Autophagy is involved in neurofibromatosis type I gene-modulated osteogenic differentiation in human bone mesenchymal stem cells

YIQIANG LI, MINGWEI ZHU, XUEMEI LIN, JINGCHUN LI, ZHE YUAN, YANHAN LIU and HONGWEN XU

Department of Pediatric Orthopedics, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, Guangdong 510623, P.R. China

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Abstract. Neurofibromatosis type I (NF1) is an autosomal dominant genetic disease that is caused by mutations in the NF1 gene. Various studies have previously demonstrated that the mTOR complex 1 signaling pathway is essential for the NF1-modulated osteogenic differentiation of bone mesenchymal stem cells (BMSCs). Additionally, the mTOR signaling pathway plays a notable role in autophagy. The present study hypothesized that NF1 could modulate the osteogenic differentiation of BMSCs by regulating the autophagic activities of BMSCs. In the present study, human BMSCs were cultured in an osteogenic induction medium. The expression of the NF1 gene was either knocked down or overexpressed by transfection with a specific small interfering RNA (siRNA) targeting NF1 or the pcDNA3.0 NF1-overexpression plasmid, respectively. Autophagic activities of BMSCs (Beclin-1, P62, LC3B I, and LC3B II) were determined using western blotting, electron microscopy, acridine orange (AO) staining and autophagic flux/lysosomal detection by fluorescence microscopy. In addition, the autophagy activator rapamycin (RAPA) and inhibitor 3-methyladenine (3-MA) were used to investigate the effects of autophagy on NF1-modulated osteogenic differentiation in BMSCs. Inhibiting NF1 with siRNA significantly decreased the expression levels of autophagy markers Beclin-1 and LC3B-II, in addition to osteogenic differentiation markers osterix, runt-related transcription factor 2 and alkaline phosphatase. By contrast, overexpressing NF1 with pcDNA3.0 significantly increased their levels. Transmission electron microscopy, AO staining and autophagic flux/lysosomal detection assays revealed that the extent of autophagosome formation was significantly decreased in the NF1-siRNA group but significantly increased in the NF1-pcDNA3.0 group when compared with the NC-siRNA and pcDNA3.0 groups, respectively. In addition, the activity of the PI3K/AKT/mTOR pathway [phosphorylated (p)-PI3K, p-AKT, p-mTOR and p-p70S6 kinase] was significantly upregulated in the NF1-siRNA group compared with the NC-siRNA group, and significantly inhibited in the NF1-pcDNA3.0 group, compared with the pcDNA3.0 group. The knockdown effects of NF1-siRNA on the autophagy and osteogenic differentiation of BMSCs were reversed by the autophagy activator RAPA, while the overexpression effects of NF1-pcDNA3.0 on the autophagy and osteogenic differentiation of BMSCs were reversed by the autophagy inhibitor 3-MA. In conclusion, results from the present study suggest at the involvement of autophagy in the NF1-modulated osteogenic differentiation of BMSCs. Furthermore, NF1 may partially regulate the autophagic activity of BMSCs through the PI3K/AKT/mTOR signaling pathway.

Introduction

Neurofibromatosis type I (NF1) is an autosomal dominant genetic disorder caused by mutations in the NF1 gene (1-3). The reported incidence of NF1 varies from 1/2,500 to 1/3,500 individuals worldwide (1-3). The clinical manifestations of NF1 are varied and include cafe-au-lait spots, hamartomas of the iris and skeletal abnormalities (2,4). It has been reported that 50% patients with NF1 have associated skeletal abnormalities, including long bone dysplasia, sphenoid wing dysplasia, scoliosis and congenital pseudarthrosis of the tibia (5,6).

Although mutations in the neurofibromin (NF1) gene are considered to be the primary cause of the occurrence of NF1 (1,7), the mechanism underlying the formation of skeletal abnormalities associated with NF1 is still not fully understood. The NF1 gene encodes a Ras GTPase that consists of 2,818 amino acids (1). Mutations in NF1 lead to the functional deficiency of neurofibromin and hyperactivation
of p21-Ras (1,7). In addition, NF1 has been documented to regulate bone mesenchymal stem cell, neuronal, and glial cell proliferation, differentiation and survival (1).

Previous studies have demonstrated the existence of significantly impaired osteogenic differentiation in human bone mesenchymal stem cells (BMSCs) in patients with NF1-associated skeletal abnormalities (8,9). Furthermore, abnormal osteoblast differentiation and proliferation have been reported to occur due to the loss of NF1 function (10-12). A previous study showed that the NF1 gene can modulate the proliferation and osteogenic differentiation of BMSCs (13). In addition, it has been demonstrated that inhibiting the expression of NF1 can activate mTOR complex 1 (mTORC1) signaling and subsequently inhibit the osteogenic differentiation of BMSCs (13).

