

## Original Article

# Wnt/ $\beta$ -catenin deficiency inhibits multiple myeloma cell growth via AMPK/mTOR signaling

Nan Su, Pingping Wang, Ying Liang, Yan Li

Department of Hematology, First Affiliated Hospital of China Medical University, Shenyang 110001, China

Received August 24, 2017; Accepted March 22, 2018; Epub June 15, 2018; Published June 30, 2018

**Abstract:** Multiple myeloma (MM) is an incurable disease with a median survival of approximately 5 years.  $\beta$ -catenin plays a central role during tumorigenesis of MM. AMP-activated protein kinase (AMPK) controls cell growth, autophagy, and proliferation by regulating the activity of mammalian target of rapamycin (mTOR), which is regularly downregulated in malignant cells. However, whether AMPK/mTOR expression is directly associated with  $\beta$ -catenin silencing-triggered apoptotic and autophagic death in MM cells has not been adequately explored. The aim of the present study was to explore the contribution of AMPK/mTOR in Wnt/ $\beta$ -catenin silencing-triggered autophagy of MM cells. To confirm the specific effect of  $\beta$ -catenin on AMPK/mTOR signaling, stable  $\beta$ -catenin-silenced MM cell lines were cultured with or without autophagy inhibitor (3-methyladenine, 3-MA; p-AMPK inhibitor compound C, AI). Our data showed that p-AMPK expression did not significantly change after 3-MA treatment of  $\beta$ -catenin-silenced RPMI 8226 MM cells. Moreover, AI-treated  $\beta$ -catenin-silenced RPMI 8226 MM cells showed no significant change in  $\beta$ -catenin expression; decreased p-AMPK, p-AMPK/AMPK, and LC3II expression as well as cell viability; and increased active caspase-3 expression as well as apoptosis. These findings suggested that Wnt/ $\beta$ -catenin silencing triggered autophagy in MM cells by activating AMPK, thus inhibiting mTOR.

**Keywords:** Multiple myeloma, apoptosis, hematological malignancy

## Introduction

Multiple myeloma (MM) is an incurable disease and the second most frequent hematological neoplastic disease, with a median survival of approximately 5 years. MM is characterized by the abnormal proliferation of bone marrow plasma cells, comprising about 1% of all cancers, > 10% of hematological cancers, and 10% of deaths caused by blood cancers. Uncontrolled growth of low-proliferation plasma cells can lead to bone marrow failure with anemia, thrombocytopenia, and leukopenia. Moreover, MM is also related to renal dysfunction [1-3]. Applications of novel therapeutics, including autologous stem cell transplantation and drugs such as bortezomib, lenalidomide, and thalidomide, have dramatically improved the median overall survival time in patients with MM [2]. However, despite the aggressive treatment of MM, it remains a clinical challenge due to the complex pathological process of MM. Therefore, to improve MM therapy, it is necessary to explore the molecular mechanism of this disease.

$\beta$ -catenin, an intracellular signal transducer of the Wnt/ $\beta$ -catenin pathway, plays a central role during embryonic development and tumorigenesis, including in MM, and has been shown to be associated with programmed cell death [4-6], which is an intracellular program-mediated cell death and innate blockade that controls cancer cells from spreading and surviving. Apoptosis was initially considered to be the only type of programmed cell death; however, autophagy is now regarded as an additional type. Nevertheless, malignant cells develop a variety of mechanisms to escape this process such as producing epigenetic variations or genetic alterations in the critical regulators of programmed cell death [7]. *In vivo*, programmed cell death participates in the compound communication between autophagy and apoptosis. Sometimes, a specific stimulus only activates one category; however, in other conditions, the same stimulus might trigger several cell death processes. Diverse mechanisms might co-exist and operate together with each other in a cell, although one mechanism will dominate the other one, eventually [8, 9].

It has been reported that inhibiting the Wnt/ $\beta$ -catenin pathway can induce apoptosis [10, 11], while autophagy can negatively modulate Wnt/ $\beta$ -catenin signaling via stimulating the disheveled degradation due to metabolic stress [12, 13]. Besides, in squamous cell carcinoma,  $\beta$ -catenin knockdown has been shown to trigger autophagic and apoptotic cell death [14].

The AMP-activated protein kinase (AMPK) is comprised of an  $\alpha$  subunit with catalytic functions as well as  $\beta$  and  $\gamma$  subunits with regulatory functions. AMPK is allosterically triggered by AMP with cellular stress or energy starvation, via binding to the AMPK $\gamma$  subunit with regulatory function, consequently encouraging pathways causing ATP creation, such as oxidation of fatty acids and blockade of anabolic processes, which consume ATP. Generally, activation of AMPK causes energy conservation for cell growth and proliferation [15]. Additionally, AMPK is the main kinase responsible for tumor suppression [16, 17]. Augmented phosphorylation of AMPK, cell cycle arrest, as well as growth inhibition were observed in MM cell lines treated with the membrane-permeable amino imidazole carboxamide ribonucleotide, and the p-AMPK inhibitor compound C (AI) could revert these effects [18]. AMPK plays an important role in controlling cell growth, autophagy, and proliferation by regulating the activity of mammalian target of rapamycin (mTOR), which is regularly downregulated in malignant cells [19].

