Beclin-1 knockdown decreases proliferation, invasion and migration of Ewing sarcoma SK-ES-1 cells via inhibition of MMP-9

CONGLIN YE*, XIAOLONG YU*, XUQIANG LIU, PING ZHAN, TAO NIE, RUNSHENG GUO, HUCHENG LIU, MIN DAI and BIN ZHANG

Department of Orthopedics, The First Affiliated Hospital of Nanchang University, Artificial Joints Engineering and Technology Research Center of Jiangxi, Nanchang, Jiangxi 330006, P.R. China

Received January 24, 2017; Accepted November 2, 2017

DOI: 10.3892/ol.2017.7667

**Abstract.** Although Beclin-1, a well-known key regulator of autophagy, has been demonstrated to serve a function in a number of disorders, including cancer, aging and degenerative diseases, its biological function in Ewing sarcoma (ES) remains unresolved. The objective of the present study was to determine the *in vitro* effect of Beclin-1 knockdown on the growth and malignant phenotype of ES SK-ES-1 cells, which have increased endogenous expression of Beclin-1 compared with RD-ES cells, and to investigate the underlying molecular mechanism. Cell proliferation, invasion and migration were investigated using CCK-8, Boyden chamber Transwell, and wound healing assays, respectively. Western blot analysis was used to detect expression levels of matrix metalloproteinase (MMP)-2 and MMP-9, which are associated with the malignant phenotype. Beclin-1 knockdown significantly inhibited proliferation, invasion and migration of SK-ES-1 cells. Western blot analysis revealed that Beclin-1 knockdown caused a significant reduction in the expression of MMP-9; no marked changes in MMP-2 expression were observed in the si-Beclin-1 group compared with the control group. The results of the present study suggest that Beclin-1 serves a function in proliferation, tumor progression and inhibition of autophagy in ES, and demonstrates it's potential as a target to increase the efficacy of anticancer agents.

**Introduction**

Ewing sarcoma (ES) is the second most common sarcoma of bone in children and young adults (1). It is an aggressive and highly metastatic tumor. In total, ~1/3 of patients with ES present with metastasis at diagnosis, with lung tissue and bone marrow being the most common sites of metastasis, resulting in poor prognosis (2). Treatment and prognosis of patients with ES are determined by the presence of metastases, among other factors. The 5-year survival rate of patients with metastases ranges between 20 and 45%, depending on location, compared with between 60 and 70% in those with localized disease (2). Thus, novel therapeutic targets, innovative approaches to therapy and improved understanding of the metastatic mechanism are necessary to improve the outcome for patients with metastatic ES.

Autophagy is a highly conserved process that contributes to maintaining cellular homeostasis via quality control of proteins and organelles. Under conditions of metabolic stress, autophagy provides nutrients and energy essential for cell survival (3-5). Beclin-1 is a B-cell lymphoma 2 (Bcl-2) homology 3 domain-only protein that is required for the formation of autophagosomes, which are utilized in the initiation of autophagy (6-8). It has been reported that cell autophagy is associated with tumor initiation and progression, and serves a function in cell signal regulation in tumors (9-11). To date, the exact effects of autophagy on the biological behavior of ES cells have not been fully resolved.

In the present study, SK-ES-1 cells were transfected with small interfering (si)RNA against Beclin-1 in order to investigate the effects of Beclin-1 knockdown on cell proliferation, invasion and migration, and to determine the underlying molecular mechanisms. To the best of our knowledge, this is the first study to report the effects of Beclin-1 knockdown on the behavior of ES cells.

**Materials and methods**

*Materials and reagents.* The SK-ES-1 and RD-ES human ES cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). RPMI-1640 medium,
fetal bovine serum (FBS), PBS, dimethyl sulfoxide and Cell Counting Kit-8 (CCK-8) were provided by Beijing Transgen Biotech Co., Ltd. (Beijing, China). Antibodies against Beclin-1 (ab207612, 1:1,000), matrix metalloproteinase (MMP)-2 (ab92536, 1:1,000), MMP-9 (ab194314, 1:1,000) and β-actin (ab8227, 1:1,000) were all purchased from Abcam (Cambridge, UK). Goat anti-rabbit IgG (H+L), horseradish peroxi-
dase-conjugated secondary antibodies (HS101-01, 1:2,000) were both purchased from Beijing Transgen Biotech Co., Ltd. Lipofectamine® 2000 and OPTI-MEM were both purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Matrigel was purchased from BD Biosciences (San Carlos, Costar, MA, USA). The Transwell invasion chambers were purchased from Costar (Cambridge, MA, USA). Crystal violet staining solution was purchased from Beyotime Institute of Biotechnology (Haimen, China). si-Beclin-1 against the BECLIN-1 gene (NM_003766) and control siRNA (si-CON) were constructed by Shanghai GeneChem Co., Ltd. (Shanghai, China).