Autophagy is an evolutionarily conserved adaptive response that takes part in numerous physiological and pathological processes (14). Previous studies have reported that autophagy serves an important role in the osteogenic differentiation of BMSCs (15,16). The PI3K/AKT/mTOR pathway is an important signaling pathway that is involved in the regulation of signal transduction and biological processes, such as cell proliferation, differentiation, apoptosis, metabolism and angiogenesis (17). The PI3K/AKT/mTOR pathway is also considered to be a classical signaling pathway for autophagy activation (18), such that mTORC1 is the main gatekeeper to autophagy that connects environmental cues to metabolic processes in order to preserve cellular homeostasis (19). In a recent study, Tan et al (20) revealed that overexpression of NF1 gene enhanced the osteogenic differentiation of BMSCs by promoting autophagy and that mTORC1 signaling was involved in this process. However, this previous study only established NF1-overexpression BMSC models, which is different from the clinical situation in patients with NF1, where the function of the NF1 gene is typically insufficient (20). Therefore, a cell model with the inhibited expression of NF1 would simulate the pathological conditions of NF1 more closely compared with one modeling the overexpression of NF1.

The present study established cell models of BMSCs that with reduced NF1 expression or overexpressed NF1, similar to the protocol followed in a previous study (13). To investigate the effect of autophagy on the osteogenic differentiation of BMSCs, a classical autophagy inhibitor 3-methyladenine (3-MA) and a specific mTOR inhibitor rapamycin (RAPA), were used. The aims of the present study were as follows: i) To evaluate the effect of NF1 on the autophagy of BMSCs; ii) to investigate the effect of autophagy on NF1-mediated osteogenic differentiation of BMSCs; and iii) to verify the effect of the PI3K/AKT/mTOR signaling pathway on NF1-mediated regulation of BMSC autophagy.

Materials and methods

Culture of human BMSCs. BMSCs were purchased from the American Type Culture Collection (cat. no. CRL-3421). They were cultured in Human Bone Marrow Mesenchymal Stem Cell Basal Medium (Cyagen Biosciences, Inc.) supplemented with 10% qualified FBS (Takara Bio, Inc.), 10% glutamine and 10% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO₂ for 14 days (13). The standard procedure to induce osteogenic differentiation of BMSCs is by culturing cells in Human Mesenchymal Stem Cell Osteogenic Differentiation Basal Medium (Cyagen Biosciences, Inc.) containing 10% FBS (Takara Bio, Inc.), 0.2% ascorbic acid (Shanghai Aladdin Biochemical Technology Co., Ltd.), 0.01% dexamethasone (Shanghai Aladdin Biochemical Technology Co., Ltd.), 1% glutamine, 100 units of penicillin-streptomycin and 1% β-glycerophosphate sodium (13).

Cell transfection and treatment. BMSC models with inhibited or overexpressed NF1 were established using a method similar to that used by a previous study (13). Briefly, a small interfering RNA (siRNA) targeting NF1 and a negative control (NC) siRNA (non-targeting sequence) were purchased from Shanghai GenePharma Co., Ltd. The siRNA targeting sequences were as follows: NF1-siRNA, 5'-ACATAACAAAGTCAGTAC T-3'; and NC-siRNA, 5'-AACAAGATGAAGACACCA-3'. Human BMSCs were grown in 6/12-well culture plates until ~80% confluence, then transfected with 100 nm siRNA-NF1 or NC-siRNA for 48 h at 37°C using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Human NF1 cDNA was amplified by PCR and inserted into pcDNA3.0 (NF1-pcDNA3.0; Invitrogen; Thermo Fisher Scientific, Inc.). Then NF1-pcDNA3.0 (1/2 µg) or pcDNA3.0 (1/2 µg) was transfected into BMSCs using Lipofectamine® 2000 in accordance with the manufacturer's protocol. Twenty-four hours later, the transfected cells were divided into the following four groups: NC-siRNA, NF1-siRNA, pcDNA3.0 and NF1-pcDNA3.0. Additionally, a control group was established containing untransfected BMSCs. The transfected BMSCs were treated with 50 nM RAPA (cat. no. AY 22989; MedChemExpress) or 5 mM 3-MA (cat. no. S2767-1; Selleck Chemicals) for 24 h at 37°C for the detection of autophagy and associated pathways. The concentrations used in the present study were based on those used in previous studies (21,22). In addition, osteogenic differentiation experiments were conducted on day 14 following transfection.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Following transfection and differentiation on day 14, total RNA was isolated from cultured cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Gene expression levels were measured in a real-time PCR detection system (Bio-Rad Laboratories, Inc.) by SYBR® Green (Bio-Rad Laboratories, Inc.) detection. Briefly, the extracted RNA was reverse transcribed in the presence of a poly (A) polymerase with an oligo-dT adaptor, using the PrimeScript™ RT Reagent kit with gDNA Eraser (Perfect Real Time; cat. no. RR047A; GenePharma Co., Ltd. The siRNA targeting sequences were as follows: NF1, runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and Osterix were quantified by qPCR using TB Green® Fast qPCR Mix (cat. no. RR430; Takara Bio, Inc.). Thermocycling conditions were as follows: Initial denaturation at 95°C for 15 min; followed by 40 cycles of 95°C for 15 sec, annealing 50-60°C for 30 sec, 72°C for 30 sec; followed by final extension at 72°C for 7 min. GAPDH was used as the internal control. The PCR primers are listed
The Cq value obtained for the gene of interest was normalized to that of the housekeeping gene GAPDH to obtain the ΔCq value. The ΔΔCq value was then obtained by subtracting the ΔCq value for each gene of interest from the ΔCq value for the control sample. The results were calculated using the equation RQ=2⁻ΔΔCq, where RQ is the relative quantity and is expressed as the fold-change relative to the corresponding gene expression level in the control sample (23).

