

# Wnt/ $\beta$ -catenin signaling pathway activation reverses gemcitabine resistance by attenuating Beclin1-mediated autophagy in the MG63 human osteosarcoma cell line

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**Abstract.** An aberrant Wnt/ $\beta$ -catenin signaling pathway is frequently implicated in tumorigenesis. However, whether the Wnt/ $\beta$ -catenin pathway plays a role in resistance to antitumor chemotherapy drugs remains unknown. In the present study, the process of autophagy was assessed following overexpression of the autophagy-associated gene Beclin 1 in gemcitabine-induced MG63 human osteosarcoma cells. Autophagy-associated gene expression was measured following activation or inhibition of the Wnt/ $\beta$ -catenin pathway in gemcitabine-induced MG63 cells using reverse transcription-quantitative polymerase chain reaction. In addition, the percentage of MG63 cell apoptosis was measured by flow cytometry following Wnt/ $\beta$ -catenin pathway activation or inhibition. The results demonstrated that Beclin 1 overexpression induced autophagy and reduced gemcitabine-induced apoptosis in MG63 human cell line. Furthermore, activation of the Wnt/ $\beta$ -catenin signaling pathway attenuated autophagy and enhanced gemcitabine-induced apoptosis. Additionally, the expression of Beclin 1 was reduced following Wnt/ $\beta$ -catenin signaling pathway activation. The present study demonstrated that activation of the Wnt/ $\beta$ -catenin signaling pathway may rescue chemotherapy drug resistance by downregulating the expression of Beclin 1.

## Introduction

Gemcitabine is a nucleoside antimetabolite that inhibits DNA synthesis (1). It is most commonly used in organ malignancies due to its functions in the promotion of cell death in several cancers including non-small cell lung cancer, colon squamous cell carcinoma, nasopharyngeal carcinoma and ovarian, breast

and pancreatic cancer (2-5). Gemcitabine-induced antitumor therapy resistance is a widely used model in studying chemotherapeutic resistance (6). Gemcitabine is used as a standard drug treatment for human osteosarcoma and has a significant therapeutic effect in osteosarcoma (7), however, the underlying mechanism requires further investigation.

Autophagy, a conserved pathway that involves the degradation of aggregated proteins and damaged organelles, serves an essential role in maintaining tissue homeostasis to support cell growth and survival (8,9). In addition, autophagy is considered to be a unique signaling pathway that influences a number of pathological conditions such as oncogenesis and cancer therapy resistance (10). Several essential signaling pathways, including mechanistic target of rapamycin, death-associated protein kinases, Beclin 1 and caspases are reported to be involved in the process of autophagy (11,12). Beclin 1 is considered to be related to the initiation and progression of autophagy. Previous studies have demonstrated that activation of autophagy may inhibit the ability of antitumor chemotherapy drugs to induce apoptosis (13,14). However, the underlying mechanism remains unknown.

The Wnt/ $\beta$ -catenin signaling pathway, which has an important role in cell proliferation and tumorigenesis, was previously reported to be aberrantly activated in the majority of tumors, including breast cancer, colon cancer as well as renal carcinomas (15,16). Evidence has also indicated that activation of the Wnt/ $\beta$ -catenin signaling pathway suppresses autophagy (17). As tumors often exhibit reduced levels of autophagy, and drug resistance is associated with abnormal apoptosis, but whether Wnt/ $\beta$ -catenin pathway inhibition is associated with antitumor chemotherapy drug resistance remains to be further determined.

The present study investigated the effect of Wnt- $\beta$ -catenin pathway activation and autophagy on drug resistance in the MG63 human osteosarcoma cell line. The results will provide novel insights into the mechanism by which the Wnt/ $\beta$ -catenin signaling pathway may regulate chemotherapy drug-induced cancer cell resistance.

## Materials and methods

**Cell culture.** The MG63 human osteosarcoma cell line (American Type Culture Collection, Manassas, VA, USA) was

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cultured in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin at 37°C in an atmosphere containing 5% CO<sub>2</sub>.