However, whether AMPK/mTOR expression is directly associated with  $\beta$ -catenin silencing-triggered apoptotic and autophagic death in MM cells has not been adequately explored. In this study, for the first time, we provide convincing evidence of the contribution of AMPK/mTOR in Wnt/ $\beta$ -catenin silencing-triggered autophagy in MM cells.

### Materials and methods

#### *Compounds and reagents*

Sheep anti-rabbit fluorescent secondary antibody REP, AI, polyvinylidene difluoride (PVDF) membranes, and enhanced chemiluminescence (ECL) solution were obtained from Merck Millipore (Darmstadt, Germany); goat anti-rabbit secondary antibody was bought from ZS-GB-BIO (Beijing, China); antibodies against p-AMPK $\alpha$  and p-mTOR were bought from Cell Signaling Technology Inc. (Danvers, MA, USA);

antibody against  $\beta$ -actin was obtained from Beijing Bioss Company (Beijing, China); 3-methyladenine (3-MA) was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA); fetal bovine serum (FBS) was bought from GE Healthcare Life Sciences Hyclone Laboratories, Inc. (Logan, UT, USA); protein marker and RPMI-1640 cell culture medium were from Thermo Fisher Scientific Inc. (Waltham, MA, USA); protein loading buffer, bovine serum albumin (BSA), and DAPI were from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China); dimethyl sulfoxide, bicinchoninic acid (BCA) Protein Quantification Kit, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) preparation reagents, 4% paraformaldehyde, and RIPA lysate were from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China); Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies, Inc. (Shanghai, China); and antibody against AMPK $\alpha$  was bought from Epitomics (Abcam) (Cambridge, USA).

#### *Cell lines and in vitro culture*

Fudan University Institutes of Biomedical Sciences Cell Bank (Shanghai, China) provided the RPMI-8826 MM cell line, which was cultured in RPMI-1640 medium supplemented with 1% penicillin/streptomycin and 10% (v/v) FBS at 37°C and 5% CO<sub>2</sub> in a humidified incubator.

The stable  $\beta$ -catenin-silenced MM cell lines of siA (RPMI 8226-LV- $\beta$ -catenin-siRNA-A cells obtained by siRNA: CCGG GCT TGG AAT GAG ACT GCT GAT CTC GAG ATC AGC AGT CTC ATT CCAAGC TTT TT) and siC (RPMI 8226-LV- $\beta$ -catenin-siRNA-C obtained by siC: CCG GAG GTG CTA TCT GTC TGC TCT ACT CGA GTAGAGCAG ACA GAT AGC ACC TTT TTT) as well as the control cell line C (RPMI 8226-LV- $\beta$ -catenin-C obtained by siC: CCG GTT CTC CGA ACG TGT CAC GTT TCA AGA GAA CGT GAC ACG TTC GGA GAA TTT TTG) were previously established in our laboratory. In brief, the different siRNAs indicated above targeting the human CTNNB1 gene (GenBank accession no. NM\_001,904, encoding the  $\beta$ -catenin protein) or scrambled siRNA were ligated with the vector of hU6-MCS-Ubiquitin-EGFP-IRES-puromycin (Genechem, Shanghai, China). Recombinant constructs were transfected to package the lentivirus. Then the plasmids were packaged into 293T cells (Genechem) with Lipofectamine 2000

(Genechem). After transfection for 48 h, the viruses were harvested for infection of the RPMI-8826 cells. After being infected with lentiviruses expressing scrambled or  $\beta$ -catenin siRNAs for 72 h, RPMI-8826 cells were treated with puromycin at a final concentration of 1  $\mu$ g/mL for 2 weeks to establish stable cells expressing  $\beta$ -catenin siRNA. Cells were then maintained in RPMI-1640 medium, as described for the parental cell line.

### *Cell viability assay*

Cell viability was measured using the CCK-8 reagent. The logarithmic phase cells ( $5 \times 10^4$  cells/well/100  $\mu$ L) were separately seeded in 96-well plates in triplicate and incubated overnight for attachment. Next, the cells were incubated with AI at a final concentration of 6  $\mu$ M for 24 h, and then 10  $\mu$ L of CCK-8 reagent/well for 2 h. A microplate reader (BioTek Instruments, Inc. Winooski, USA) was used to detect the absorbance at a wavelength of 450 nm.

### *Cell apoptosis analysis*

The logarithmic phase cells ( $10^6$  cells/100  $\mu$ L) were separately added into a test tube for flow cytometric analysis in triplicate. Cells were collected after treatment with AI (6  $\mu$ M) for 24 h, washed twice with PBS by centrifugation at 1000 rpm for 3 min each time, and then resuspended in  $1 \times$  Binding Buffer at a concentration of  $10^6$  cells/mL. Annexin V-FITC (5  $\mu$ L) and propidium iodide (PI; 5  $\mu$ L) were mixed with 100  $\mu$ L of the cell suspension prepared above and cultured at room temperature in the dark for 15 min. After adding 400  $\mu$ L of  $1 \times$  Binding Buffer, cell apoptosis was analyzed by flow cytometry (BD Biosciences). The left lower quadrant (Q3) represents live cells (Annexin V<sup>-</sup>, PI<sup>-</sup>), the upper left quadrant (Q1) represents dead cells (Annexin V<sup>+</sup>, PI<sup>+</sup>), the upper right quadrant (Q2) represents late apoptotic cells (Annexin V<sup>+</sup>, PI<sup>+</sup>), and the right lower quadrant (Q4) represents early apoptotic cells (Annexin V<sup>+</sup>, PI<sup>-</sup>).