Western blotting. Transfected and treated BMSC proteins were extracted using lysis buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM PMSF and 0.2% aprotinin (Beyotime Institute of Biotechnology). A BCA™ Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used for quantification of protein samples. Equal amounts of protein samples (30 µg per lane) were separated on 15% SDS-PAGE gels and transferred onto PVDF membranes. After blocking in 5% BSA serum (Roche Diagnostics GmbH) for 1 h at 37˚C, the blocked membranes were incubated with the corresponding primary antibodies overnight at 4˚C. The membranes were then washed in Tris-buffered saline (Tris 20 mM, NaCl 137 mM, pH 7.6) containing 0.1% Tween-20 (TBST; cat. no. P2287; Sigma-Aldrich; Merck KgaA) three times and incubated for 1 h at room temperature with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). The membranes were incubated with ECL reagent (Immun-Star HRP Substrate kit; cat. no. C3006; Beyotime Institute of Biotechnology). A BCA™ Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used for quantification of protein samples. Equal amounts of protein samples (30 µg per lane) were separated on 15% SDS-PAGE gels and transferred onto PVDF membranes. After blocking in 5% BSA serum (Roche Diagnostics GmbH) for 1 h at 37˚C, the blocked membranes were incubated with the corresponding primary antibodies overnight at 4˚C. The membranes were then washed in Tris-buffered saline (Tris 20 mM, NaCl 137 mM, pH 7.6) containing 0.1% Tween-20 (TBST; cat. no. P2287; Sigma-Aldrich; Merck KgaA) three times and incubated for 1 h at room temperature with appropriate secondary antibodies conjugated to horseradish peroxidase (HRP). The membranes were incubated with ECL reagent (Immun-Star HRP Substrate kit; cat. no. 1705040; Bio-Rad Laboratories, Inc.). The signals were then visualized and analyzed using Image Lab™ software 5.2 (Bio-Rad Laboratories, Inc.). For the present study, primary antibodies against NF1 (1:1,000; cat. no. ab128054; Abcam), Beclin-1 (1:1,000; cat. no. ab210498; Abcam), p62 (1:1,000; cat. no. ab109012; Abcam), LC3B/I (1:1,000; cat. no. ab192890; Abcam), GAPDH (1:1,000; cat. no. ab8245; Abcam), ALP (1:1,000; cat. no. ab229126; Abcam), Runx2 (1:1,000; cat. no. ab236639; Abcam) and Osterix (1:1,000; cat. no. ab209484; Abcam) were used. Antibodies against phosphorylated (p)-mTOR (1:1,000; cat. no. 5536; Cell Signaling Technology), total (t)-mTOR (1:1,000; cat. no. 2983; Cell Signaling Technology), p-p70S6 kinase (p70S6K; 1:1,000; cat. no. 9204; Cell Signaling Technology), t-p70S6K (1:1,000; cat. no. 2708; Cell Signaling Technology), AKT (1:1,000; cat. no. 9272; Cell Signaling Technology), p-AKT (1:1,000; cat. no. 4060; Cell Signaling Technology), P13K (1:1,000; cat. no. 4249; Cell Signaling Technology) and p-P13K (1:1,000; cat. no. 17366; Cell Signaling Technology) were also used. HRP-conjugated anti-mouse IgG (1:5,000; cat. no. ab6728; Abcam) and HRP-conjugated anti-rabbit IgG (1:5,000; cat. no. ab6721; Abcam) were used as secondary antibodies.