**Treatment.** The cultured cells were randomly divided into the following groups: Control (untreated); Beclin 1 over-expression treatment; Gemcitabine treatment; XAV939 treatment; wnt3 $\alpha$  treatment; Beclin 1 + Gemcitabine treatment; Beclin 1 + Gemcitabine + XAV939 treatment; Beclin 1 + Gemcitabine + Wnt3 $\alpha$  treatment. The pcDNA3-Beclin 1 plasmid was purchased from Addgene Inc. (#21150; Cambridge, MA, USA). XAV939, Wnt3 $\alpha$  and gemcitabine were purchased from Sigma-Aldrich (Merck-KGaA, Darmstadt, Germany).

**Overexpression of Beclin 1 in MG63 cells.** pcDNA3.1 (empty vector) and pcDNA3-Beclin 1 over-expressing Beclin 1 gene were transfected into MG63 cells using FugeneHD (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. Following incubation for 2 days, the cell transfection efficiency was determined by western blot analysis.

**Confocal microscopy.** To visualize the induction of autophagy, the MG63 cell line was transfected with the pQXI-DsRed-LC3-GFP puromycin-encoding plasmid (#31183; Addgene, Inc.). MG63 cells cultured in serum-free medium for 3 days were used as a positive control. While DsRed was constitutively expressed in all treatment groups, the induction of autophagy resulted in cleavage of the GFP domain from DsRed-LC3-GFP (18). Therefore, the percentage of autophagy-positive cells was determined by the amount of GFP-negative cells under a Zeiss laser scanning confocal fluorescence microscope.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Following incubation of cells with 10  $\mu$ g/ml Gemcitabine, 10  $\mu$ g/ml Gemcitabine + 5  $\mu$ M Beclin 1, 50  $\mu$ g/ml Gemcitabine, 50  $\mu$ g/ml Gemcitabine + 5  $\mu$ M Beclin 1, 20  $\mu$ M XAV939 or 15  $\mu$ M Wnt3 $\alpha$  for 2 days, the expression of autophagy-related (ATG)4, ATG5, ATG12, Beclin 1, B-cell lymphoma 2 (BCL-2), cellular FLICE-inhibitory protein (c-FLIP), caspase-3, caspase-7, cyclin D1 and lysosomal-associated membrane protein 1 (LAMP1) was determined by RT-qPCR. Total cellular RNA was extracted using TRIzol<sup>®</sup> reagent (Invitrogen, Thermo Fisher Scientific, Inc.) and cDNA was synthesized using AMV reverse transcriptase according to the manufacturer's protocol (Promega Corporation). qPCR was performed using SYBR-Green Real-Time PCR in the 7900HT Fast Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc.) The PCR program included initial denaturation for 10 min at 95°C, followed by 40 cycles of denaturation for 15 sec at 95°C, annealing for 1min at 55°C and extension for 40 sec at 72°C. Expression levels were measured by calculating the threshold cycle (Cq) of target genes following normalization against the Cq value of GAPDH using the 2<sup>- $\Delta\Delta$ Cq</sup> method (19). Experiments were repeated three times. The primers were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and

synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Primer sequences were as follows: GAPDH forward, 5'-CAC CATCTTCCAGGAGCGAG-3' and reverse, 5'-TCACGCCAC AGTTTCCCGGA-3'; BCL-2 forward, 5'-CCGATCAGTGGA GCTGAAGAA-3' and reverse, 5'-GCCACAGGATGTTCT CGTCA-3'; cyclin D1 forward, 5'-CAAGGCCTGAACCTG AGGAG-3' and reverse, 5'-CTTGGGGTCCATGTTCTG CT-3'; c-FLIP forward, 5'-GAGTGCCGGCTATTGGACTT-3' and reverse, 5'-GCGCTTCTCTCCTACACCTC-3'; caspase-3 forward, 5'-GCGGTTGTAGAAGTTAATAAAGGT-3' and reverse, 5'-TACCAGACCGAGATGTCAATTC-3'; caspase-7 forward, 5'-CGTGGGAACGGCAGGAAGT-3' and reverse, 5'-CGGGTGCTTTGATGGATCG-3'; ATG4 forward, 5'-TACAGCATTTTCACAGAGAAGGACG-3' and reverse, 5'-CTCCAGCAGGGAACCCATTAC-3'; ATG5 forward, 5'-TGGGCCATCAATCGGAAACTC-3' and reverse, 5'-TGC AGCCACAGGACGAAACTC-3'; ATG12 forward, 5'-TTG GAGGCATAGACAGACAC-3' and reverse, 5'-TATGTGTAT TCCGTGCCATC-3'; Beclin 1 forward, 5'-GTTGCCGTT ATACTGTTCTCGT-3' and reverse, 5'-CCTCCAGTGTCTTCA ATG-3'; LAMP1 forward, 5'-GTGTCAGCTGGACGAGAA CA-3' and reverse, 5'-GCGCTAGATGGTCTGGTAGC-3'.