Cell culture and transfection. SK-ES-1 and RD-ES cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. All cells used in the present study were subjected to <20 cell passages. SK-ES-1 cells at logarithmic phase were seeded at a density of 3x10⁴ cells/well in a 6-well plate for 24 h prior to transfection. Lipofectamine 2000 (10 µl diluted in 250 µl OPTI-MEM) was used for the transfection of 4 µg si-Beclin-1 or empty vector diluted in 250 µl OPTI-MEM, followed by incubation of the samples for 20 min at room temperature. The plasmid DNA-Lipofectamine 2000 complex was then added into 500 µl OPTI-MEM and incubated at 37°C with 5% CO₂ in an incubator for 6 h. Subsequently, the medium was replaced and the cells were incubated for 24 or 48 h in RPMI-1640 medium supplemented with 10% FBS prior to use in the corresponding experiments, which included a blank control group (non-transfected SK-ES-1 cells), a negative control group (SK-ES-1 cells transfected with blank plasmid, i.e., si-CON) and an experimental group (SK-ES-1 cells transfected with si-Beclin-1). Transfected cells were collected at 24 or 48 h post-transfection and used in subsequent experiments.

Cell proliferation assay. Cell growth was determined using the CCK-8 assay. In brief, cells infected with si-Beclin-1 or si-CON and non-transfected SK-ES-1 cells were incubated in 96-well plates at a density of 3x10⁴ cells/well. Cells were treated with 10 µl CCK-8 reagent at 24, 48 and 72 h and then measured at 450 nm using a Universal Microplate reader (EL800; Bio-Tek Instruments Inc., Winooski, VT, USA).

Boyden chamber Transwell assays. The invasive capacity of SK-ES-1 cells was detected via Matrigel-coated Transwell cell culture chambers (8 µm pore size). Following transfection for 24 h, SK-ES-1 cells of the three different groups were collected and suspended in serum-free medium. Isolated cells were then added to the upper chamber of the Transwell insert at a density of 4x10⁴ cells/well and the lower wells were filled with complete growth medium supplemented with 10% FBS. All samples were incubated for 24 h in a CO₂ incubator. Non-invading cells (on the upper membrane surface) were removed using a cotton swab and invading cells (on the lower membrane surface) were fixed with 95% ethanol for 15 min at 25°C, stained with 0.1% crystal violet staining solution for 20 min at 25°C, then counted under a phase-contrast microscope in three random fields (magnification, x200).

Wound healing assays. Migration of SK-ES-1 cells was measured using wound healing assays. After 24 h of transfection, SK-ES-1 cells of the three different groups were seeded at a density of 5x10⁴ cells/well in a 6-well culture plate to form a confluent monolayer. Cells were wounded with a sterile 100 µl pipette tip. All cells in the plates were incubated in fresh RPMI-1640 medium with 10% FBS for 24 h. Then scratch wounds were observed using a phase-contrast microscope and images were captured of each wound.

Western blot analysis. Following transfection, SK-ES-1 cells of the three different groups were seeded in 6-well plates at a concentration of 3x10³ cells/well and incubated in RPMI-1640 medium with 10% FBS for 48 h. The cells were collected and lysed in radioimmunoprecipitation assay buffer containing phenylmethane sulfonyl fluoride and phosphatase inhibitor cocktail (Sigma Aldrich; Merck KGaA, Darmstadt, Germany). Each sample was centrifuged at 17,105.6 x g for 10 min at 4°C using a Universal 320R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttingen, Germany), to remove cell debris and collect the supernatant for immunoblotting. Protein concentra-
tions were calculated using a bicinchoninic acid assay kit (Beijing Transgen Biotech Co., Ltd.) according to the manufac-
turer’s instructions with bovine serum albumin as the relative standard. Proteins (10 µl) were loaded and separated using SDS-PAGE (10% gel, 100 V for 2 h under reducing conditions). Following electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes in a tris-glycine transfer buffer and incubated with antibodies against β-actin, Beclin-1, MMP-2 and MMP-9 overnight at 4°C. The PVDF membranes were washed in Tris-buffered saline Tween-20 (TBST) three times. Secondary HRP-conjugated antibodies were added at 1:2,000 dilution and incubated for 2 h at 25°C. The PVDF membranes were washed a further three times in TBST. Immunoreactive proteins were detected using an enhanced chemiluminescence system (GE Healthcare, Chicago, IL, USA) according to the manufacturer’s instruc-
tions followed by exposure to X-ray films. Western blotting data was quantified using ImageJ software (version 7.0; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data were analyzed using the SPSS package for Windows (version 19.0; IBM Corp., Armonk, NY, USA). Quantitative data are expressed as mean ± standard deviation. Statistical analysis was performed using a one-way analysis of variance with the Student-Newman-Keuls method as a post hoc test. P<0.05 was considered to indicate a statisti-
cally significant difference.