Acridine orange (AO) staining. After conditioning, BMSCs were harvested and suspended in PBS at 1x10⁶ cells/ml. Next, 95 µl of this cell suspension was considered and 5 µl AO staining solution (Sigma-Aldrich; Merck KGaA) was added. The reaction was allowed to proceed for 10 min in the dark at room temperature. Subsequently, 5 ml PBS was added and the suspension was then centrifuged on a conventional centrifuge at 150 x g at room temperature for 5 min. The supernatant was then discarded and washed twice with PBS. The cells suspended in PBS were pipetted onto slides and sealed with cover glass. Autophagy was visualized under a fluorescence microscope (magnification, x40; Leica DMIRB; Leica Microsystems GmbH).

Autophagic flux/lysosomal detection. To track and observe the formation of autophagosomes and autophagic flux, 1x10⁶ cells/ml were grown to ~80% confluence, infected with Ad-GFP-LC3B (cat. no. C3006; Beyotime Institute of Biotechnology) at 7 log₁₀ PFU/ml for 24 h and cultured in a 6-well plate on cover glass to monitor autophagy flux. Furthermore, the slides were washed with PBS and 3% paraformaldehyde was added into each well. The slides were then placed in the dark for 20 min at room temperature. The slides were washed three times with PBS before 2 ml PBS was then added to each well. The plate was incubated with shaking at room temperature for 10 min. The coverslips were mounted with an anti-fade mounting solution (cat. no. P0126; Beyotime Institute of Biotechnology) and dried for 1 min at room temperature. Subsequently, the slides were observed with a fluorescence microscope.

### Table I. Primers used in the present study.

| ID  | Orientation | Sequence (direction, 5'-3') |
|-----|-------------|---------------------------|
| NF1 | F           | GTATTGAATGACCATTTTGGTGG   |
| NF1 | R           | CCGCCCAAGGCTCCCCCAG       |
| ALP | F           | CCAACTCTTTTGTGCAGAGA      |
| ALP | R           | GGCTACATTGGTGTAGCTTTT     |
| Runx2| F        | GACTTGGTTTTCATAACAGCGGA   |
| Runx2| R        | ACCTACCCATCTGACCTTGTCT    |
| Osterix| F    | CTGCCCACATATTCCACTG       |
| Osterix| R     | AGGGTGGTGTGAACGGATTG      |
| GAPDH| F         | GGGGTCGTTGATGGCAACCA      |

NF1, neurofibromatosis type I; ALP, alkaline phosphatase; Runx2, runt-related transcription factor 2; F, forward; R, reverse.
(magnification, x40; Leica DMIRB; Leica Microsystems GmbH). During this procedure, GFP-LC3 was combined with autophagosomes, and detected using fluorescence microscopy. Under the fluorescence microscope, the GFP-LC3 combined autophagosomes were indicated by granular green fluorescence.

**ALP staining.** BMSCs were inoculated into a 12-well culture plate at a density of 1x10^4 cells per well at 37°C in 5% CO2. After 24 h, the medium was replaced with the osteogenic induction medium (cat. no. CTCC-Y001, PH Biomedicine) and cultured at 37°C in 5% CO2. The medium was changed every 2-3 days and removed after 14 days. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. The paraformaldehyde was then removed and the cells were washed three times with ddH2O and an ALP staining solution (Shanghai Gefan Biotechnology Co., Ltd.) was added for 30 min at room temperature. The ALP staining solution was removed, the cells were washed three times with ddH2O and visualized under a microscope (magnification, x40; Leica DMIRB; Leica Microsystems GmbH); in addition, images were captured.

**Alizarin red staining.** BMSCs were cultured with osteogenic induction medium for 14 days before examination of Alizarin red staining. The culture medium was discarded and the cells (1x10^4) were fixed with 4% paraformaldehyde for 15-20 min at room temperature, following which they were washed three times with PBS. Alizarin red staining solution (ScienCell Research Laboratories, Inc.) was prepared in advance and was added to the culture plate and placed in the incubator for 15 min at room temperature. The staining solution was then discarded, the plate was washed three times with PBS solution and placed under a differential interference contrast microscope (magnification, x40; Leica DMIRB; Leica Microsystems GmbH) to capture images.