**Western blotting.** Cells (1x10<sup>6</sup>) were washed twice with PBS and incubated with pyrolysed solution containing 0.1 M phenylmethylsulfonyl fluoride. Cell lysates were centrifuged at 13,000 x g for 20 min at 4°C. Proteins were quantified by Bradford assay and 10  $\mu$ g were separated by 12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Following blocking with 5% non-fat dry milk in TBS for 1 h at room temperature, membranes were incubated with rabbit anti-Beclin 1 (1:500 dilution, ab55878), anti-GAPDH antibody (1:1,000 dilution, ab9485) or rabbit anti-microtubule-associated protein 1A/1B-light chain 3 (LC3)B (1:1,000 dilution, ab81785) (all from Abcam, Cambridge, UK) primary antibodies at 4°C overnight. Following five washes with TBS with 0.5% Tween-20, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2,000 dilution, ab6717; Abcam) for 30 min at room temperature. Specific bands were observed using enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Inc.) and detected using a Bio-Rad ChemiDoc XRS image system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Flow cytometry.** To determine cell apoptosis, 1x10<sup>6</sup> MG63 cells were treated with Beclin 1, Gemcitabine, Beclin 1 + Gemcitabine, Beclin 1 + Gemcitabine + XAV939, or Beclin 1 + Gemcitabine + Wnt3 $\alpha$  for 2 days. Cells were then fixed with 2% paraformaldehyde and permeabilized by PBS containing 0.5% Triton X-100 (both from Sigma Aldrich; Merck KGaA, Darmstadt, Germany). Cells were subsequently resuspended in 1 ml Annexin V binding buffer. A fluorescein isothiocyanate-conjugated Annexin V antibody at 1:500 (5  $\mu$ l; RUO-556419; BD Biosciences, San Diego, CA, USA) was added and incubated for 20 min at room temperature. The cells were washed 3 times with PBS for 5 min, and at 20 min prior to flow cytometry analysis, 5  $\mu$ l propidium iodide was added to visualize DNA and washed 3 times with PBS for 5 min. Data was acquired by BD FACSCalibur<sup>™</sup> 3C (BD Biosciences,

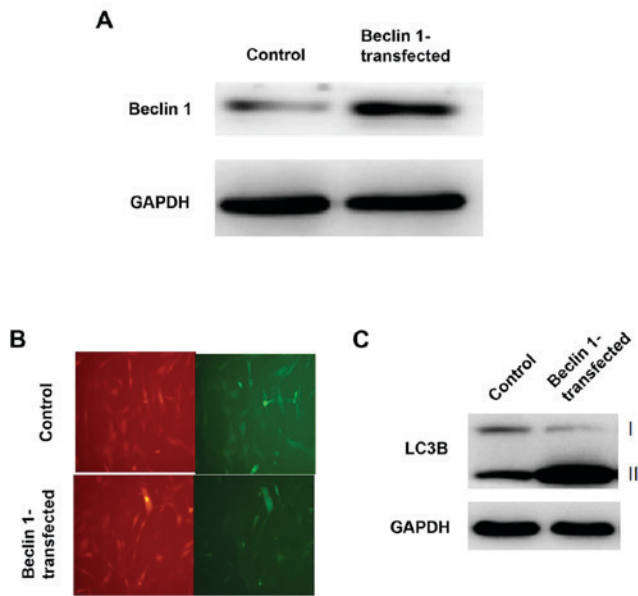


Figure 1. Confirmation of Beclin 1 overexpression and detection of autophagy. (A) Representative Western blot images of Beclin 1 protein expression in control and Beclin 1-transfected cells. GAPDH served as an internal control. (B) Confocal microscopy of autolysosome formation was performed. (C) Representative western blot images of LC3B-I and -II protein expression levels, normalized to GAPDH levels. LC3B, microtubule-associated protein 1A/1B-light chain 3 B.

