Autophagy alleviates the decrease in proliferation of amyloid β_{1-42}-treated bone marrow mesenchymal stem cells via the AKT/mTOR signaling pathway

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Abstract. Alzheimer's disease (AD) and osteoporosis (OP) are 2 common progressive age-associated diseases, primarily affecting the elderly worldwide. Accumulating evidence has demonstrated that patients with AD are more likely to suffer from bone mass loss and even OP, but whether it is a pathological feature of AD or secondary to motor dysfunction remains poorly understood. The present study aimed to investigate whether amyloid-β_{1-42} ($A\beta_{1-42}$), the typical pathological product of AD, exhibited a negative effect on the proliferation of bone marrow mesenchymal stem cells (BMSCs) and the role of autophagy. The proliferation of BMSCs was measured using a Cell Counting Kit-8 assay, cell cycle analysis and 5-ethynyl-2'-deoxyuridine (EdU) staining. The autophagy-associated proteins microtubule-associated proteins 1A/1B light chain 3B and sequestosome 1 (p62) were evaluated by western blot analysis and autophagosomes were detected by transmission electron microscopy and immunofluorescence. The activity of the protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway was measured using western blot analysis, and the autophagy inducer rapamycin (RAPA), inhibitor 3-methyladenine (3-MA) and the AKT activator SC79 were also used to investigate the role of AKT/mTOR signaling pathway and autophagy in the proliferation of BMSCs. The results suggested that the proliferation of BMSCs treated with $A\beta_{1-42}$ was inhibited, with the autophagy level increasing following treatment with $A\beta_{1-42}$ in a dose-dependent manner, while the AKT/mTOR signaling pathway participated in the regulation of the autophagy level. Activation of autophagy using RAPA inhibited the decrease in proliferation of BMSCs, while suppression of autophagy by 3-MA and activation of the AKT/mTOR signaling pathway increased the decrease in proliferation of BMSCs caused by $A\beta_{1-42}$. It was concluded that $A\beta_{1-42}$, as an external stimulus, suppressed the proliferation of BMSCs directly and that the AKT/mTOR signaling pathway participated in the regulation of the level of autophagy. Concomitantly, autophagy may serve as a resistance mechanism in inhibiting the decreased proliferation of BMSCs treated with $A\beta_{1-42}$.

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Introduction

As the average age of the population increase, the prevalence of neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) increases, while an increased prevalence of osteoporosis (OP) has paralleled the increase in these neurodegenerative disorders over previous decades (1). A prospective study in China has demonstrated that in males and females, decreased bone mineral density (BMD) and an increased rate of bone loss were associated with a higher risk of AD (2). Compared with the general population, BMD appeared to be decreased and the OP rate was increased in patients suffering from PD (3). Although studies investigating OP in patients with ALS are scarce, bone mineral loss has been noted in ALS (4). In a cohort of young men with MS, 80% presented with bone mass loss and of these, 37% exhibited overt OP (5). Although evidence has indicated that OP is closely associated with the progression of neurodegenerative diseases, evidence to confirm the causes is lacking. In the present study, the association between AD and OP was investigated and the aim was to examine how amyloid-β_{1-42} ($A\beta_{1-42}$), the typical pathological product of AD, induced a negative effect on the proliferation of BMSCs.

AD and OP are 2 slowly-progressing but common age-associated diseases primarily affecting the elderly worldwide, and severely decreasing their quality of life. Decreases in cognitive competence, behavioral disorders and gradual loss of autonomy are frequently observed in patients suffering from AD. While OP is a systemic disease caused by a number of etiological factors, a decrease in BMD, impaired bone

microstructure, increased bone fragility and fracture risk are frequently observed. AD and OP appear to be 2 independent diseases, but they share certain common risk factors including alcohol and tobacco consumption (6-8). Increasing evidence has indicated that the decrease in BMD is associated with the development of AD (2)and that OP and hip fractures are common complications observed in patients with AD, but whether these phenomena are part of a pathological process during the development of AD or are a 'by-product' of disuse OP caused by neurological function disorders of patients with AD remains poorly understood.

A previous study based on amyloid precursor protein (APP)/PS1 transgenic mice has demonstrated that bone microstructure was poorer in these AD model mice compared with a negative control (9), and mRNA and protein levels of Aβ were increased in the bone tissue of patients with OP (10), indicating that dementia may result in adverse effects to the skeletal system. Amyloid-β (Aβ) peptides are typical pathological products of AD and serve an important role in the development of AD; the toxic effect of $A\beta_{1-42}$ is the more notable (11). Bone marrow mesenchymal stem cells (BMSCs), possessing key properties including self-renewal and pluripotency, have been extensively studied and are acknowledged to serve a key role in bone metabolism. Proliferation of BMSCs, independent of their differentiation potential, is also associated with the bone formation processes essential for repair and renewal of old and dead cells. At present, the effect of $A\beta_{1-42}$ on the proliferation of BMSCs remains unclear and requires additional study.

Autophagy, which depends upon the formation of autophagosomes, is regarded as an essential process for the elimination of damaged organelles and biomacromolecules to maintain cellular homeostasis. As a cell regulatory process, autophagy serves an important role in regulating BMSC function (12,13). Autophagy has been demonstrated not only to participate in the formation, but also the elimination, of Aβ (14). However, the effect of autophagy on the proliferation of $A\beta_{1-42}$-treated BMSCs remains unclear. Protein kinase B (AKT) and mechanistic target of rapamycin (mTOR), key regulatory factors within the AKT/mTOR signaling pathway may be phosphorylated and serve a critical role in regulating multiple cell functions.The AKT/mTOR signaling pathway is associated with cell growth (15) and autophagy (16), but whether this pathway participates in the regulation of autophagy in BMSCs following treatment with $A\beta_{1-42}$ remains unknown.

The aim of the present study was to determine the effect on proliferation of BMSCs treated with $A\beta_{1-42}$ *in vitro*, and the potential role of the AKT/mTOR signaling pathway and autophagy in this process.