### *Cell immunofluorescence assay*

Coverslips soaked in absolute ethanol were placed in a six-well plate, and siC cells ( $10^6$  cells in 200  $\mu$ L) with or without AI treatment were added dropwise onto the coverslip. The cells were fixed using 4% paraformaldehyde for 15 min and then washed three times with PBS. Next, the cells were allowed to permeate

the membrane for 20 min with 0.4% TritonX-100, washed three times using PBS, blocked at room temperature for 1 h using 1% BSA, cultured with rabbit anti-human LC3 antibody (diluted with 1% BSA at a ratio of 1:200) in a box supplied with water at 4°C overnight, and washed three times with PBS. Then the cells were incubated with rhodamine-labeled goat anti-rabbit secondary antibody for 50 min, washed three times with PBS, further stained with DAPI for 2 min, and washed three times with PBS. The coverslips were then sealed with anti-fluorescence quenching mounting solution and observed under a fluorescence microscope.

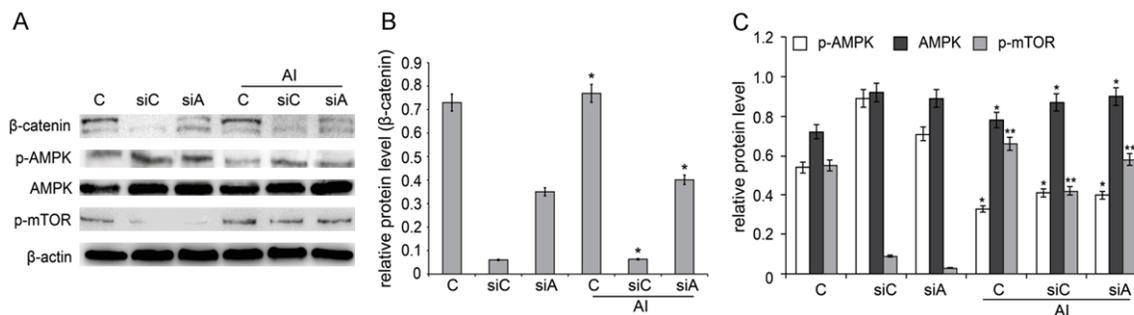
### *Western blot assay*

Logarithmic phase cells, with or without AI treatment, were harvested and washed twice with PBS, lysed for 30 min on ice with 150-250  $\mu$ L of RIPA lysate containing 1 mM phenylmethane sulfonyl fluoride. The supernatant was collected after centrifugation at 12,000 rpm and 4°C for 20 min.

The BCA protein assay kit was used to detect the protein concentrations, according to the manufacturer's protocol. In brief, the standard preparation was diluted by multiple proportion, and the test samples were diluted 10 times. Then, the optical density (OD) was detected at a wavelength of 462 nm using a microplate reader (BioTek Instruments, Inc.; Winooski, VT, USA). A standard curve was made using the OD values of the multiple proportion-diluted standard preparation. Protein concentrations were calculated according to the standard curve.

A total of 30  $\mu$ g of protein lysate for each sample was separated using 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were incubated with primary antibodies (1:1000) at 4°C overnight and reblotted with the corresponding horseradish peroxidase-labeled secondary antibody (1:8000) to the primary antibody for 1 h at room temperature. The membrane was then developed with ECL reagents and recorded using the chemiluminescence gel imaging system (MicroChemi, Beijing, China). For quantitative analysis, the gray value of each target band was determined by ImageJ 1.44 software (National Institutes of Health, Bethesda, MD, USA), and the relative content of the target protein was determined by normalization to  $\beta$ -actin.

## β-catenin/AMPK/mTOR in multiple myeloma



**Figure 1.** β-catenin silencing upregulates the expression of AMPK/mTOR in RPMI 8226 multiple myeloma cells. A. β-catenin-silenced cells, including siA and siC, and control (C) cells were cultured for 24 h in the presence or absence of the p-AMPK inhibitor compound C (AI) at a final concentration of 6 μM. Western blotting demonstrated that the protein expression level of p-AMPK was upregulated and mTOR was downregulated after β-catenin silencing. β-catenin expression could not be regulated by AMPK, indicating that β-catenin was upstream of AMPK signaling. B and C. Quantification of each test band of the western blot was performed by assessing the relative expression levels of β-catenin, p-AMPK, AMPK, and mTOR (normalized to the expression of β-actin) using Image J software. Representative data are shown from three independent experiments with similar results. In the C, siC, and siA cells treated with AI at a final concentration of 6 μM for 24 h, p-AMPK expression was decreased by 0.40 ± 0.05-fold, 0.54 ± 0.03-fold, and 0.42 ± 0.06-fold, respectively; p-AMPK/AMPK was decreased by 0.44 ± 0.02-fold, 0.51 ± 0.04-fold, and 0.45 ± 0.05-fold, respectively; and p-mTOR expression was increased by 0.20 ± 0.04-fold, 3.67 ± 0.86-fold, and 18.33 ± 1.30-fold, respectively ( $P < 0.05$ ), compared with the corresponding cells without AI treatment. One-way analysis of variance (ANOVA) was used. \* $P < 0.05$ , \*\* $P < 0.01$ , versus the corresponding cells without AI treatment.

### Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Measurement data were expressed as the mean ± standard deviation. A *t* test was used to compare the difference between the paired data, and differences between groups for the multiple group data were compared by one-way analysis of variance (ANOVA). The differences with statistical significance were \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

### Results

#### β-catenin silencing upregulates the expression of AMPK and downregulates mTOR in RPMI 8226 MM cells

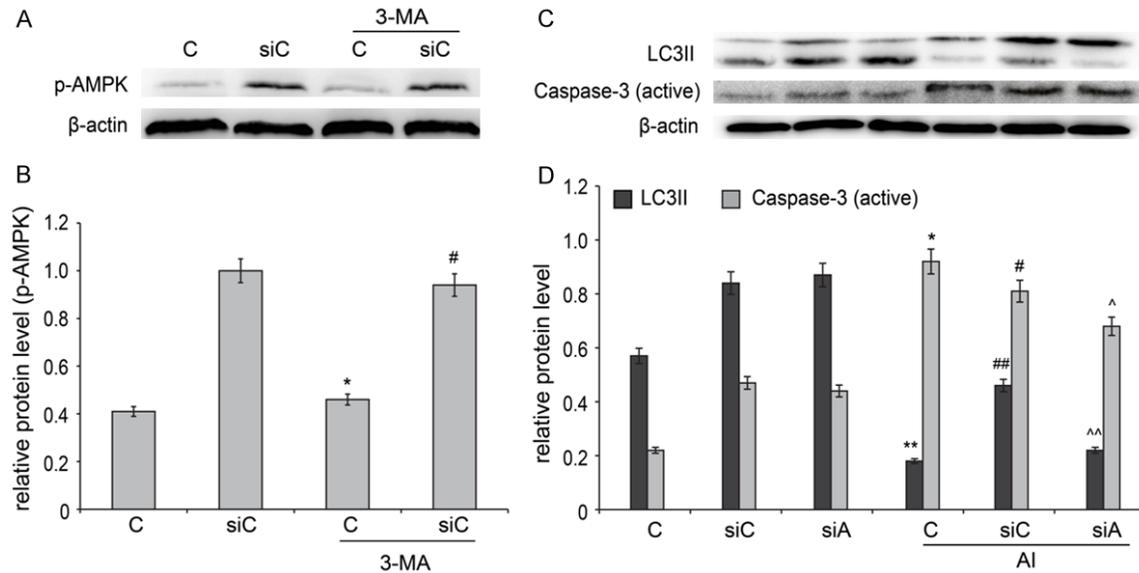
The protein expression level of p-AMPK was upregulated after β-catenin silencing in RPMI 8226 MM cells, while p-mTOR was downregulated by β-catenin knockdown. To confirm that p-AMPK and p-mTOR were specifically regulated by β-catenin, β-catenin-silenced siA and siC cells as well as C control cells were cultured with or without AI, and the protein expression levels were identified by western blot. The results showed that no significant change in the expression of β-catenin appeared after AI treatment of the cells for 24 h at a final concentration of 6 μM (Figure 1A and 1B) ( $P > 0.05$ ).

Therefore, these data suggested that AMPK signaling could be regulated by β-catenin; however, β-catenin could not be regulated by AMPK, indicating that β-catenin was upstream of AMPK signaling and could specifically regulate AMPK signaling. In the C, siC, and siA cells treated with AI at a final concentration of 6 μM for 24 h, p-AMPK expression was decreased by 0.40 ± 0.05-fold, 0.54 ± 0.03-fold, and 0.42 ± 0.06-fold, respectively; p-AMPK/AMPK was decreased by 0.44 ± 0.02-fold, 0.51 ± 0.04-fold, and 0.45 ± 0.05-fold, respectively; and p-mTOR expression was increased by 0.20 ± 0.04-fold, 3.67 ± 0.86-fold, and 18.33 ± 1.30-fold, respectively ( $P < 0.05$ ), compared with the corresponding cells without AI treatment (Figure 1A and 1B). These results further confirmed that silencing of β-catenin activated p-AMPK and inhibited mTOR. β-catenin silencing triggered autophagy in MM cells by activating AMPK, thus inhibiting mTOR.