**Transmission electron microscopy.** BMSCs were cultured in 6-well plates and transfected with NFI-pcDNA3.0 for 48 h, and then collected and fixed with a mixture of 2.5% glutaraldehyde and 1% acetic acid for 2 h at room temperature. Samples were then processed following a standard protocol (24). Briefly, samples were dehydrated using ethanol, stained for 2 h using uranyl acetate and alkaline lead citrate at room temperature, embedded using Epon resin at 37°C overnight, and cut into 500 nm-thick sections using an automatic microwave sample processor (Leica EM AMW; Leica Microsystems GmbH) at room temperature. Observation and imaging were then performed by using a JEM-1400 transmission electron microscope (JEOL, Ltd.).

**Statistical analysis.** All experiments were repeated ≥ three times. The results are expressed as the mean ± standard deviation. Data were processed using the SPSS 10.0 statistical software (SPSS, Inc.). For between-group comparisons, data were analyzed using an unpaired Student’s t-test. One-way analysis of variance followed by a post hoc test of LSD was used to analyze the data among the three groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**NFI regulates the autophagic activity of BMSCs.** To detect the effect of NFI on the autophagic activity of BMSCs, cell models of BMSCs with NFI knockout using NFI-siRNA or NFI overexpression (NFI-pcDNA3.0) were established. RT-qPCR and western blotting results showed that the mRNA (Fig. 1A) and protein (Fig. 1B and C) expression levels of NFI were significantly decreased in the NFI-siRNA group compared with those in NC siRNA, but significantly increased in the NFI-pcDNA3.0 group compared with those in the pcDNA3.0 group. Western blotting results indicated that in the NFI-siRNA group, the expression of Beclin-1 was significantly decreased, whereas that of LC3B-I and p62, a marker of autophagosome degradation (25), were significantly increased compared with that in the NC siRNA group (Fig. 1B and C). In addition, in the NFI-pcDNA3.0 group, the expression of autophagy markers Beclin-1, was significantly increased, whereas that of LC3B-I and p62 was significantly decreased compared with that in the pcDNA3.0 group (Fig. 1B and C).

Furthermore, autophagic activity was detected using transmission electron microscopy. The results revealed that overexpression of NFI(NFI-pcDNA3.0) increased the formation of autophagosomes, whilst inhibiting the expression of NFI(NFI-siRNA) significantly reduced the formation of autophagosomes when compared with groups pcDNA3.0 and NC-siRNA, respectively (Fig. 2). Furthermore, AO staining and autophagic flux/lysosomal detection were performed (Fig. 3A). AO staining demonstrated that the formation of autophagolysosomes (indicated by yellow-red or orange fluorescence in Fig. 3A) was increased in the NFI-pcDNA3.0 group but decreased in the NFI-siRNA group when compared with groups NC-siRNA and pcDNA3.0. The autophagic flux/lysosomal detection assay also revealed similar results (Fig. 3B). During the formation of autophagosomes, GFP-LC3 protein transferred to the membrane of autophagosomes, and the autophagosomes were indicated by green puncta using fluorescence microscopy. The results of the present study indicated that the autophagic flux/lysosomal was significantly increased in the NFI-pcDNA3.0 group but decreased in the NFI-siRNA group when compared with the pcDNA3.0 and NC-siRNA groups, respectively.

Collectively, these results suggest that overexpression of NFI promoted autophagic activity of BMSCs, whilst knockdown of NFI expression decreased the level of autophagy in BMSCs.

**NFI modulates osteogenic differentiation by influencing the autophagic activity of BMSCs.** The results of RT-qPCR and western blotting indicated that the expression levels of osteogenic differentiation markers Osterix, Runx2 and ALP were markedly decreased in the NFI-siRNA group, whereas they were increased in the NFI-pcDNA3.0 group compared with those in the NC siRNA group (Fig. 4A-D). To investigate the effects of autophagy on osteogenic differentiation of BMSCs, an activator (RAPA) and inhibitor (3-MA) of autophagy were added to the NFI-siRNA and NFI-pcDNA3.0 groups, respectively. As a result, the levels of the osteogenic differentiation markers Osterix, Runx2 and ALP increased markedly in the RAPA-treated group compared with that in
Figure 1. *NF1* regulates the autophagic activity of bone mesenchymal stem cells. (A) Reverse transcription-quantitative PCR demonstrated significant down-regulation of *NF1*-mRNA expression in the *NF1*-siRNA group and upregulation of *NF1*-mRNA expression in the *NF1*-pcDNA3.0 group. (B) Western blotting and subsequent (C) quantification indicated that the protein levels of *NF1*, Beclin-1 were significantly decreased in the *NF1*-siRNA group and increased in the *NF1*-pcDNA3.0 group, whereas the changes in the expression of p62 and LC3B-I revealed the opposite pattern. *P*<0.05 vs. NC-siRNA; *P*<0.05 vs. *NF1*-pcDNA3.0. *NF1*, neurofibromin-1; si-, small interfering; NC, negative control.