Materials and methods

Cell line and primary reagents. Sprague-Dawley rat BMSCs were purchased from Cyagen Biosciences (RASMX-01001, Guangzhou, China) Inc. Based on the cell descriptions provided by the supplier, the BMSCs were positive for the cell surface markers cluster of differentiation (CD)29, CD44 and CD90, and negative for CD11, CD34 and protein tyrosine phosphatase receptor type, C. BMSCs were cultured with L-Dulbecco's modified Eagle's medium (10567-014; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (SH30070.03; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and 1% penicillin-streptomycin (SV30010.10, Hyclone; GE Healthcare Life Sciences), and were placed in a cell incubator with a humidified 5% CO_2 atmosphere at 37˚C. The media was changed every other day. Only cells in the 6th generation or younger were used in the present study. $A\beta_{1-42}$ peptide freeze-dried powder (A9810), the autophagy inducer rapamycin (RAPA; V900930) and the inhibitor 3-methyladenine (3-MA; M9281) were all obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The cell cycle analysis kit (C1052) was purchased from Beyotime Institute of Biotechnology (Shanghai, China) and the 5-ethynyl-2'-deoxyuidine (EdU) cell proliferation kit (C-10310-3) was obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). All autophagy-associated antibodies used in the present study, including microtubule-associated proteins 1A/1B light chain 3B (LC3B-II; ab48394; 1: 3,000) and sequestosome 1 (p62; ab56416; 1:1,000), were purchased from Abcam (Cambridge, MA, USA), and the AKT/mTOR signaling pathway-associated antibodies AKT (2920; 1:1,000), mTOR (2983; 1:1,000), phosphorylated (p)-AKT (4060; 1:1,000) and p-mTOR (5536; 1:1,000), were obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA). The AKT/mTOR signaling pathway activator SC79 (HY-18749) was obtained from MedChemExpress, Monmouth Junction, NJ, USA. Each experiment in the present study was repeated independently 3 times.

Preparation of $A\beta_{1-42}$. The $A\beta_{1-42}$ used in the present study was prepared as previously described (17).

Cell viability assay. Cell viability of BMSCs was determined using the Cell Counting Kit-8 (CCK-8) assay (C0037; Beyotime Institute of Biotechnology). Cells were seeded into 96-well plates at a density of $5x10^3$ cells/well and cultured for 48 h, then divided into different groups, and each group consisted of 3 wells in parallel. Following addition of the 10 μl CCK-8 reagent and incubation at 37˚C for 1 h, optical density was evaluated using a microplate reader at a wavelength of 450 nm.

Cell cycle analysis. BMSCs were seeded into 6-well plates at a density of $1x10^4$ cells/well. When cell confluence reached 40%, media with or without increasing concentrations of $A\beta_{1-42}$ (1, 2.5 and 5 μM/l) and media with or without $A\beta_{1-42}$ (5 μM/l), $A\beta_{1-42}$ (5 μM/l) + 3-MA (2 mM/l), $A\beta_{1-42}$ (5 μM/l) + SC79 (4 μg/ml) and $A\beta_{1-42}$ (5 μM/l) + SC79 (4 μg/ml) + RAPA (3 μM/l) were added. After 48 h; culture, cells were collected. Following washing with cold PBS twice, 70% alcohol was added for fixation for 2 h at 4˚C. Subsequent to the addition of 500 μl pre-prepared operating fluid consisting of RNase A and propidium iodide (5 μg/ml, Beyotime Institute of Biotechnology) and incubation for 60 min in the dark at room temperature, samples were then immediately analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) at a wavelength of 488 nm and ModFit LT version 5 (Verity Software House, Topsham, ME, USA) was used to analyze the data.

EdU cell proliferation analysis. As an efficient and well-known method for detecting cell proliferation, EdU analysis was used

in the present study. BMSCs were seeded into 96-well plates at a density of $5x10^3$ cells/well and divided into different groups. According to the protocol of the manufacturer, cells were incubated with the pre-prepared EdU solution for 2 h, then following washing with PBS twice, 4% paraformaldehyde was used for fixation for 15 min at room temperature, followed by washing with 2 mg/ml glycine solution. Preparation of Apollo staining was performed and cells were incubated for 30 min at room temperature in the dark. Then 100 μl 0.5% Triton X-100 solution was used for permeation. Subsequent to washing with PBS, 100 μl 1X Hoechst33342 solution was added for staining of DNA for 30 min at room temperature in the dark. The results were then immediately detected using a wide-field fluorescence microscope (IX71; Olympus Corporation, Tokyo, Japan; magnification, x400) and the number of EdU positive cells was counted.

Western blot analysis. BMSCs were collected following treatment with or without increasing concentrations of $A\beta_{1-42}$ (1, 2.5 and 5 μM/l) and with or without $A\beta_{1-42}$ (5 μM/l), $A\beta_{1-42}$ (5 μM/l) + 3-MA (2 mM/l), $A\beta_{1-42}$ (5 μM/l) + SC79 (4 μg/ml) and $A\beta_{1-42}$ (5 μM/l) + SC79 (4 μg/ml) + RAPA (3 μM/l) for 48 h, lysed with RIPA lysis buffer for 30 min on ice, and centrifuged at 12,000 x g for 30 min at 4˚C. Following total protein quantification using a bicinchoninic acid protein assay (P0010s; Beyotime Institute of Biotechnology), samples containing 30 μg total protein were resolved by SDS-PAGE (5% stacking gel and 10% separating gel) under a voltage of 80 V, and transferred onto polyvinylidene difluoride membranes by electroblotting at 110 mA for 60 min. Membranes were blocked by incubating with 5% bovine serum albumin (BSA; Beyotime Institute of Biotechnology) for 2 h at room temperature, and then membranes were incubated with anti LC3B-II (ab48394; 1:3,000; Abcam, Cambridge, MA, USA), p62 (ab56416; 1:1,000 Abcam), AKT (2920; 1:1,000 Cell Signaling Technology, Inc., Danvers, MA, USA), mTOR (2983; 1:1,000; Cell Signaling Technology, Inc.), p-AKT (4060; 1:1,000; Cell Signaling Technology, Inc.) and p-mTOR (5536; 1:1,000; Cell Signaling Technology, Inc.) antibodies at 4˚C overnight followed by incubation with secondary antibodies conjugated to horseradish peroxidase (ZB-2306; 1:1,000; OriGene Technologies, Inc., Beijing, China) for 2 h at room temperature. The EC3 imaging system (UVP; Analytik Jena AG, Jena, Germany) was used to detect the proteins and ImageJ version 1.44P (National Institutes of Health, Bethesda, MD, USA) was used to perform the densitometric analysis.