#### β-catenin silencing upregulates expression of autophagy- and apoptosis-related proteins

Our data above confirmed that β-catenin silencing could directly upregulate p-AMPK and downregulate p-mTOR. Since AMPK/m-TOR signaling is closely related with cell autophagy and apoptosis, in our current study, we further investi-

## β-catenin/AMPK/mTOR in multiple myeloma



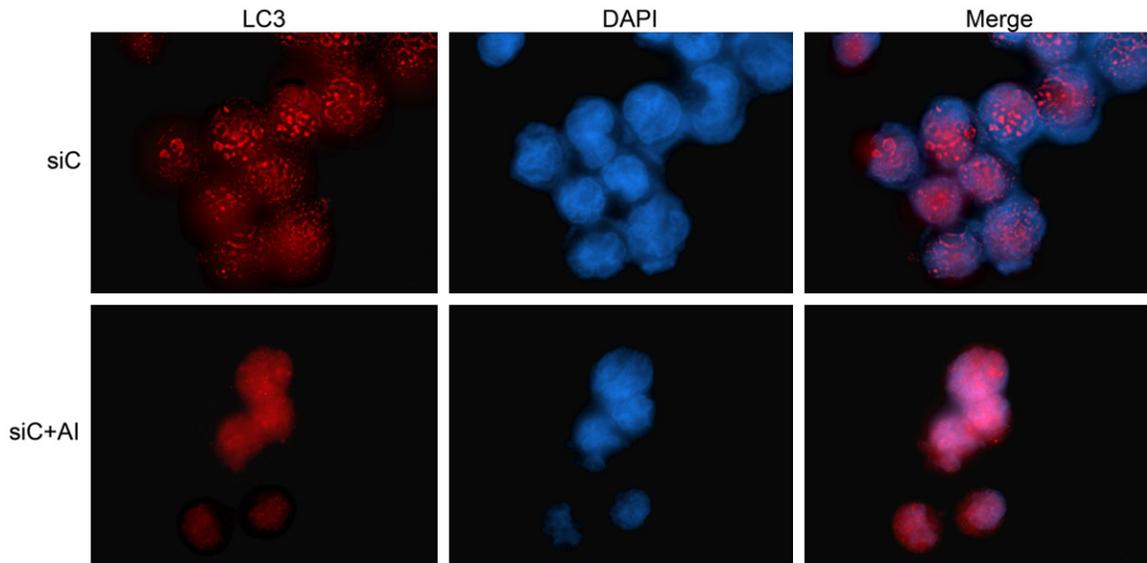
**Figure 2.** β-catenin silencing upregulates the expression of key factors in the apoptosis and autophagy of RPMI 8226 multiple myeloma cells. **A.** β-catenin-silenced siC cells and control (C) cells were cultured in the presence or absence of the autophagy inhibitor 3-methyladenine (3-MA) for 8 h at a final concentration of 5 mM, and the protein expression of p-AMPK was detected using western blot analysis, with β-actin as a loading control. **B.** Quantification of each test band of the western blot was performed by assessing the relative expression levels of p-AMPK (normalized to the expression of β-actin) using Image J software. p-AMPK expression did not significantly change after 3-MA treatment of the cells for 8 h at a final concentration of 5 mM. **C.** β-catenin-silenced siA and siC cells as well as control (C) cells were cultured for 24 h in the presence or absence of the p-AMPK inhibitor compound C (AI) at a final concentration of 6 μM, and the protein expression levels of caspase-3 (a key factor of cell apoptosis) and LC3II (a key factor of autophagy) were detected using western blot analysis, with β-actin as a loading control. **D.** Quantification of each test band of the western blot was performed by assessing the relative expression levels of caspase-3 and LC3II (normalized to the expression of β-actin) using Image J software. Representative data are shown from three independent experiments with similar results. One-way analysis of variance (ANOVA) was used. In **Figure 2B**, compared with C, \* $P > 0.05$ ; compared with siC, # $P > 0.05$ . In **Figure 2D**, compared with C, \*\* $P < 0.01$ ; compared with siC, # $P < 0.05$ , ## $P < 0.01$ ; compared with siA, ^ $P < 0.05$ , ^^ $P < 0.01$ . The expression of caspase-3 was increased by  $3.18 \pm 0.05$ -fold,  $0.72 \pm 1.25$ -fold, and  $0.55 \pm 1.09$ -fold, respectively; while the expression of LC3II was downregulated by  $0.69 \pm 0.08$ -fold,  $0.45 \pm 0.14$ -fold, and  $0.75 \pm 0.05$ -fold in C, siC, and siA cells treated with AI at a final concentration of 6 μM for 24 h, compared with the corresponding cells without AI treatment.

gated whether the autophagic effect induced by β-catenin silencing was related to activation of AMPK/mTOR. β-catenin-silenced siC cells and C control cells were cultured with or without the autophagy inhibitor 3-MA at a final concentration of 5 mM for 8 h, and the protein expression of p-AMPK was measured by western blot. The results showed that p-AMPK expression did not significantly change after 3-MA treatment of the cells for 8 h at a final concentration of 5 mM (**Figure 2A** and **2B**) ( $P > 0.05$ ).

