downstream targets of ferritin are differentially expressed through neural differentiation mechanism

To confirm the ferritin-dependent roles of IRP-1 and cofilin in neural differentiation, we used western blotting to detect protein expression levels of IRP1 and cofilin. IRP-1 and cofilin were differentially expressed in control cells and ELFEF exposed cells (Figure 4 (A)); both targets were upregulated in the ELFEF-exposed cells. However, IRP-1 and cofilin levels decreased in the DFO-induced ferritin degradation cells. These data suggest that IRP-1 and cofilin are downstream candidates

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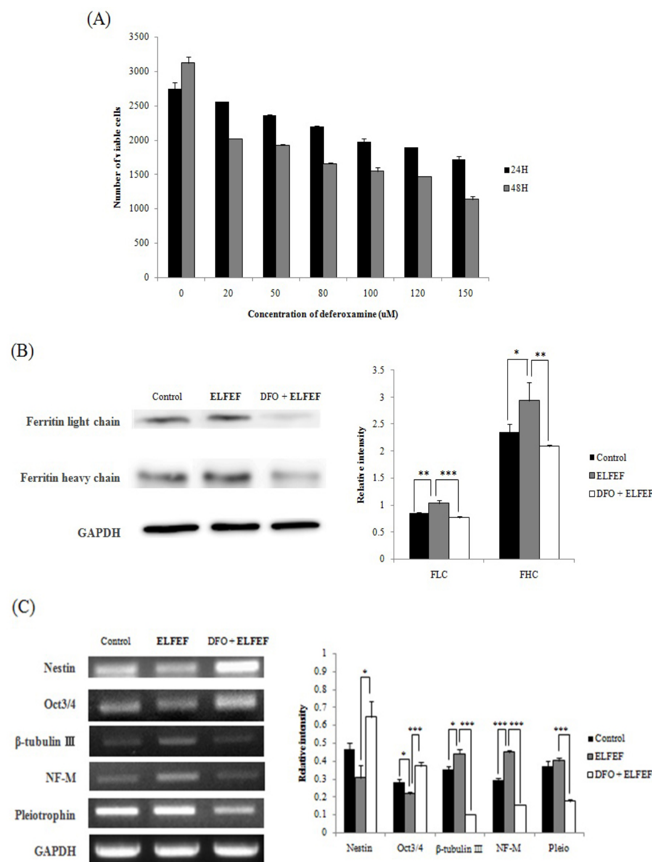

Figure 3. Effects of DFO treatment on ferritin degradation. (A) Viability of BM-MSCs was detected after time-dependent DFO treatment. (B) Ferritin expression levels decreased after DFO treatment. Western blot analysis indicates degradation of FLC and FHC. (C) Using RT-PCR, gene expression levels of neural differentiation and stemness markers in BM-MSCs were identified in control, ELFEF-exposed, and DFO-treated ELFEF-exposed cells. GAPDH mRNA was used to normalize the variability in template loading. Each bar represents mean ± SEM. (*P < 0.05, **P < 0.01, ***P < 0.001).

of ferritin during ELFEF-induced neural differentiation.

ATP is required for neural differentiation, including neurite outgrowth. To verify the difference in ATP levels among the different cells, ATP analysis was performed. In ELFEF-exposed cells, the ATP concentration was significantly upregulated. The ATP level of DFO-treated, ELFEF-exposed cells decreased (Figure 4 (B)). This result suggests that ferritin also is involved in ATP production, especially during ELFEF-induced neural differentiation.

Discussion

A number of studies have evaluated the effect of low-frequency EMFs on humans; however, few studies have evaluated its effect at the cellular level (20). In our previous study to determine the effects of ELFEF-induced neural differentiation at cellular level, proteomic analysis was performed on BM-MSCs exposed to 50 Hz, 1 mT ELFEF for 12 days and compared to control cells. The result indicated eight differentially expressed proteins, including the upregulation of FLC (11). Consistent with the earlier report, Helena Skalnikova et al. found out that FLC overexpressed in the differentiation of neural stem cells (21). Thus, here, we focused on the role of ferritin in ELFEF-induced neural differentiation and the