Autophagosome analysis with transmission electron microscopy (TEM) and immunofluorescence. For TEM (H-7650; Hitachi, Ltd., Tokyo, Japan), BMSCs from the control and 5 μM/l $A\beta_{1-42}$-treated groups were collected using a cell scraper following culture for 48 h, and cells were washed twice with cold PBS and then fixed in 5% glutaraldehyde at 4˚C overnight. Dehydration, saturation, sectioning at 70 nm and staining of the samples were conducted according to standard procedures by the electron microscopy room of Department of Cell Biology, China Medical University, and autophagosomes were observed using a transmission electron microscope and counted in every 10 fields.

For immunofluorescent staining, 4% paraformaldehyde (P0099; Beyotime Institute of Biotechnology) was used for the fixation of cells from different groups at room temperature for 15 min. Following washing with PBS, 0.2% Triton X-100 was added to permeabilize the cells for 10 min, then they were blocked in 5% BSA in blocking buffer for 1 h at 37˚C followed by incubation with anti-LC3 antibody (ab48394; 1:200; Abcam) overnight at 4˚C. The goat anti-rabbit secondary antibody labeled with fluorescein (ZF-0511; 1:500; OriGene Technologies, Inc.) was then applied for 2 h. Following staining with 10 μg/ml 4',6-diamidino-2-phenylindole (C1006; Beyotime Institute of Biotechnology) for 10 min in the dark at room temperature and rinsing with PBS again, a wide-field fluorescence microscope (IX71; Olympus Corporation; magnification, x600) was used to detect the autophagosomes. The number of LC3 puncta was counted visually among 3 randomly-selected fields.

Statistical analysis. Data are presented as the mean ± standard error of the mean of 3 independent experiments performed in triplicate and the GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Statistically significant differences between two groups were analyzed using Student's t-test. Differences between multiple groups were analyzed with one-way analysis of variance, followed by a Student-Newman-Keuls post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

$A\beta_{1-42}$ exhibits an adverse effect on the proliferation of BMSCs. To determine the effect of $A\beta_{1-42}$ on the proliferation of BMSCs, cells were divided into different groups as follows: Control, 1, 2.5 and 5 μM/l $A\beta_{1-42}$, and treated for 48 h. The proliferation of BMSCs was measured by cell cycle analysis, CCK-8 assay and EdU staining. It was demonstrated that the S phase BMSCs detected by flow cytometry decreased gradually with the increasing concentrations of $A\beta_{1-42}$ (Fig. 1A and B). The CCK-8 assay indicated that the viability of BMSCs was impaired directly by $A\beta_{1-42}$ (Fig. 1C). EdU staining, a rapid and sensitive method for detecting proliferation, demonstrated a similar result to the cell cycle analysis and CCK-8 assay (Fig. 1D and E).

Autophagy level increases in BMSCs treated with $A\beta_{1-42}$. As demonstrated, LC3 and p62 are positively and negatively associated with the level of autophagy, respectively. According to the results of the present study, the protein level of LC3 increased gradually with increasing concentrations of $A\beta_{1-42}$ (1, 2.5 and 5 μM/l), and the expression of p62 decreased accordingly (Fig. 2A and B). Fluorescence microscopy was also used to detect the number of LC3 puncta at the cellular level and the results indicated that the number of LC3 puncta increased with the increasing concentrations of $A\beta_{1-42}$ (Fig. 2C and D). TEM, a reliable method for detecting autophagy, was used to demonstrate the occurrence of autophagy induced by 5 μM/l $A\beta_{1-42}$ compared with the control group, and an increased number of autophagosomes were detected in 5 μM/l $A\beta_{1-42}$ group compared with the control group (Fig. 2E and F).