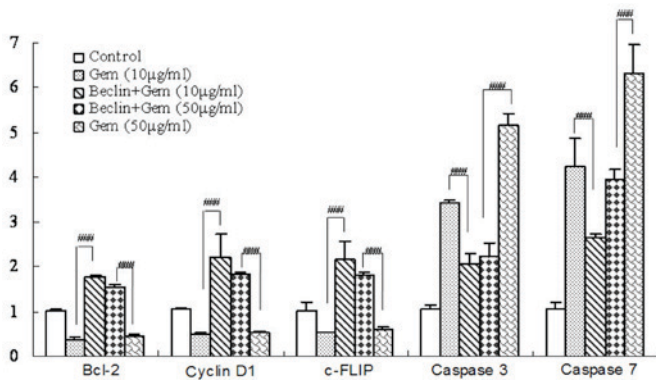


Figure 2. Beclin 1 overexpression inhibited gemcitabine-induced apoptosis. BCL-2, cyclin D1, c-FLIP, caspase-3 and caspase-7 gene expression was measured by RT-qPCR after the MG63 cell line was treated with 10  $\mu$ g/ml Gemcitabine, 10  $\mu$ g/ml Gemcitabine + 5  $\mu$ M Beclin 1, 50  $\mu$ g/ml Gemcitabine, 50  $\mu$ g/ml Gemcitabine + 5  $\mu$ M Beclin 1. Expression was normalized to GAPDH expression. Data are expressed as the mean  $\pm$  standard deviation. ###P<0.001. Gem, gemcitabine; c-FLIP, cellular FLICE-inhibitory protein.

Franklin Lakes, NJ, USA) and analyzed by FlowJo software 7.6.1 (Tree Star, Inc., Ashland, OR, USA).

**Cell viability.** An MTT assay was used to measure cell viability according to the manufacturer's protocol. Cells were plated at a density of  $5 \times 10^4$ /well into 96-well plates and treated with Beclin 1, Beclin 1 + XAV939 or Beclin 1 + wnt3 $\alpha$  for 24 h, and then stimulated with 20, 40 or 60  $\mu$ g/ml Gemcitabine for 2 days. Subsequently, 10  $\mu$ l MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to each well and cells were incubated at 37°C for 4-6 h. Formazan crystals dissolved in 150  $\mu$ l DMSO were added for 10 min with agitation. Absorbance was

measured at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** All statistical analyses were performed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean  $\pm$  standard deviation. One-way analysis of variance was performed to determine statistical significance for multiple comparisons, followed by a least-significant difference or Tamhane test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Beclin 1 gene overexpression induces autophagy in MG63 human osteosarcoma cell line.** Previous reports have demonstrated that Beclin 1 is required for initiating autophagosome formation (20). To validate the autophagic role of Beclin 1, it was over-expressed in the MG63 cell line, and DsRed-LC3-GFP reporters were used to monitor autophagy under a fluorescence microscope. Western blotting demonstrated that the Beclin 1 gene was successfully over-expressed in MG63 cells (Fig. 1A). Furthermore, Beclin 1 overexpression was associated with enhanced autophagy, which was identified by dampened green fluorescence observed in MG63 cells transfected with Beclin 1 compared with the control cells treated with empty vector (Fig. 1B). LC3B is a specific marker of the steady-state levels of autophagosomes. Therefore, western blot analysis was performed to detect protein expression levels of LC3B conversion and progression of autophagy. Consistent with the fluorescence microscope results, increased conversion of LC3B was detected following Beclin-1 overexpression compared with control cells, which indicated increased autophagy in cells over-expressing Beclin 1 (Fig. 1C).

