Front. Med. 2022, 16(6): 883−895
https://doi.org/10.1007/s11684-022-0919-0

RESEARCH ARTICLE

Superenhancers activate the autophagy-related genes Beclin1 and LC3B to drive metastasis and drug resistance in osteosarcoma

Hongyi Wang[1,2,*], Zhuochao Liu[1,*], Jun Wang[2], Fangqiong Hu[2], Qi Zhou[2], Li Wei[2], Qiyuan Bao[1], Jizhuang Wang[2], Jing Liang[2], Zhihong Liu (✉)[1,2], Weibin Zhang (✉)[1]

[1]*Department of Orthopedics, Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China;* [2]*Shanghai Institute of Traumatology and Orthopedics, Shanghai 200025, China*

Abstract **Metastasis and drug resistance are the leading causes of poor prognosis in patients with osteosarcoma. Identifying the relevant factors that drive metastasis and drug resistance is the key to improving the therapeutic outcome of osteosarcoma. Here, we reported that autophagy was highly activated in metastatic osteosarcoma. We found increased autophagolysosomes in metastatic osteosarcoma cell lines by using electron microscopy, Western blot, and immunofluorescence experiments. We further examined the expression of the autophagy-related genes Beclin1 and LC3B in 82 patients through immunohistochemistry and found that Beclin1 and LC3B were highly related to unfavorable prognosis of osteosarcoma. Knockdown of Beclin1 and LC3B reduced invasion, metastasis, and proliferation in metastatic osteosarcoma cells. *In vitro* and *in vivo* studies also demonstrated that inhibiting by 3-MA inhibited cell growth and metastasis. Moreover, we demonstrated that autophagy-related genes were activated by SEs and that the inhibition of SEs by JQ-1 decreased the metastasis of osteosarcoma. Overall, our findings highlighted the association of autophagy with osteosarcoma progression and shed new light on autophagy-targeting therapy for osteosarcoma.**

Keywords osteosarcoma; autophagy; metastasis; drug resistance; Beclin1; LC3B

Introduction

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents [1]. With the development of surgical techniques and chemotherapy, the survival rate of osteosarcoma has increased by nearly two decades. Studies show that the 5-year overall survival rate of osteosarcoma is approximately 70% [1–3]. However, the clinical outcome of osteosarcoma with metastasis and local recurrence remains unsatisfactory, and the current 5-year overall survival rate of metastatic or relapsed osteosarcoma is only approximately 20% [4–6]. Thus, identifying the factors contributing to the metastasis and local recurrence of osteosarcoma is key to improving the overall therapeutic outcome.

Received June 15, 2021; accepted December 21, 2021

Correspondence: Weibin Zhang, zhangweibin10368@163.com;
Zhihong Liu, drliuzhihong@163.com

*These authors contribute equally to this work.

Autophagy is a common cellular catabolic degradation machinery in response to nutrient starvation or stress conditions [7,8]. The main function of this process is to maintain metabolic homeostasis through the selective degradation of unfolded or aggregated proteins and damaged or excess organelles [8]. Autophagy is a complicated regulatory process and is controlled by many proteins. Generally, autophagy is initiated by cellular stress and AMPK activation. The class III phosphatidylinositol 3-kinase (PI3K) VPS34, Beclin1, ATG14, and VPS15 help form autophagosomes [9–11]. The cytosolic form of microtubule-associated protein 1 light chain 3 LC3 (MAP1LC3), also known as LC3-I, conjugates to phosphatidylethanolamine and forms an LC3-phosphatidylethanolamine conjugate (LC3-II) [12,13]. LC3-II is recruited to the membranes of autophagosomes, which then fuse with lysosomes, form autophagolysosomes, and complete the degradation of proteins inside autophagolysosomes, including LC3 itself. Initiation of the autophagosome, fusion of lysosomes with autophagosomes, and degradation in autophagolysosomes induce

autophagic flux [14].

Currently, the functions of autophagy in cancer remain controversial [15–17]. On one hand, autophagy is known as type II programmed cell death [18], and overactivated autophagy may trigger the death program and lead to cell death. On the other hand, emerging evidence has demonstrated that autophagy contributes greatly to the progression and drug resistance of malignant disease and that targeting autophagy contributes to cancer therapy [17]. Tumor cells usually undergo stressful conditions, such as hypoxia and limited nutrition [19,20]. Activated autophagy may help tumor cells overcome such situations. Radiotherapy and chemotherapy, as common neoadjuvant treatments for osteosarcoma, can also cause tumor-cell stress [21], such as disorders in protein folding, mitochondrial damage, and endoplasmic reticulum stress. Autophagy may contribute to maintaining the organization of cellular function and confer resistance to anticancer therapy.