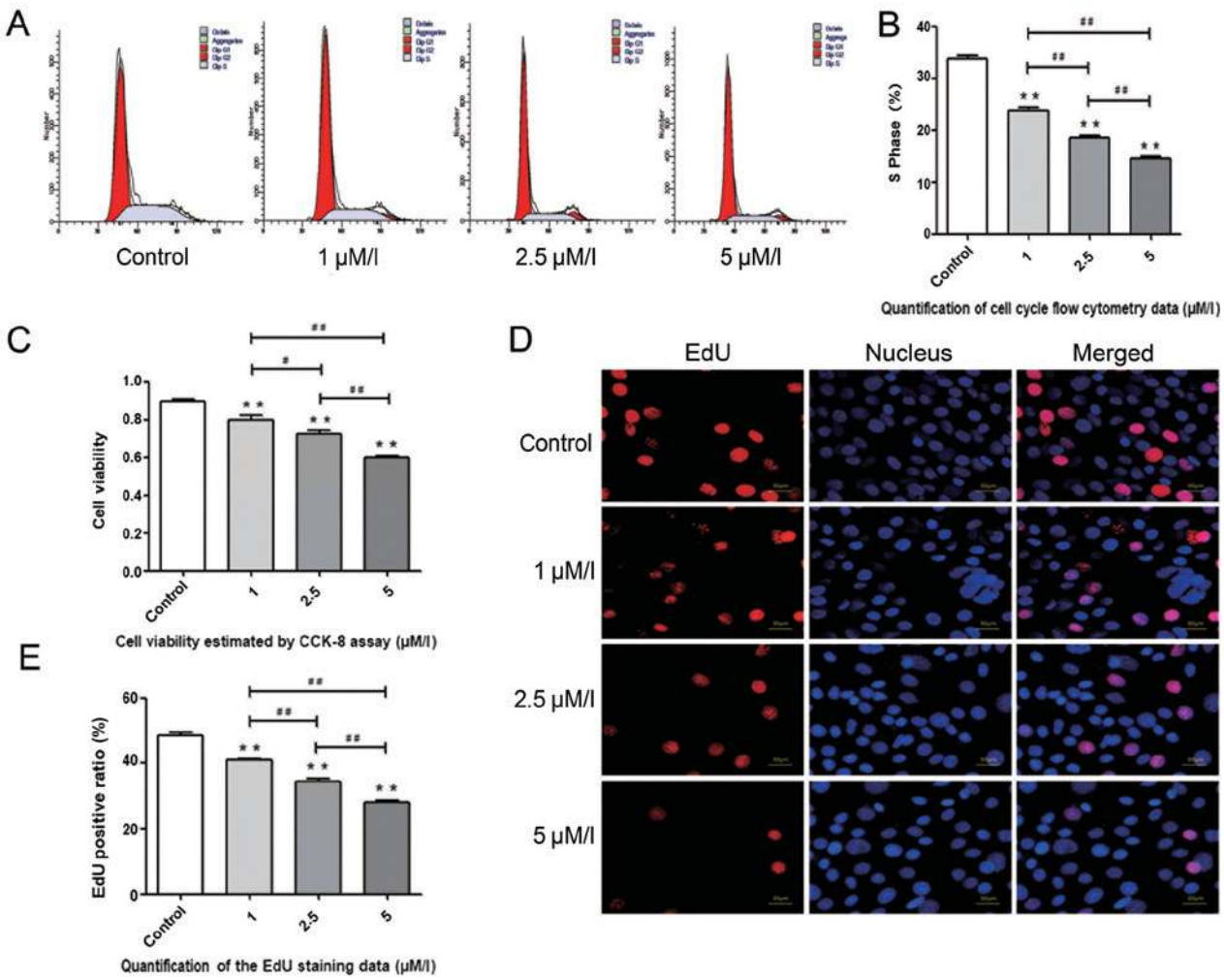

Figure 1. Effect of $A\beta_{1-42}$ on the proliferation of BMSCs. BMSCs were treated with $A\beta_{1-42}$ at various concentrations for 48 h. (A) Cell cycle was examined by flow cytometry. (B) Quantification of cell cycle flow cytometry data. **P<0.01 vs. the control group. $^{\#\#}$P<0.01. n=10 per group. (C) Cell viability was estimated using a Cell Counting Kit-8 assay. **P<0.01 vs. the control group. $^{\#}$P<0.05 and $^{\#\#}$P<0.01. n=10 per group. (D) Cell proliferation was additionally detected by EdU staining. Scale bar=50 μM. (E) Quantification of the EdU staining data. **P<0.01 vs. the control group. $^{\#\#}$P<0.01. n=10 per group. All values are presented as the mean ± standard error of the mean from 3 independent experiments. Aβ, amyloid β; BMSC, bone mesenchymal stem cells; EdU, 5-ethynyl-2'-deoxyuridine.

Autophagy of BMSCs induced by $A\beta_{1-42}$ is mediated via the AKT/mTOR signaling pathway. The present study demonstrated that the AKT/mTOR signaling pathway, which is negatively associated with autophagy, was suppressed following treatment with 5 μM/l $A\beta_{1-42}$ following 48 h culture. To additionally assess whether the AKT/mTOR signaling pathway participated in the regulation of autophagy in $A\beta_{1-42}$-treated BMSCs, 2 mM/l 3-MA, 4 μg/ml SC79, and 4 μg/ml SC79 +3 μM/l RAPA was added following treatment with 5 μM/l $A\beta_{1-42}$, and western blot analysis was performed 48 h later. The results indicated that, compared with the $A\beta_{1-42}$ group, the activation of AKT and inhibition of autophagy initiated the AKT/mTOR signaling pathway, while the activation of autophagy using RAPA suppressed the expression of mTOR compared with the $A\beta_{1-42}$ + SC79 group (Fig. 3A and B). Accordingly, the expression of LC3 and p62 demonstrated that the autophagy level decreased in the $A\beta_{1-42}$ +3-MA and $A\beta_{1-42}$ + SC79 groups compared with the $A\beta_{1-42}$ group, and increased in $A\beta_{1-42}$ + SC79 + RAPA group compared with the $A\beta_{1-42}$ + SC79 group (Fig. 3A and C). These results indicated that the AKT/mTOR signaling pathway was directly involved in the regulation of autophagy induced by $A\beta_{1-42}$.

Autophagy alleviates the decrease in proliferation of BMSCs treated with $A\beta_{1-42}$. To determine the potential role of autophagy induced by $A\beta_{1-42}$, the proliferation of BMSCs was examined accordingly. Based on the inhibitory effect of $A\beta_{1-42}$ on the proliferation of BMSCs, fewer S phase cells were detected in the 2 Mm/l autophagy inhibitor 3-MA group and 4 μg/ml AKT agonist SC79 group by flow cytometry, but this decrease was partly reversed by 3μM/l RAPA, the autophagy inducer (Fig. 4A and B). The CCK-8 assay demonstrated that the cell viability of BMSCs was decreased following treatment with 3-MA and SC79 compared with the $A\beta_{1-42}$ group, while RAPA inhibited this decrease (Fig. 4C). EdU staining revealed that the suppression of DNA replication was more pronounced when autophagy was inhibited or when AKT was activated compared with the effect of $A\beta_{1-42}$ alone, while the activation of autophagy with RAPA alleviated the decrease in DNA replication induced by SC79 (Fig. 4D and E).