**Beclin 1 gene overexpression reduces gemcitabine-induced apoptosis.** To confirm whether activation of autophagy may inhibit the induction of apoptosis by antitumor chemotherapy drugs, the apoptosis of cells overexpressing Beclin 1 following treatment with gemcitabine was analyzed by investigating the mRNA expression levels of pro- and anti-apoptotic genes. It was revealed that gemcitabine alone induced apoptosis, which was determined by the increased expression of apoptosis-associated genes caspase-3 (P<0.001) and 7 (P<0.001), and the reduced expression of anti-apoptotic Bcl-2 (P<0.005), cyclin D1 (P<0.005) and c-FLIP (P<0.005) (Fig. 2). As expected, Beclin 1 overexpression significantly reduced the expression of pro-apoptotic caspase-3 (P<0.001) and caspase-7 (P<0.001) induced by 50  $\mu$ g/ml gemcitabine compared with gemcitabine-treated cells without Beclin 1 stimulation. The results demonstrated that overexpression of Beclin 1 was associated with a downregulation in gemcitabine-induced apoptosis.

**Activation of the Wnt/ $\beta$ -catenin signaling pathway attenuates autophagy.** The present study also investigated the potential mechanisms of how Beclin 1 overexpression may reduce gemcitabine-induced apoptosis. It has been previously reported that suppression of the Wnt/ $\beta$ -catenin signaling pathway enhanced breast cancer stem-like cell autophagy (21). The autophagic role of the Wnt/ $\beta$ -catenin signaling pathway

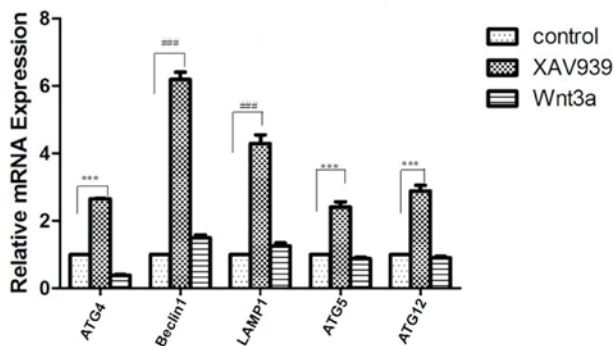


Figure 3. Activation of the Wnt/ $\beta$ -catenin signaling pathway attenuates the expression of autophagy-associated genes. The expression of autophagy-associated genes, ATG4, Beclin 1, LAMP1, ATG5 and ATG12 was analyzed by RT-qPCR after cells were treated with XAV939 or Wnt3a. Expression was normalized to GAPDH expression. Data are expressed as the mean  $\pm$  standard deviation. \*\*\* $P < 0.05$ ; ### $P < 0.001$ . ATG, autophagy-related; LAMP1, lysosomal-associated membrane protein 1; Wnt3a, activator of the Wnt/ $\beta$ -catenin signaling pathway; XAV939, inhibitor of the Wnt/ $\beta$ -catenin signaling pathway.