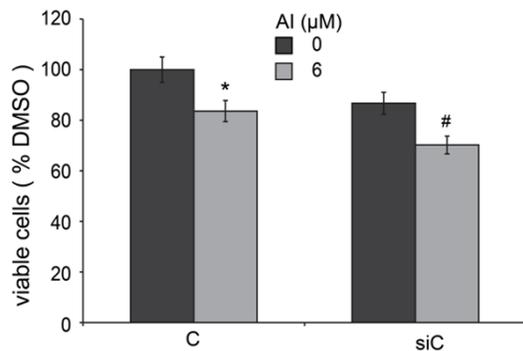
Moreover, β-catenin-silenced siA and siC cells as well as C control cells were cultured with or without AI, and the protein expression levels of the key factors of autophagy and apoptosis were detected using western blot. The results indicated that in C, siC, and siA cells treated

with AI at a final concentration of 6 μM for 24 h, the expression of caspase-3, which is a key factor of cell apoptosis, was increased by  $3.18 \pm 0.05$ -fold,  $0.72 \pm 1.25$ -fold, and  $0.55 \pm 1.09$ -fold, respectively; while the expression of LC3II, a key factor of autophagy, was downregulated by  $0.69 \pm 0.08$ -fold,  $0.45 \pm 0.14$ -fold, and  $0.75 \pm 0.05$ -fold, respectively, compared with the corresponding cells without AI treatment (**Figure 2C** and **2D**).

The change of LC3II expression was further confirmed by double immunofluorescence staining of siC cells in the presence or absence of AI, with antibodies against LC3 and DAPI. The results showed that the fluorescence in siC cells without AI treatment was spotty or massively distributed (**Figure 3**, upper panel), indicating the formation of LC3II and the activation



**Figure 3.** Immunofluorescence assay for LC3 expression. LC3 expression in the siC cells before and after treatment with the p-AMPK inhibitor compound C (AI) at a final concentration of 6  $\mu$ M for 24 h was detected by LC3-rhodamine antibody immunofluorescence staining ( $\times$  400). The fluorescence in siC cells without AI treatment (the control) was spotty or massively distributed (upper panel), while the fluorescence was scattered with less spot-like or massive distribution after siC cells were treated with AI (lower panel).



**Figure 4.** Cell viability as detected by the cell counting kit-8 (CCK-8) assay. Cell viability of control (C) and siC cells before and after treatment with the p-AMPK inhibitor compound C (AI) at a final concentration of 6  $\mu$ M for 24 h was detected by the CCK-8 assay. N = 3; one-way analysis of variance (ANOVA) was used. Compared with the C cells, the viability of the siC cells was significantly decreased. Compared with the siC cells without AI treatment, the viability of the siC cells with AI treatment was significantly decreased. \* $P < 0.05$ , compared with C; # $P < 0.05$ , compared with siC.

of autophagy, as evidenced by increased red fluorescence due to plaque formation. Meanwhile, the fluorescence was scattered with less spot-like or massive distribution after siC cells were treated with AI, indicating that LC3II formation and autophagy activity were inhibited

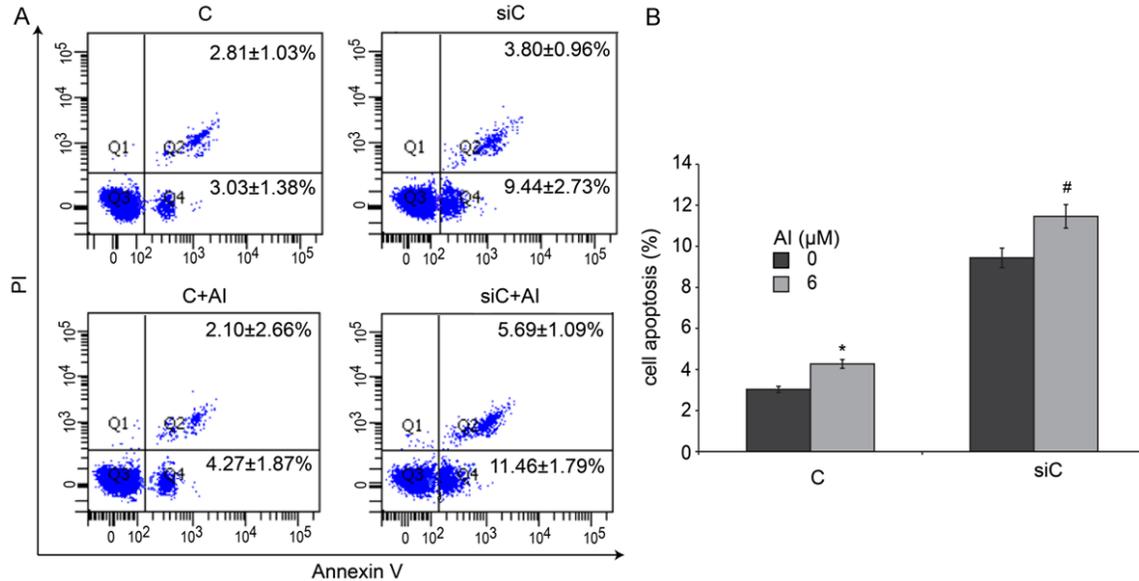
(Figure 3, lower panel), as evidenced by diffuse red fluorescence. Thus, our data suggested that  $\beta$ -catenin silencing could upregulate LC3II formation and caspase-3 via the AMPK/m-TOR pathway.