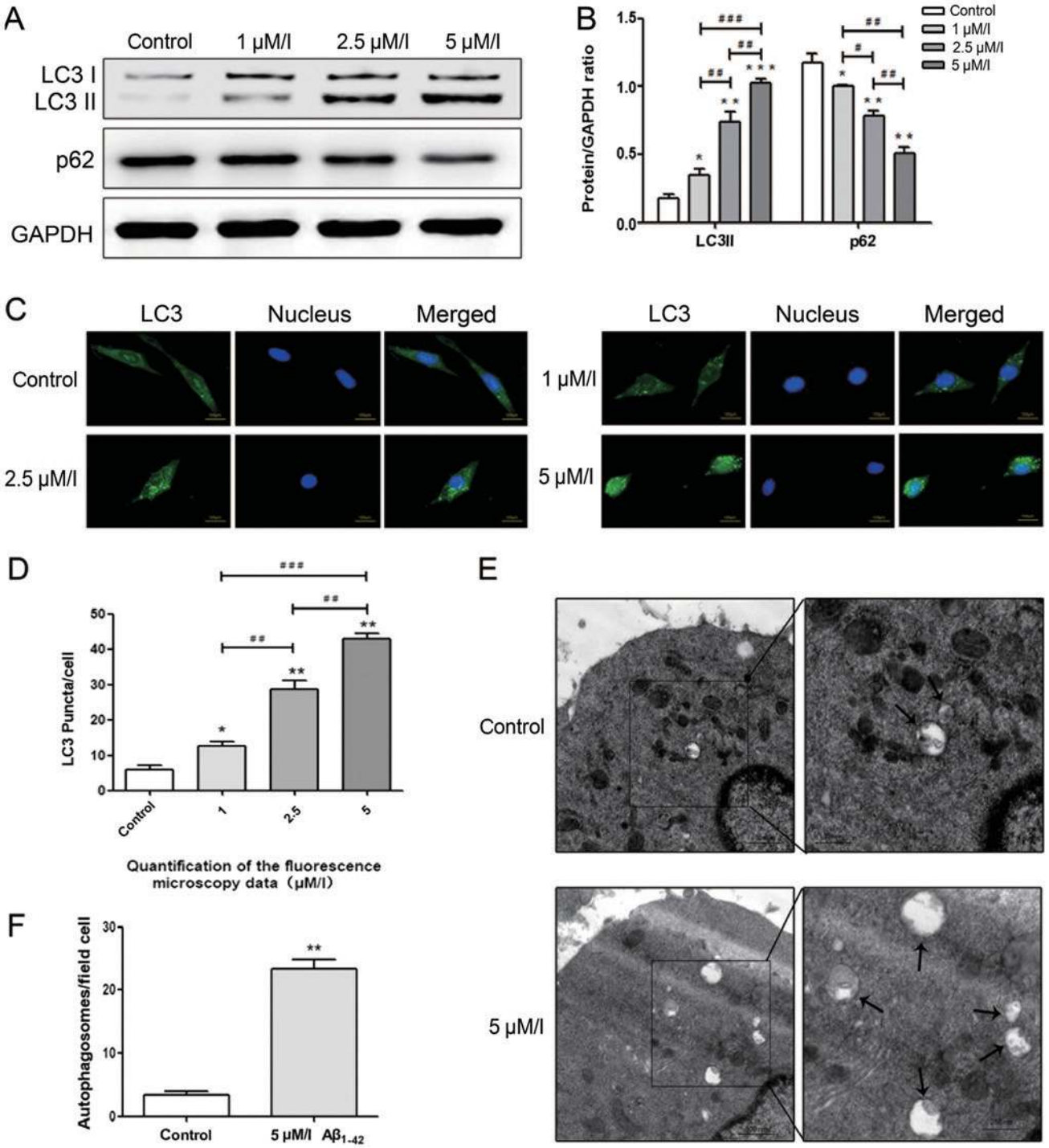

Figure 2. Autophagy is involved in the effect of $A\beta_{1-42}$-treated BMSCs. (A) Western blot analysis demonstrating protein levels of LC3 and p62 following treatment for 48 h with different concentrations of $A\beta_{1-42}$. (B) Quantification of the western blot analysis data. *P<0.05, **P<0.01 and ***P<0.001 vs. the control group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001. n=10 per group. (C) Fluorescence microscopy demonstrating LC3 puncta with green fluorescence in BMSCs treated with $A\beta_{1-42}$ at various concentrations. (D) Quantification of the fluorescence microscopy data. *P<0.05 and **P<0.01 vs. the control group, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001. n=10 per group. (E) TEM images indicating the difference in autophagosomes (arrows) between the control and 5 μM/l $A\beta_{1-42}$ groups. (F) Quantification of the TEM data. **P<0.01 vs. the control group. n=10 per group. All values are presented as the mean ± standard error of the mean from 3 independent experiments. Aβ, amyloid β; BMSC, bone mesenchymal stem cells; LC3, microtubule-associated proteins 1A/1B light chain 3B; LC3 II, lipid-modified LC3; p62, sequestosome 1; TEM, transmission electron microscopy.

Discussion

The causes of neurodegenerative disorders and OP remain unclear, and the association between neurodegenerative diseases and OP is also unknown. It has been hypothesized that an environmental toxicant may contribute to the development of neurodegenerative disorders, for example, free copper (Cu) ions may mediate the aggregation of Aβ in AD brains (18), and overexposure to Cu from the environment is a risk for AD (19). Iron (Fe) has been demonstrated to participate in the pathological process of PD (20). Aluminum, Cu, zinc and a number of other ions have been demonstrated to be significantly