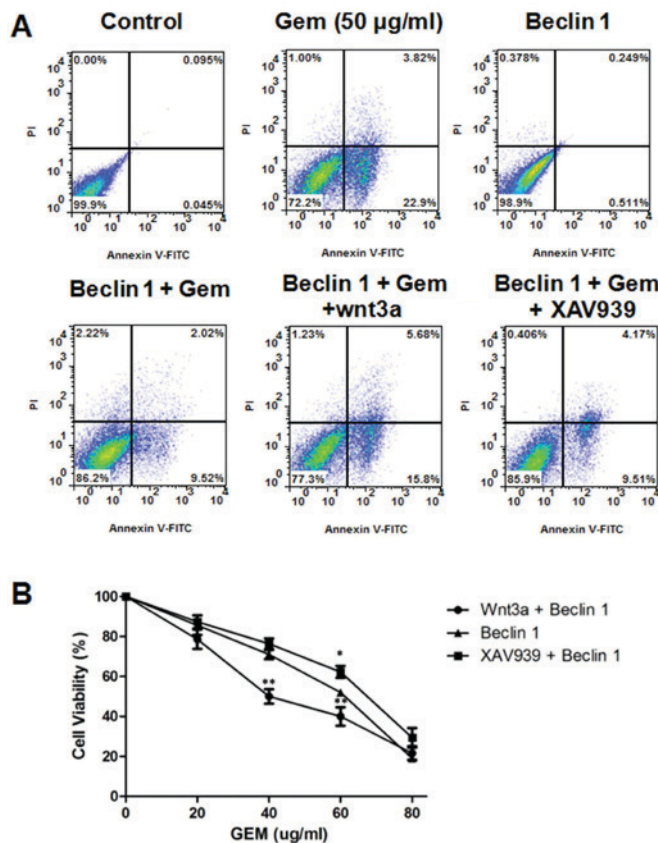


Figure 4. Activation of the Wnt/ $\beta$ -catenin signaling pathway inhibited autophagy and reduced resistance of Beclin 1 over-expressing MG63 cells to gemcitabine treatment. (A) Cell apoptosis was determined by flow cytometry using Annexin V-FITC and PI dual labeling in different treatment groups. (B) Cell viability was measured by MTT assay. \* $P < 0.05$ ; \*\* $P < 0.001$ . Gem, gemcitabine; FITC, fluorescein isothiocyanate; PI, propidium iodide; Wnt3a, activator of the Wnt/ $\beta$ -catenin signaling pathway; XAV939, inhibitor of the Wnt/ $\beta$ -catenin signaling pathway.

in the MG63 human osteosarcoma cell line was investigated using RT-qPCR. As demonstrated in Fig. 3, very little change

was observed in the relative mRNA expression of several autophagy-associated genes including ATG4, Beclin 1, ATG5, ATG12 and LAMP1 in Wnt3a-treated cells (activator of the Wnt/ $\beta$ -catenin signaling pathway) compared with the control ( $P > 0.05$ ). However, the expression of ATG4 ( $P < 0.05$ ), ATG5 ( $P < 0.05$ ), ATG12 ( $P < 0.05$ ), Beclin 1 ( $P < 0.01$ ), and LAMP1 ( $P < 0.01$ ) was increased in XAV939-treated cells (inhibitor of the Wnt/ $\beta$ -catenin signaling pathway) compared with the control. Particularly, Beclin 1 expression was significantly increased following the inhibition of the Wnt/ $\beta$ -catenin signaling pathway by XAV939, indicating that altered Beclin 1 expression may be involved in the Wnt/ $\beta$ -catenin signaling pathway-attenuated autophagy.

*Activation of the Wnt/ $\beta$ -catenin signaling pathway inhibits autophagy and reduces the resistance of Beclin 1-overexpressing MG63 cells to gemcitabine-induced apoptosis.* To further investigate the role of the Wnt/ $\beta$ -catenin signaling pathway in Beclin 1 overexpression-induced autophagy, Beclin 1-transfected MG63 human osteosarcoma cells were treated with either a Wnt/ $\beta$ -catenin signaling activator (Wnt3a) or a inhibitor (XAV939). The rate of apoptosis was measured by flow cytometry, and the percentage of Annexin V-positive cells presented in Fig. 4A. It was suggested that Beclin 1 overexpression reduced apoptosis induced by gemcitabine, and the activation of the Wnt/ $\beta$ -catenin signaling pathway by Wnt3a reversed this reduction. Furthermore, Beclin 1 significantly reduced cell viability induced by gemcitabine following the activation of the Wnt/ $\beta$ -catenin signaling pathway, whereas Beclin 1 promoted cell viability induced by gemcitabine after the inhibition of the Wnt/ $\beta$ -catenin signaling pathway ( $P < 0.01$ ; Fig. 4B). The results demonstrated that the Wnt/ $\beta$ -catenin signaling pathway may inhibit autophagy and increase gemcitabine-induced apoptosis in the MG63 cell line by downregulating the expression of Beclin 1.

## Discussion

In addition to the roles in development, oncogenesis, cardiovascular, metabolic and neurodegenerative diseases, autophagy is an important tumor resistance mechanism that eliminates damaged proteins and organelles, and protects cells from apoptosis (14). However, the mechanisms by which autophagy affects drug-induced resistance in tumor cells requires further investigation.