Figure 2. Transmission electron microscopy results. It demonstrated that the formation of autophagosomes was decreased in the *NF1*-siRNA group and increased in the *NF1*-pcDNA3.0 group (indicated by the arrows). Scale bars, 2 µm. NC, negative control; si-, small interfering; *NF1*, neurofibromin-1.
the NF1-siRNA group alone. By contrast, the expression levels decreased markedly in the 3-MA-treated group compared with those in NF1-pcDNA3.0 group alone (Fig. 4A-D).

ALP staining indicated that ALP activity was decreased by knocking down NF1 expression (NF1-siRNA) and increased by NF1 overexpression (NF1-pcDNA3.0) when compared
with NC-siRNA and pcDNA3.0 groups, respectively. In addition, RAPA and 3-MA treatment reversed the changes in ALP activity in the NF1-siRNA and NF1-pcDNA3.0 groups, respectively (Fig. 5). Alizarin red staining, representing calcium deposition, also revealed similar results (Fig. 6). After application of RAPA and 3-MA, the decreased and increased calcium deposition in the NF1-siRNA and NF1-pcDNA3.0 groups, respectively, were restored to the levels in the control groups (Fig. 6). These results suggest that NF1 could modulate the osteogenic differentiation of BMSCs by regulating the autophagic activity of BMSCs.

NF1 partially regulates the autophagic activity of BMSCs via the PI3K/AKT/mTOR pathway. The present study verified whether NF1 could regulate autophagy in BMSCs through the PI3K/AKT/mTOR pathway. Western blotting results demonstrated that the PI3K/AKT/mTOR pathway was significantly activated in the NF1-siRNA group, as indicated by increased levels of p-PI3K, p-AKT, p-mTOR and p-p70S6K compared with those in the NC-siRNA group (Fig. 7A-C). By contrast, the PI3K/AKT/mTOR pathway was significantly inhibited in the NF1-pcDNA3.0 group, as indicated by decreased levels of p-PI3K, p-AKT, p-mTOR and p-p70S6K compared with those in the pcDNA3.0 group (Fig. 7A-C). RAPA was used to activate whereas 3-MA was used to inhibit the autophagy of BMSCs in the NF1-siRNA and NF1-pcDNA3.0 groups, respectively. The results of western blot analysis indicated that the autophagic activity was significantly increased in group NF1-siRNA+RAPA and decreased in group NF1-pcDNA3.0+3-MA, compared with the NF1-siRNA and group NF1-pcDNA3.0, respectively (Fig. 7D-F). Transmission electron microscopy, AO staining...
and autophagic flux/lysosomal detection indicated that the formation of autophagosomes were significantly increased in the NF1-siRNA + RAPA group compared with the NF1-siRNA, whilst it was significantly decreased in the NF1-pcDNA3.0 + 3-MA group compared with that in the NF1-pcDNA3.0 group (Figs. 8 and 9). The levels of p-mTOR, p-p70S6K, p-PI3K, and p-AKT were significantly decreased in the NF1-siRNA + RAPA group compared with those in the NF1-siRNA group (Fig. 7A-C). Additionally, the levels of p-PI3K, p-AKT, p-mTOR and p-p70S6K were all significantly decreased in the NF1-pcDNA3.0 + 3-MA group compared with those in the NF1-pcDNA3.0 group (Fig. 7A-C). These results indicated that NF1 could partially regulate the autophagic activity of BMSCs via the PI3K/AKT/mTOR signaling pathway.

**Discussion**

Data from the present study indicate that NF1 regulated the autophagy of BMSCs. Overexpression of NF1 promoted the autophagic activity of BMSCs, whilst autophagic activity was inhibited by the downregulation of NF1. Several studies have reported the effect of autophagy on NF1-deficient malignant peripheral nerve sheath tumors (MPNSTs) (26,27). In particular, Yang et al (26) found that NF1-deficient MPNST samples exhibit high mobility group protein A2