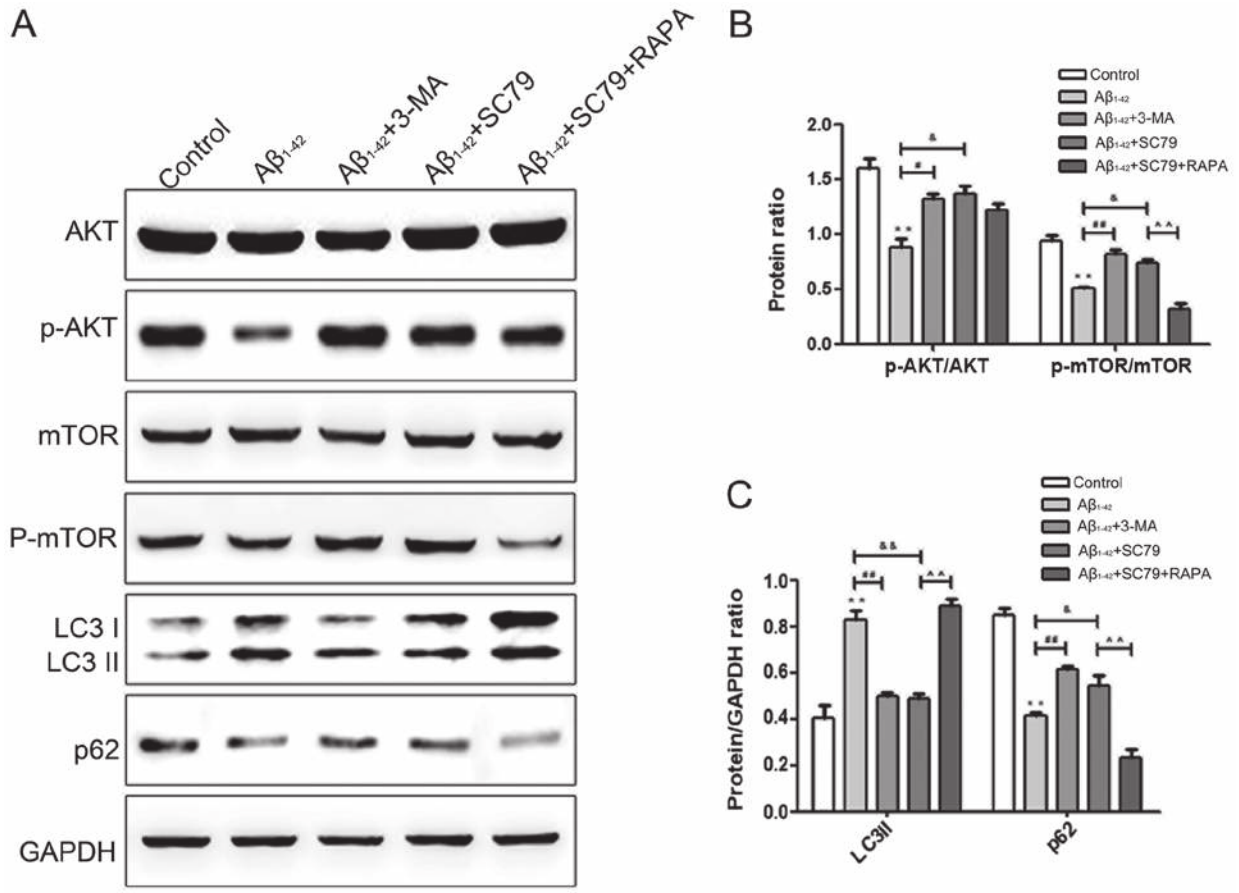

Figure 3. Autophagy is mediated via the AKT/mTOR signaling pathway in $A\beta_{1-42}$-treated BMSCs. (A) Western blot analysis demonstrating the protein levels of LC3, p62, AKT, p-AKT, mTOR and p-mTOR following treatment with $A\beta_{1-42}$, 3-MA, SC79 and SC79 + RAPA for 48 h, and the expression of the AKT/mTOR signaling pathway-associated proteins of AKT, p-AKT, mTOR and p-mTOR following treatment with $A\beta_{1-42}$, 3-MA, SC79 and SC79 + RAPA for 48 h. (B) Quantification of the AKT, p-AKT, mTOR and p-mTOR western blot analysis data. $^{**}P<0.01$ vs. the control group. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs. the $A\beta_{1-42}$ group, $^{\&}P<0.05$ vs. the $A\beta_{1-42}$ group. $^{\wedge\wedge}P<0.01$ vs. the $A\beta_{1-42}$ + SC79 group. n=10 per group. (C) Quantification of the western blot analysis data demonstrating the expression of the AKT/mTOR signaling pathway-associated proteins AKT, p-AKT, mTOR and p-mTOR. $^{**}P<0.01$ vs. the control group. $^{\#\#}P<0.01$ vs. the $A\beta_{1-42}$ group. $^{\&}P<0.05$ and $^{\&\&}P<0.01$ vs. the $A\beta_{1-42}$ group. $^{\wedge\wedge}P<0.01$ vs. the $A\beta_{1-42}$ + SC79 group. n=10 per group. All values are presented as the mean ± standard error of the mean from 3 independent experiments. Aβ, amyloid β; BMSC, bone mesenchymal stem cells; mTOR, mammalian target of rapamycin; AKT, protein kinase B; p, phosphorylated; RAPA, rapamycin; LC3, microtubule-associated proteins 1A/1B light chain 3B; LC3 II, lipid-modified LC3; p62, sequestosome 1; 3-MA, 3-methyladenine.

increased in the cerebrospinal fluid of patients with ALS (21), and accumulation of Fe is also an early event in MS (22). In the pathology of OP, environmental cadmium exposure is associated with an increased loss of BMD in males and females, leading to OP and increased risk of fractures, particularly in the elderly and females (23,24). All the aforementioned evidence has indicated that the external environment, in particular metal ions, participate in the pathological processes of these two neurodegenerative diseases and OP, but whether the internal factors of neurodegenerative diseases, including typical pathological products, affect the process of OP remains unknown. In the present study, it was demonstrated that $A\beta_{1-42}$, an endogenous pathological product of AD, inhibited the proliferation of BMSCs, which provided additional evidence for the occurrence of AD-associated OP.