#### *$\beta$ -catenin silencing decreases the viability of RPMI 8226 MM cells via AMPK/m-TOR signaling*

To investigate whether the antiproliferative effect of  $\beta$ -catenin silencing on RPMI 8226 MM cells is associated with AMPK signaling, the logarithmic phase C and siC cells were cultured in the presence or absence of AI at a final concentration of 6  $\mu$ M for 24 h, and the relative numbers of viable cells were analyzed using a CCK-8 kit. As shown in Figure 4, compared with the C cells, the viability of the siC cells was significantly decreased ( $P < 0.05$ ) (Figure 4), indicating that  $\beta$ -catenin silencing decreased the viability of MM cells via AMPK/m-TOR signaling *in vitro*.

#### *$\beta$ -catenin silencing increases the apoptosis of RPMI 8226 MM cells via AMPK/m-TOR signaling*

To investigate whether the apoptosis induced by  $\beta$ -catenin silencing in RPMI 8226 MM cells is



**Figure 5.** Apoptosis as detected by flow cytometry. Apoptosis of control (C) and siC cells before and after treatment with the p-AMPK inhibitor compound C (AI) at a final concentration of 6  $\mu$ M for 24 h was detected by flow cytometry. The apoptosis rate of RPMI 8226 cells was increased from 3.03 (C) to 9.44 (siC) after  $\beta$ -catenin silencing; the apoptosis rate of RPMI 8226 cells with  $\beta$ -catenin silencing and AI treatment was increased from 9.44% (siC) to 11.46% (siC+AI). N = 3; one-way analysis of variance (ANOVA) was used. \* $P < 0.05$ , compared with C; # $P < 0.01$ , compared with siC. A. Representative figures of flow cytometric analysis. B. Quantitative analysis of flow cytometric analyses with statistical tags.

associated with AMPK signaling, we used the stable  $\beta$ -catenin-deficient SiC MM cells and C control cells as the target cells. The logarithmic phase C and siC cells were cultured in the presence or absence of AI at a final concentration of 6  $\mu$ M for 24 h, and cell apoptosis was analyzed using flow cytometry. As shown in **Figure 5**, the apoptosis rate of RPMI 8226 cells increased from 3.03 to 9.44 after  $\beta$ -catenin silencing and the apoptosis rate increased from 9.44% to 11.46% ( $P < 0.01$ ), indicating that the  $\beta$ -catenin silencing-induced apoptosis in RPMI 8226 MM cells is associated with AMPK signaling (**Figure 5**).

### Discussion

Although recent advances in various therapeutic approaches have been used to treat MM, e.g., autologous stem cell transplantation and drugs such as bortezomib, lenalidomide, and thalidomide, it is still an incurable hematological malignancy with the highest mortality rate. Moreover, the pathological process of MM is complex, and the molecular mechanism of MM has not yet been fully elucidated [20, 21]. Therefore, there is an urgent need for a deeper

understanding of the molecular mechanism in order to improve MM therapy.

The present study aimed to investigate the involvement of AMPK/mTOR in  $\beta$ -catenin silencing-induced apoptosis and autophagy in RPMI 8226 MM cells. In brief, the expression of p-AMPK was detected using western blot analysis in  $\beta$ -catenin-silenced RPMI 8226 MM cells with or without 3-MA treatment. In addition, the expression levels of  $\beta$ -catenin, p-AMPK, and the key factors of autophagy (LC3) and apoptosis (active caspase-3) were detected using western blot analysis. The expression of LC3 was further observed by fluorescence microscopy, and cell viability was detected by the CCK-8 method. Finally, the apoptosis rate was detected by flow cytometry in  $\beta$ -catenin-silenced RPMI 8226 MM cells with or without AI treatment.

In the present study, parallel activation of autophagy and apoptosis by  $\beta$ -catenin silencing in MM cells, both of which are related to cell death, was observed [22]. Our results showed that p-AMPK expression did not significantly change after 3-MA treatment of the  $\beta$ -catenin-silenced RPMI 8226 MM cells. Meanwhile,

AI-treated β-catenin-silenced RPMI 8226 MM cells showed no significant change in β-catenin expression, but demonstrated decreased p-AMPK, p-AMPK/AMPK, and LC3II expression as well as cell viability, and increased active caspase-3 expression and apoptosis. Our findings suggested that silencing of β-catenin stimulated both apoptosis and autophagy in MM cells, potentially via the AMPK/mTOR signaling pathway.

In conclusion, our current study confirmed that β-catenin silencing-triggered MM cell autophagy and apoptosis is closely related with activation of the AMPK/mTOR signaling pathway. Thus, simultaneously targeting both the β-catenin and AMPK/mTOR signaling pathways could be a potential strategy to develop therapeutics for the treatment of MM.

However, there were some limitations in our study. For example, only one cell line was used in our current study and no animal model was used to confirm our discovery. Therefore, further studies using MM cell-prepared xenograft animal models and patient-derived tumor tissues are essential to clarify the specific cross-talk of β-catenin-knockdown and AMPK/mTOR signaling associated with activation of autophagy and apoptosis for MM prevention *in vivo*.