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Figure 7. NF1 regulates autophagy in bone mesenchymal stem cells by inhibiting the PI3K/AKT/mTOR pathway. (A-C) Western blotting demonstrated that NF1-siRNA significantly decreased the expression of Beclin-1 and LC3B-II and increased the expression of P62 and LC3B-I, while RAPA reversed the effects of NF1-siRNA. NF1-pcDNA3.0 significantly increased the expression of Beclin-1 and LC3B-II while decreased the expression of P62 and LC3B-I, while 3-MA reversed the effects of NF1-pcDNA3.0. (D-F) Western blotting indicated that the levels of proteins in the PI3K/AKT/mTOR pathway (p-PI3K, p-AKT, p-mTOR and p-P70S6K) were upregulated in the NF1-siRNA group and downregulated in the NF1-pcDNA3.0 group when compared with groups NC-siRNA and pcDNA3.0, respectively. RAPA downregulated PI3K/AKT/mTOR pathway in BMSCs with NF1-siRNA, while 3-MA upregulated PI3K/AKT/mTOR pathway in BMSCs with NF1-pcDNA3.0. Quantified levels of (B) Beclin-1, (C) p62, LC3B-I, LC3B-II, (E, F) p-PI3K, p-AKT, p-mTOR and p-P70S6K. *P<0.05 vs. pcDNA3.0; **P<0.05 vs NC-siRNA; ***P<0.05 vs NF1-siRNA; ****P<0.05 vs NF1-pcDNA3.0. NF1, neurofibromatosis type 1; si-, small interfering; NC, negative control; RAPA, rapamycin; 3-MA, 3-methyladenine; p-, phosphorylated; P70S6K, p70S6 kinase.
(HMGA2) expression levels and that HMGA2 knockdown inhibited autophagy, which subsequently promotes MPNST cell death. However, to the best of our knowledge, few studies have reported the effects of NF1 on autophagy in BMSCs. Tan et al (20) revealed that autophagic activity and osteogenic differentiation were significantly enhanced in NF1-overexpressing BMSCs, consistent with the results of the present study. However, Tan et al (20) only established NF1-overexpression BMSC models, which are different from the clinical situation in patients with NF1 mutations, where the function of NF1 is insufficient (8). Therefore, a cell model with inhibited expression of NF1 can simulate the pathological conditions of NF1 more closely compared with one with overexpression of NF1. The present study established cell models of BMSCs with the inhibition or overexpression of NF1, which is more translational for investigating the effects of NF1 on the autophagy of BMSCs (26,27).

The present study also demonstrated that autophagy served a notable role in NF1-modulated osteogenic differentiation of BMSCs. Knockdown of NF1 inhibited the autophagic activity of BMSCs and decreased the osteogenic differentiation of BMSCs whilst overexpression of NF1 resulted in the opposite effects. In addition, an autophagy activator (RAPA) and an autophagy inhibitor (3-MA) reversed the effects of NF1-knockdown and overexpression, respectively, on the osteogenic differentiation of BMSCs. A number of studies have demonstrated the involvement of NF1 in osteogenic differentiation of BMSCs (8,10,11,28). In particular, a previous study demonstrated that downregulation and upregulation of NF1 respectively inhibited and promoted osteogenic differentiation of BMSCs, respectively (13). Leskelä et al (8) cultured mesenchymal stem cells of patients with NF1 and revealed impaired osteoblast differentiation. Conversely, loss of NF1 resulted in increased osteoblast proliferation (10,11). Kolanczyk et al (10) established a mouse model with conditional inactivation of NF1 in the limb skeleton and tested the effect of NF1 on osteoblast proliferation. They revealed that the osteoblast cell division rate was significantly increased in inactivated NF1 mutant cells when compared with that in controls. Additionally, other studies have demonstrated that autophagy can serve an important role in the osteogenic differentiation process (15,16,29-32). Wan et al (15) investigated the lumbar BMSCs of patients with osteoporosis and determined