Results

Endogenous expression of Beclin-1 in the human ES cell lines. Endogenous expression of Beclin-1 in the human ES SK-ES-1
and RD-ES cell lines was evaluated using western blot analysis. As presented in Fig. 1A and B, expression of Beclin-1 was significantly increased in the SK-ES-1 cell line compared with the RD-ES cell line (P<0.05). Thus, the SK-ES-1 cell line was used for Beclin-1 knockdown.

si-Beclin-1 significantly decreases the expression of Beclin-1 in SK-ES-1 cells. Protein levels of Beclin-1 were determined using western blotting following transfection of SK-ES-1 cells with si-Beclin-1 or si-CNT vectors for 48 h. As presented in Fig. 1C and D, Beclin-1 expression was significantly decreased in the si-Beclin-1 group compared with the blank control group (P<0.01).

Beclin-1 knockdown inhibits the proliferation of SK-ES-1 cells. The effect of Beclin-1 knockdown on SK-ES-1 cell growth was determined using a CCK-8 assay. As presented in Fig. 2A, knockdown of Beclin-1 significantly suppressed the growth of SK-ES-1 cells (P<0.05 at 24 h and P<0.01 at 48 h).

Beclin-1 knockdown represses the invasion and migration of SK-ES-1 cells. Transwell and wound healing assays were conducted to confirm the effect of Beclin-1 knockdown on the invasion and migration of SK-ES-1 cells. Representative micrographs of Transwell filters are presented in Fig. 2B. The invasive cell count, also presented in Fig. 2B, demonstrated that the invasive potential was significantly decreased in the si-Beclin-1 group relative to the blank control group (P<0.01). Furthermore, Beclin-1 knockdown resulted in a decrease in migration capability, as presented in Fig. 2C and D (P<0.01).

A western blot assay was performed to investigate the effect of Beclin-1 knockdown on the expression of MMP-2 and MMP-9, since it is generally acknowledged that they serve functions in tumor invasion, and metastasis (12-14). As presented in Fig. 3A and B, MMP-9 expression was significantly decreased in the si-Beclin-1 group compared with the Con group (P<0.01); however, no significant difference in the expression of MMP-2 was observed between the two groups. These results suggest that Beclin-1 knockdown may inhibit invasion and metastasis of SK-ES-1 cells via downregulation of the expression of MMP-9.

**Discussion**

ES is an aggressive bone and soft tissue malignant tumor that primarily affects children, and young adults (15). In previous years, the overall survival rate has risen markedly for patients who present with localized disease, owing to development of multi-agent systemic chemotherapy and aggressive local control methods, which have resulted in five-year event-free survival rates of 70-80% in these patients (15,16). However, for the ~25% of patients who present with metastatic disease, the prognosis is poor and event-free survival rate for these patients remains <25% (15). Thus, novel therapeutic targets and increased understanding of the metastatic mechanism of ES are required to achieve an improved outcome for these patients.
The function of autophagy in cancer has been highlighted in previous years. Autophagy serves a function in cell cycle regulation, apoptosis, angiogenesis and other aspects of tumor initiation and progression (17). Increasing evidence suggests that autophagy contributes to the malignant phenotype in a number of tumors, including lung adenocarcinoma, ovarian carcinoma and esophageal squamous cell carcinoma (18-20). Autophagy-related protein six, known as Beclin-1, the first confirmed mammalian autophagic gene, has been demonstrated to initiate autophagosome formation through binding
Matrix metalloproteinase 9 (MMP-9) is generally established to be closely associated with tumor invasion and metastasis, suggested that Beclin-1 knockdown inhibited invasion and migration of SK-ES-1 cells through downregulating the expression of MMP-9. Taken together, the results of the present study suggest that Beclin-1 knockdown may suppress the growth and malignant phenotype of SK-ES-1 cells by inhibition of MMP-9. Thus Beclin-1 is a promising therapeutic target for treatment of ES, particularly in patients that present with metastasis. Further experiments on the in vitro effects of Beclin-1 knockdown on apoptosis of ES cells may further resolve its viability as a therapeutic target.