In the present study, Beclin 1 overexpression enhanced autophagy and reduced gemcitabine-induced apoptosis in the MG63 human osteosarcoma cell line, and the activation of the Wnt/ $\beta$ -catenin pathway attenuated autophagy by down-regulating Beclin 1 gene expression. The results of the current study also demonstrated that Beclin 1 overexpressing MG63 cells were more resistant to gemcitabine-induced apoptosis. However, Wnt/ $\beta$ -catenin activation reversed the inhibition. The results revealed the potential novel mechanisms of how autophagy may regulate drug-induced resistance in antitumor chemotherapy. A number of studies have demonstrated that Beclin 1 expression regulates autophagy, and that Beclin 1 upregulation was associated with the increased autophagosome numbers (22,23). In addition, knockdown of Beclin 1 in tumor cell lines or in mice was observed to inhibit

autophagy (24,25). Downregulated Beclin 1 expression has been previously demonstrated in a number of tumor types, including human breast carcinoma, brain tumors and prostatic carcinoma (26,27). In the present study, to confirm the autophagic role of Beclin 1, a Beclin 1 gene plasmid was transfected into the MG63 human osteosarcoma cell line and the process of autophagy was monitored. As expected, enhanced expression of LC3B and activity of autophagy following overexpression of Beclin 1 was observed, indicating that Beclin 1 functions promoted the autophagy process in MG63 tumor cell lines.

The purpose of chemotherapy in tumors is to trigger the apoptosis of tumor cells. Osteosarcoma, a cancerous tumor of the bone, is an aggressive malignant neoplasm (28). Surgery plus chemotherapy is the primary clinical treatment choice for osteosarcoma (29). Gemcitabine, an antitumor chemotherapy drug that functions by inhibiting DNA synthesis, has demonstrated significant clinical benefits in osteosarcoma (30). A previous study revealed that enhanced autophagy was associated with the resistance to gemcitabine-induced tumor cell apoptosis (31). In the current study, consistent with previous conclusions, the increased expression of apoptosis-associated genes caspase-3 and -7, and downregulated expression of anti-apoptotic genes BCL-2, cyclin D1 and c-FLIP by gemcitabine treatment was observed in MG63 cell lines. However, Beclin 1 overexpression reversed gemcitabine-induced resistance by upregulating anti-apoptotic genes. The results demonstrated that Beclin 1-induced autophagy may affect chemotherapy-induced apoptosis in tumor cells.

Aberrant activation of the Wnt/ $\beta$ -catenin signaling pathway is frequently observed in cancer cases. It was previously revealed that the Wnt/ $\beta$ -catenin pathway also serves as a negative regulator of autophagy (12,32,33). To further examine the role of the Wnt/ $\beta$ -catenin signaling pathway in chemotherapy resistance in tumor cells, the MG63 cell line was treated with either a Wnt/ $\beta$ -catenin activator (Wnt3a) or an inhibitor (XAV939), and the expression of autophagy-associated genes and the apoptosis of gemcitabine-treated tumor cells were investigated. Significantly enhanced autophagy-associated-gene expression was observed following the inhibition of Wnt/ $\beta$ -catenin pathway. Furthermore, the results demonstrated that Wnt/ $\beta$ -catenin signaling pathway activation increased the percentage of gemcitabine-induced apoptotic cells in the MG63 human osteosarcoma cell line compared with cells only transfected with Beclin 1. Beclin 1 mRNA expression levels were greatly inhibited following Wnt/ $\beta$ -catenin activator treatment, compared with inhibitor XAV939 treatment. The results demonstrated that Wnt/ $\beta$ -catenin signaling reversed gemcitabine resistance by attenuating Beclin 1-mediated autophagy.

In conclusion, the results of the current study provided a novel insight into resistance to antitumor chemotherapy drugs. Aberrant Wnt/ $\beta$ -catenin signaling may enhance Beclin 1-mediated autophagy, thus preventing chemotherapy drug-induced cell apoptosis. These results may provide evidence for enhancing the sensitivity of tumors to apoptosis induced by chemotherapy drugs, and by modulating aberrant Wnt/ $\beta$ -catenin signaling-associated autophagy and autophagy proteins.

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