![Figure 8. Results of transmission electron microscopy. Treatment with RAPA and 3-MA reversed the effects of NF1-siRNA and NF1-pcDNA3.0 transfection, respectively, on the autophagy activity of BMSCs (autophagosome was indicated by the arrows). Scale bars, 2 µm. NF1, neurofibromin-1; si-, small interfering; NC, negative control; RAPA, rapamycin; 3-MA, 3-methyladenine.](image-url)
that the autophagy level in these BMSCs was significantly decreased, which was accompanied by inhibited osteogenic differentiation. Furthermore, it was identified that an autophagy activator (RAPA) significantly increased the osteogenic differentiation of BMSCs (15). Ma et al (29) also determined that the autophagy levels in young male mice were higher compared with those in aged male mice. In addition, treatment with an autophagy inhibitor significantly suppressed the osteogenic differentiation of BMSCs (29). Nuschke et al (30) found that autophagy levels in BMSCs were significantly increased during the early stages of osteogenic differentiation, whilst it was significantly decreased after differentiation into mature osteocytes. In addition, Liu et al (31), Gómez-Puerto et al (32) and Zhou et al (16) reported that autophagy served an important role in osteogenic differentiation of BMSCs. In brief, the majority of previous studies aforementioned reported that an increase in autophagic activity of BMSCs was partially regulated by NF1 via the PI3K/AKT/mTOR signaling pathway. The PI3K/AKT/mTOR pathway was activated in NF1-knockdown BMSCs, whilst it was inhibited in NF1-overexpressing BMSCs. It has been previously reported that the PI3K/AKT/mTOR pathway serves a notable role in regulating autophagy (18,33,34). It is a classical signaling pathway for autophagy activation and the main gateway to autophagy (18,19). In particular, various studies have reported activation of the PI3K/AKT/mTOR signaling pathway in NF1-related MPNSTs (35,36). Previous studies by Tan et al (20) and Li et al (13), combined with the present study, demonstrate that regulation of autophagy facilitates NF1-mediated modulation of osteogenic differentiation of BMSCs via the PI3K/AKT/mTOR signaling pathway.

The present study also indicated that the autophagic activity of BMSCs was partially regulated by NF1 via the PI3K/AKT/mTOR signaling pathway. The PI3K/AKT/mTOR pathway was activated in NF1-knockdown BMSCs, whilst it was inhibited in NF1-overexpressing BMSCs. It has been previously reported that the PI3K/AKT/mTOR pathway serves a notable role in regulating autophagy (18,33,34). It is a classical signaling pathway for autophagy activation and the main gateway to autophagy (18,19). In particular, various studies have reported activation of the PI3K/AKT/mTOR signaling pathway in NF1-related MPNSTs (35,36). Previous studies by Tan et al (20) and Li et al (13), combined with the present study, demonstrate that regulation of autophagy facilitates NF1-mediated modulation of osteogenic differentiation of BMSCs via the PI3K/AKT/mTOR signaling pathway.

The mTORC1 signaling pathway has been reported to be an important regulator of autophagy (19). Unc-51-like autophagy activating kinase (ULK) is a key initiator of autophagy (37,38). Furthermore, mTORC1 inhibits the ULK complex by phosphorylating its components, including autophagy-related gene 13 and ULK1/2 (37,38). Additionally, mTORC1 regulates the Vps34 class III PI3K complex, which is needed for autophagosome formation (37,38). In the present
study, the autophagic activity in the NF1-pcDNA3.0 group was significantly decreased after an autophagy inhibitor (3-MA) was applied. It is known that 3-MA is an inhibitor of PI3K (39) and that it suppresses autophagy by inhibiting the class III PI3K (40). Additionally, 3-MA inhibits AKT by inhibiting the class I PI3K (40,41) and subsequently leads to mTORC1 inactivation (18). As aforementioned, mTORC1 signaling inhibits autophagosome formation (37,38). The inhibition of autophagy and osteogenic differentiation of BMSCs in the NF1-pcDNA3.0 + 3-MA group in the present study suggests that autophagy may partially regulate osteogenic differentiation of BMSCs through other mechanisms that are independent of mTOR signaling. It has been reported that AMP-activated protein kinase and the Wnt/β-catenin signaling pathway may also mediate important roles in regulating autophagy in BMSCs (42,43). Therefore, further study is required to investigate the mechanism by which NF1 modulates osteogenic differentiation of BMSCs through autophagy.

There are still some limitations in the present study. Firstly, our study only established BMSCs models with knockdown or overexpression of NF1 by siRNA or pcDNA3.0, which is different from clinical condition (NF1 mutation). Secondly, the present study is a cell experiment, further animal studies are required to confirm the role of autophagy on NF1-modulated growth of bone.

In conclusion, the present study demonstrated that autophagy played a significant role in NF1-mediated osteogenic differentiation of BMSCs. Downregulation of NF1 inhibited autophagy to decrease osteogenic differentiation of BMSCs, whereas upregulation of NF1 activated autophagy to increase osteogenic differentiation. NF1 may partially regulate the autophagic activity of BMSCs via the PI3K/AKT/mTOR signaling pathway. The present study could guide a new direction for elucidating the etiology of NF1-associated skeletal abnormalities and provide a novel theoretical basis for the treatment of NF1.

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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

Conceptualization, methodology, supervision and writing (review and editing) were performed by YQL, HWX and XML. Original draft and funding acquisition were performed by YQL. HWX and YQL provided resources. XML, MWZ, JCL, ZY and YHL performed the experiments. YQL and MWZ analyzed the data. YQL and HWX confirm the authenticity of all the raw data. All 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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