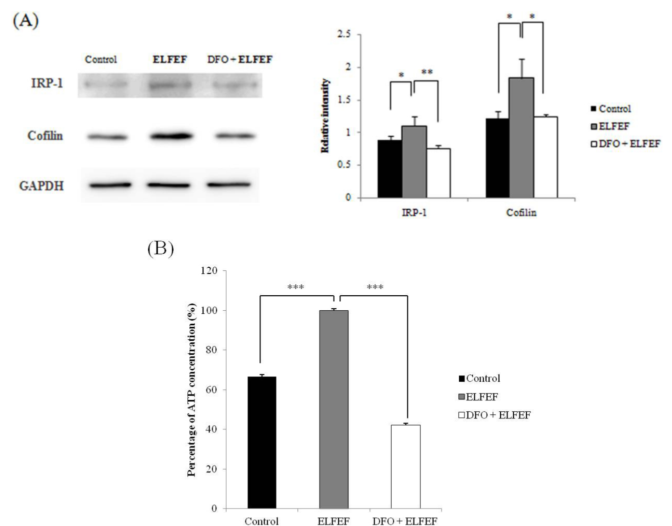

Figure 4. Verification of ATP-related ferritin mechanism after DFO treatment. (A) Western blot analysis indicating downregulation of IRP-1 and cofilin compared with GAPDH loading control in DFO-treated ELFEF-exposed cells. (B) ATP assay was performed in control, ELFEF-exposed, and DFO-treated ELFEF-exposed cells for 12 days. Each bar represents mean ± SEM. (*P < 0.05, **P < 0.01, ***P < 0.001).

underlying mechanisms.

In this study, neural differentiation of BM-MSCs was induced by exposure to 50 Hz, 1 mT ELFEF for 12 days. The expression levels of specific neural markers, including nestin, β-tubulin III, NF-M, and pleiotrophin, and the stemness marker, oct 3/4 were measured using RT-PCR. To confirm the role of ferritin in ELFEF-exposed BM-MSCs, BM-MSCs were treated with DFO to induce ferritin degradation. DFO-induced ferritin degradation significantly affected the gene expression levels of both neural markers and stemness markers. These results suggest that ferritin plays a critical role in neural differentiation induced by ELFEF exposure.

The proteins IRP-1 and cofilin influence ATP production, actin reorganization, and neural morphogenesis. In particular, the synthesis of ferritin is regulated by IRP-1. IRP-1 also acts as an aconitase in the TCA cycle. Through this cycle, ATP is produced and used for a number of biological functions. The reorganization of actin cytoskeleton is one of the processes by which biological energies are consumed. Cofilin plays an important role in actin cytoskeleton organization associated with neurite growth.

To investigate the downstream molecules of ferritin in neural differentiation, IRP-1 and cofilin were targeted as its downstream molecules. IRP-1 was upregulated in ELFEF-exposed cells. When ferritin degradation was induced by DFO, IRP-1 expression decreased, and the expression level was comparable to the level in control cells. Cofilin had a similar expression pattern, suggesting that both IPR-1 and cofilin are downstream targets of ferritin in ELFEF-induced neural differentiation. These results suggest that IRP-1 and cofilin are connected to ferritin during neural differentiation.

Recent studies on neural differentiation have shed light on the function of cofilin. Actin reorganization is required during the process of neural differentiation. In this situation, cofilin plays a critical role in actin filament assembly, and cofilin activity is also important for maintaining neurite outgrowth, growth cone dynamics,

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pathfinding, and the establishment of neuronal polarity (19, 22-24). Interestingly, during neurite outgrowth, the concentration of ATP is highly upregulated (25). Our study also indicated that the level of ATP increased significantly in ELFEF-exposed cells with enhanced neurite outgrowth. These findings are consistent with those of the previous study. Moreover, the concentration of ATP decreased in ferritin downregulated cells. Taken together, these results suggest that an increased level of ATP is related to the upregulation of ferritin and IRP-1. However, the mechanisms of events downstream of ferritin still need to be elucidated. Likewise, additional studies are required to determine the effects of ferritin in normal cells.

In summary, the results of this study provide useful information for understanding the mechanism of neural differentiation after ELFEF stimulation, which has beneficial effects on various physiological functions in humans and animals. Upregulated ferritin promotes neural differentiation of BM-MSCs through regulation of actin reorganization, particularly by regulation of cofilin activity. Although further studies are necessary to identify the effectiveness and mechanism of ferritin, ferritin-based therapies may be used to treat various neural diseases.

Acknowledgements
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