Clinically, OP is frequently perceived to occur concurrently with the development of AD. Previous studies have demonstrated that the level of hip BMD is decreased and risk of hip fracture is increased in patients with AD (25,26). A study involving an AD mouse model expressing a Swedish mutation of APP indicated that impaired bone mass was detected (27), and that such suppression of osteoblastogenesis and bone formation in Tg2576 mice, a breed of AD model mice, was triggered by reactive oxygen species induced by mutant APP (28). Furthermore, our previous study also demonstrated that excessive Aβ was identified in the bone tissue of APP/PS1 transgenic mouse, and bone mineral loss was more serious compared with the control group (9). In addition, Aβ has been suggested to enhance the function of osteoclasts (OCs) (10), and gene knockout experiments and the use of Tg2576 mice have identified a role for Aβ in the activation of OCs (29,30), Aβ also enhanced receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells ligand-induced OC activation through calcium oscillation signaling pathways (31). An OC is a regulatory cell in bone resorption, and serves a key role in the development of OP. These data have demonstrated that OP may occur secondary to AD. AD is characterized pathologically by synapse loss and the presence of Aβ plaques and tau tangles (32). Aβ, a peptide consisting of 36-43 amino acids, is generated via sequential proteolysis of APP by β-secretase and γ-secretase. Aβ is known to be specifically toxic to neurons (33), while the noxious effect

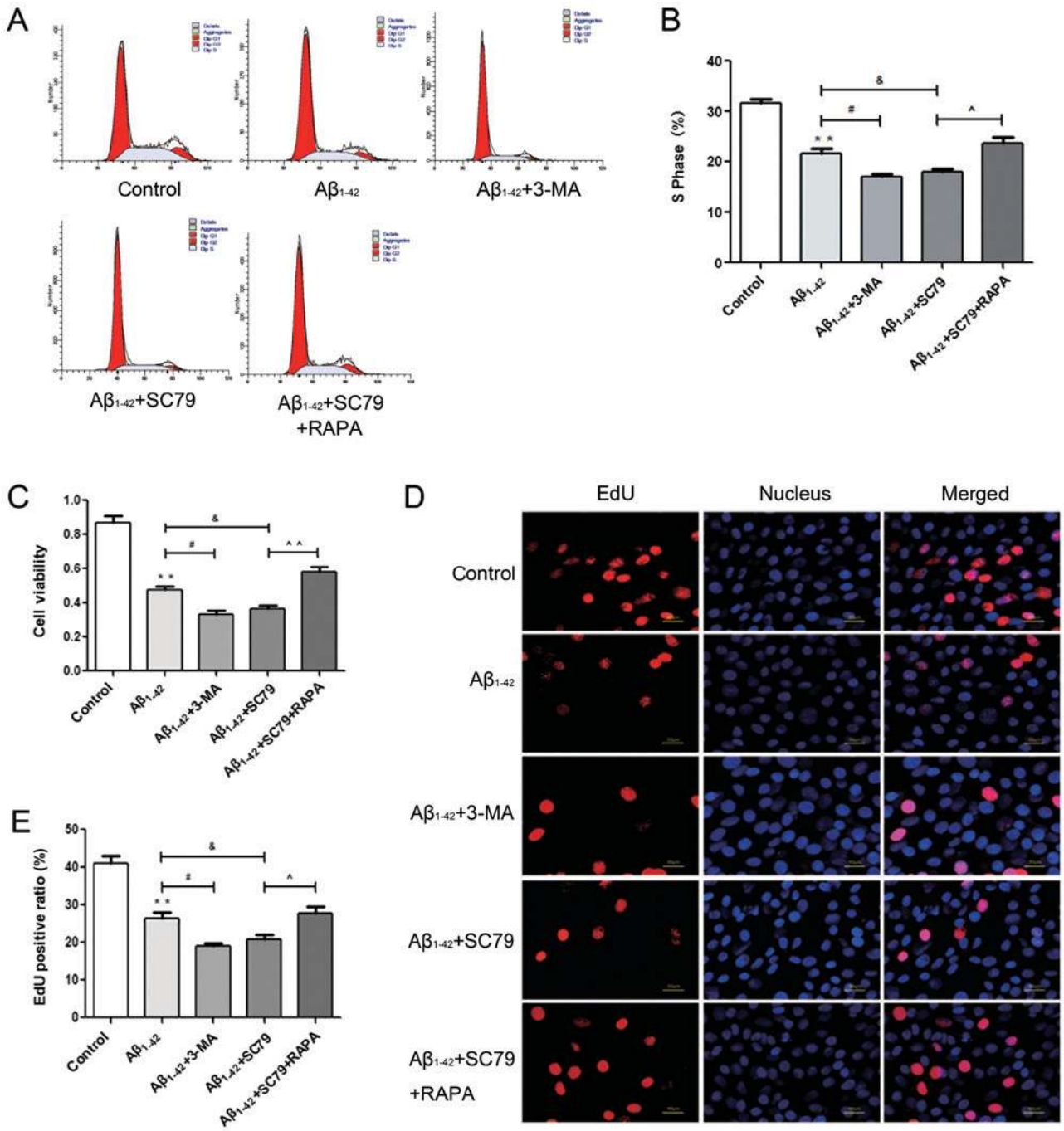

Figure 4. Autophagy serves a protective role against the decrease of proliferation induced by $A\beta_{1-42}$. (A) BMSCs were treated with $A\beta_{1-42}$, 3-MA, SC79 and SC79 + RAPA for 48 h and the cell cycle analysis was performed using flow cytometry. (B) Quantification of cell cycle flow cytometry data. **P<0.01 vs. the control group. $^{\#}$P<0.05 vs. the $A\beta_{1-42}$ group. $^{\&}$P<0.05 vs. the $A\beta_{1-42}$ group. $^{\wedge}$P<0.05 vs. the $A\beta_{1-42}$ + SC79 group. n=10 per group. (C) Cell viability was evaluated by Cell Counting Kit-8 assay. **P<0.01 vs. the control group. $^{\#}$P<0.05 vs. the $A\beta_{1-42}$ group. $^{\&}$P<0.05 vs. the $A\beta_{1-42}$ group. $^{\wedge\wedge}$P<0.01 vs. the $A\beta_{1-42}$ + SC79 group. n=10 per group. (D) EdU staining was used for detection of cell proliferation. (E) Quantification of the EdU staining data. **P<0.01 vs. the control group. $^{\#}$P<0.05 vs. the $A\beta_{1-42}$ group. $^{\&}$P<0.05 vs. the $A\beta_{1-42}$ group. $^{\wedge}$P<0.05 vs. the $A\beta_{1-42}$ + SC79 group. n=10 per group. All values are presented as the mean ± standard error of the mean from 3 independent experiments. Aβ, amyloid β; BMSC, bone mesenchymal stem cells; 3-MA, 3-methyladenine; RAPA, rapamycin; EdU, 5-ethynyl-2'-deoxyuridine.