Acknowledgements

The present study was supported by The Foundation of Health Department of Jiangxi Province (grant no. 2016A073) and Gan-Po Talents Project 555 of Jiangxi Province.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Gaspar N, Hawkins DS, Dirksen U, Lewis JJ, Ferrari S, Le Deley MC, Koval H, Grимер R, Whelan J, Claude L, et al: Ewing sarcoma: Current management and future approaches through collaboration. J Clin Oncol 33: 3036-3046, 2015.
3. Levine B and Klionsky DJ: Development by self-digestion: Molecular mechanisms and biological functions of autophagy. Dev Cell 6: 463-477, 2004.
4. Mizushima N: Autophagy: Process and function. Genes Dev 21: 2861-2873, 2007.
5. Mathew R, Karantza-Wadsworth V and White E: Role of autophagy in cancer. Nat Rev 7: 961-967, 2007.
6. Liang XH, Kleeman LK, Jiang HH, Gordon G, Goldman JE, Berry G, Herman B and Levine B: Protection against fatal Sindbis virus encephalitis by beclin1, a novel Bcl-2-interacting protein. J Virol 72: 8586-8596, 1998.
7. Oberstein A, Jeffrey PD and Shi Y: Crystal structure of the Bcl-XY-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. J Biol Chem 282: 13123-13132, 2007.
8. Liu B, Bao JK, Yang JM and Cheng Y: Targeting autophagic pathways for cancer drug discovery. Chin J Cancer 32: 113-120, 2013.
9. Meijer AJ and Codogno P: Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol 36: 2445-2462, 2004.
10. Sun Y, Liu JH, Jin L, Lin SM, Yang Y, Sui YX and Shi H: Over-expression of the Beclin1 gene upregulates chemosensitivity to anti-cancer drugs by enhancing therapy-induced apoptosis in cervix squamous carcinoma CaSki cells. Cancer Lett 294: 204-210, 2010.
11. Jin S and White E: Role of autophagy in cancer: Management of metabolic stress. Autophagy 3: 28-31, 2007.
12. Li H, Zhang K, Liu LH, Ouyang Y, Bu J, Guo HB and Xiao T: A systematic review of matrix metalloproteinase 9 as a biomarker of survival in patients with osteosarcoma. Tumour Biol 35: 5487-5491, 2014.
13. Wang J, Shi Q, Yuan TX, Song QL, Zhang Y, Wei Q, Zhou L, Luo J, Zuo G, Tang M, et al: Matrix metalloproteinase 9 (MMP-9) in osteosarcoma: Review and meta-analysis. Clin Chim Acta 433: 225-231, 2014.
14. Shang HS, Cheng JB, Lin JH, Lin JP, Hsu SC, Liu CM, Liu JY, Wu PP, Lu HF, AU MK and Chung JG: Deguelin inhibits the migration and invasion of U-2 OS human osteosarcoma cells via the inhibition of matrix metalloproteinase-2/9 in vitro. Molecules 19: 16588-16608, 2014.
15. Balamuth NJ and Womer RB: Ewing's sarcoma. Lancet Oncol 11: 184-192, 2010.
16. Gorlick R, Janeway K, Lessnick S, Randall RL and Marin A: COG Bone Tumor Committee: Children's Oncology Group's 2013 blueprint for research: Bone tumors. Pediatr Blood Cancer 60: 1009-1015, 2013.
17. Filippi-Chiela EC, Villodre ES, Zamin LL and Lenz G: Autophagy interplay with apoptosis and cell cycle regulation in the growth inhibiting effect of resveratrol in glioma cells. PLoS One 6: e20849, 2011.
18. Pan B, Chen D, Huang J, Wang R, Feng B, Song H and Chen L: HMGB1-mediated autophagy promotes docetaxel resistance in human lung adenocarcinoma. Mol Cancer 13: 165, 2014.
19. Liu B, Bao JK, Yang JM and Cheng Y: Targeting autophagic proteins LC3 and Beclin-1 impacts susceptibility to anti-cancer drugs by enhancing therapy-induced apoptosis in pancreatic ductal adenocarcinoma. Pancreas 42: 829-835, 2013.
20. Tong Y, You L, Liu H, Li L, Meng H, Qian Q and Qian W: Potent antitumor activity of oncolytic adenovirus expressing beclin-1 via induction of autophagic cell death in leukemia. Oncotarget 4: 960-874, 2013.
21. Bai W, Ji X, Hu X, Cao Y, Chen Z, Hou X, Xiao S, Zhao Z, Guo W and Tan B: Autophagy-related proteins LC3 and Beclin-1 impact the efficacy of chemoradiation on esophageal squamous cell carcinoma. Pathol Res Pract 209: 562-567, 2013.