#### Disclosure of conflict of interest

None.

**Address correspondence to:** Yan Li, Department of Hematology, First Affiliated Hospital of China Medical University, Shenyang 110001, China. Tel: 86-24-83282501; E-mail: beeperchild@163.com

#### References

- [1] Palumbo A and Anderson K. Multiple myeloma. *N Engl J Med* 2011; 364: 1046-1060.
- [2] Hideshima T, Mitsiades C, Tonon G, Richardson PG and Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer* 2007; 7: 585-598.
- [3] Shvartsur A, Givechian KB, Garban H and Bonavida B. Overexpression of RKIP and its cross-talk with several regulatory gene products in multiple myeloma. *J Exp Clin Cancer Res* 2017; 36: 62.
- [4] Morin PJ. β-catenin signaling and cancer. *Bioessays* 1999; 21: 1021-1030.
- [5] Zhan T, Rindtorff N and Boutros M. Wnt signaling in cancer. *Oncogene* 2017; 36: 1461-1473.
- [6] Xu Q, Krause M, Samoylenko A and Vainio S. Wnt signaling in renal cell carcinoma. *Cancers (Basel)* 2016; 8.
- [7] Su Z, Yang Z, Xu Y, Chen Y and Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. *Mol Cancer* 2015; 14: 48.
- [8] Long JS and Ryan KM. New frontiers in promoting tumour cell death: targeting apoptosis, necroptosis and autophagy. *Oncogene* 2012; 31: 5045-5060.
- [9] Bursch W, Ellinger A, Gerner C, Frohwein U and Schulte-Hermann R. Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? *Ann N Y Acad Sci* 2000; 926: 1-12.
- [10] Chen S, Guttridge DC, You Z, Zhang Z, Fribley A, Mayo MW, Kitajewski J and Wang CY. Wnt-1 signaling inhibits apoptosis by activating β-catenin/T cell factor-mediated transcription. *J Cell Biol* 2001; 152: 87-96.
- [11] Hseu YC, Thiyagarajan V, Tsou HT, Lin KY, Chen HJ, Lin CM, Liao JW and Yang HL. In vitro and in vivo anti-tumor activity of CoQO against melanoma cells: inhibition of metastasis and induction of cell-cycle arrest and apoptosis through modulation of Wnt/β-catenin signaling pathways. *Oncotarget* 2016; 7: 22409-22426.
- [12] Gao C, Cao W, Bao L, Zuo W, Xie G, Cai T, Fu W, Zhang J, Wu W, Zhang X and Chen YG. Autophagy negatively regulates Wnt signalling by promoting dishevelled degradation. *Nat Cell Biol* 2010; 12: 781-790.
- [13] He C and Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. *Annu Rev Genet* 2009; 43: 67-93.
- [14] Chang HW, Lee YS, Nam HY, Han MW, Kim HJ, Moon SY, Jeon H, Park JJ, Carey TE, Chang SE, Kim SW and Kim SY. Knockdown of β-catenin controls both apoptotic and autophagic cell death through LKB1/AMPK signaling in head and neck squamous cell carcinoma cell lines. *Cell Signal* 2013; 25: 839-847.
- [15] Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol* 2007; 8: 774-785.
- [16] Tiainen M, Vaahtomeri K, Ylikorkala A and Makela TP. Growth arrest by the LKB1 tumor suppressor: induction of p21 (WAF1/CIP1). *Hum Mol Genet* 2002; 11: 1497-1504.
- [17] Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ and Thompson CB. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell* 2005; 18: 283-293.
- [18] Baumann P, Mandl-Weber S, Emmerich B, Straka C and Schmidmaier R. Activation of adenosine monophosphate activated protein ki-

## $\beta$ -catenin/AMPK/mTOR in multiple myeloma

- nase inhibits growth of multiple myeloma cells. *Exp Cell Res* 2007; 313: 3592-3603.
- [19] Chapuis N, Tamburini J, Green AS, Willems L, Bardet V, Park S, Lacombe C, Mayeux P and Bouscary D. Perspectives on inhibiting mTOR as a future treatment strategy for hematological malignancies. *Leukemia* 2010; 24: 1686-1699.
- [20] Qiang YW, Chen Y, Brown N, Hu B, Epstein J, Barlogie B and Shaughnessy JD Jr. Characterization of Wnt/beta-catenin signalling in osteoclasts in multiple myeloma. *Br J Haematol* 2010; 148: 726-738.
- [21] Sukhdeo K, Mani M, Zhang Y, Dutta J, Yasui H, Rooney MD, Carrasco DE, Zheng M, He H, Tai YT, Mitsiades C, Anderson KC and Carrasco DR. Targeting the beta-catenin/TCF transcriptional complex in the treatment of multiple myeloma. *Proc Natl Acad Sci U S A* 2007; 104: 7516-7521.
- [22] Su N, Wang P and Li Y. Role of Wnt/beta-catenin pathway in inducing autophagy and apoptosis in multiple myeloma cells. *Oncol Lett* 2016; 12: 4623-4629.