of $A\beta_{1-42}$, the major component of senile plaques, is the most remarkable. Despite this, conclusive evidence to demonstrate the effect of Aβ on bone metabolism is lacking. BMSCs, the progenitor cells of osteoblasts, participate indirectly in the homeostasis of bone formation and absorption. In addition to differentiation, proliferation is also an important function of BMSCs and it is required for BMSCs to expand cell populations to perform certain functions. As demonstrated previously, Aβ inhibits the proliferation of neural stem cells (NSCs) (34) and serves a crucial role in the development of AD due to its toxic effects. In the present study, it was demonstrated that $A\beta_{1-42}$ decreased cell viability, the number of cells in S phase and the level of DNA replication of BMSCs in a dose-dependent manner; these results provided direct evidence that $A\beta_{1-42}$ may exert a negative effect on the proliferation of cells from the brain, particularly on cells from the skeletal system, and that

they had a similar effect to that of Aβ on the proliferation of NSCs, indicating that $A\beta_{1-42}$ may also serve a critical role in the development of AD-associated OP.

Autophagy, since its identification, has been recognized as an essential process by which damaged organelles and biomacromolecules are eliminated (35,36). This degradation pathway depends upon the formation of autophagosomes with double-layered membranes, which combine with lysosomes and result in degradation of the contents, and is associated with various human disorders, including neurodegenerative diseases, cancer and infectious diseases (37). Autophagy may be activated in response to adverse environmental conditions including nutrient deprivation, exposure to toxic agents and a number of other stress signals (38-41) and serves as a survival mechanism to maintain cell functions. As recommended techniques for detecting autophagy (42), western blot analysis, immunofluorescence and TEM were employed, and demonstrated that the autophagy level increased with increasing concentration of $A\beta_{1-42}$. These results were similar to the phenomenon that Aβ upregulated the autophagy level in the brain and PC12 cells (43). Notably, this upregulation in autophagy level was accompanied by a decrease in proliferation in BMSCs following treatment with $A\beta_{1-42}$. However, additional studies are required to investigate the underlying mechanism of autophagy induced by $A\beta_{1-42}$ and the role of autophagy. mTOR, in particular the mTOR complex 1, is a key regulator of autophagy and cell proliferation. mTOR receives inputs from different signaling pathways. In the present study, it was demonstrated that alternations to AKT, an upstream modulator, were consistent with the variation tendency of mTOR. The phosphorylation of AKT and mTOR decreased following treatment with $A\beta_{1-42}$, suggesting that the AKT/mTOR signaling pathway was involved in this regulatory process. Furthermore, the use of autophagy inhibitor 3-MA and AKT activator SC79 increased the suppression of the AKT/mTOR signaling pathway induced by $A\beta_{1-42}$. Accordingly, the autophagy level also decreased, while treatment with RAPA, an autophagy inducer, resulted in marked decreases in the level of p-mTOR, while the level of autophagy increased. As a result, it was determined that autophagy induced by $A\beta_{1-42}$ was mediated via the AKT/mTOR signaling pathway. The phosphoinositol 3-kinase/AKT/mTOR signaling pathway serves a critical role in the central nervous system, particularly in the pathology of AD (44,45); the results of the present study provided evidence that this pathway may also serve a role in AD-associated OP.

The effects of autophagy may be two-fold: Knockout of autophagy-related gene resulted in a higher rate of cell death (46), while it has been demonstrated that autophagy plays a regulatory role in human tumor cell death and cell death control in numerous studies (47,48) As an essential and highly-conserved intracellular degradation process, autophagy serves a significant role in eukaryotic cell growth, cell death, infection and homeostasis (49,50). Autophagy may increase cell proliferation in conditions of external stress, including hypoxia (51). Although conflicting results exist, the majority of studies consider autophagy as a protective mechanism in AD pathology (52,53). In the present study, when autophagy induced by $A\beta_{1-42}$ was suppressed using 3-MA, or the AKT/mTOR signaling pathway was activated by SC79, the proliferation of BMSCs increased. Following treatment with the autophagy inducer RAPA, it was demonstrated that the proliferation of BMSCs increased even following treatment with SC79. These results indicated that autophagy was likely to be beneficial for $A\beta_{1-42}$-treated BMSCs. The results of the present study are consistent with previous studies that focused on the role of autophagy induced by Aβ in brain tissue; for example, autophagy enhanced by RAPA rescued dysfunctions in AD model mice (52,54). The present study hypothesized that autophagy may also exhibit a protective role in AD-associated OP.

In conclusion, the present study demonstrated that $A\beta_{1-42}$ inhibited the proliferation of BMSCs and upregulated the autophagy level simultaneously. The present study also suggested that the AKT/mTOR signaling pathway was involved in $A\beta_{1-42}$-induced autophagy, and that this autophagy served a protective role in confronting the negative effects of $A\beta_{1-42}$. These data provide an improved understanding of the pathogenesis of AD-associated OP, and regulating the autophagy level may be a novel therapeutic target.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BY and MY conceived the present study. BY and ZC performed the experiments and wrote the paper. WZhang, DY and WZhao helped with data analysis. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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