Several studies have revealed that autophagy is involved in osteosarcoma regulation, but the conclusion is controversial. On one hand, autophagy provides osteosarcoma cells with energy and nutrients in the stressful microenvironment of tumors, such as hypoxia and starvation. Additionally, autophagy is activated during chemotherapy, such as with cisplatin, in osteosarcoma [22]. Some studies have found that autophagy inhibition by rapamycin leads to autophagy-mediated cell death in osteosarcoma [23,24]. On the other hand, other studies have shown that autophagy induction, such as treatment with apatinib, also leads to cell death in osteosarcoma [25]. Apparently, the perturbation of autophagy, whether induction or decrement, leads to cell death of osteosarcoma, which may depend on the basal autophagic status of osteosarcoma cells. However, current studies on autophagy and osteosarcoma have focused mostly on drug innovation, and the basal status of autophagy in osteosarcoma and its relationship with disease progression and prognosis remain unclear.

In the present study, we reported that autophagy was activated in osteosarcoma and that metastatic osteosarcoma showed higher levels of autophagic flux. We also reported that high levels of autophagy were associated with poor prognosis of osteosarcoma and that autophagy inhibition sensitized osteosarcoma patients to chemotherapy. We further reported that osteosarcoma-related genes were activated by superenhancers and that the inhibition of these superenhancer decreased metastasis in osteosarcoma.

Materials and methods

Enrollment

Eighty-two patients with osteosarcoma were enrolled in this study. Tumor tissues were obtained after surgical removal. This study was approved by the ethics committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine and was conducted according to the *Declaration of Helsinki* and its amendments. Informed consent of the patients was acquired.

Cell lines

U2OS, SaOS2, HOS, and 143B cells were provided by American Type Culture Collection. These cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (04-001-1ACS, Biological Industries, Beit Haemek, Israel). NW5 was a primary osteosarcoma cell line established by our laboratory. In a typical procedure, three pieces of fresh osteosarcoma tumor tissue after surgical operation were cut and washed five times with PBS. Single-cell suspensions were obtained and transferred onto a 6 cm culture plate. Cells were cultured in DMEM (SH30022.01B, HyClone, Logan, UT, USA) supplemented with 10% fetal calf serum, amphotericin B, and penicillin/streptomycin. After five passages, the cell mix was seeded onto a 96-well plate to obtain a single clone. Six clones were collected, and metastatic ability was assessed. The cells with the highest metastatic ability were named NW5 cells.

Western blot

Cultured cells were washed with PBS for three times, and 1×10^5 cells were lysed in 100 μL 2× loading buffer (100 mmol/L Tris-Cl (pH6.8), 200 mmol/L DTT, 4% SDS, 20% glycerol, 0.1% bromphenol blue sodium salt). Whole-cell lysates were heated three times at 100 °C for 5 min. Whole-cell lysates were used for Western blot experiments. Antibodies against LC3B (CST3868S, Cell Signaling Technology, USA), Beclin1 (CST3738S, Cell Signaling Technology), GAPDH (SC25778, Santa Cruz Technology), and P-ULK1 (CST5869S, Cell Signaling Technology) were used.

siRNA-mediated knockdown of LC3B and Beclin1

siRNA targeting LC3B and Beclin1 was transduced into 143B cells with Lipofectamine 2000 (11668027, Thermo Fisher, USA) according to the manufacturer's instructions. The sequences of the siRNAs were as follows: si-LC3B-sense, 5′-GCCCUCUACUGAUUG UUAATT-3′; si-LC3B-antisense, 5′- UUAACAAUCA GUAGAGGGCTT-3′; si-Beclin1-sense, 5′-CAGUUUGG CACAAUCAAUA-3′; and si-Beclin1-antisense, 5′-UAUUGAUUGUGCCAAACUG-3′.

Immunofluorescence and immunochemistry (IHC) staining

IHC experiments were performed as previously described [26]. Immunofluorescence experiments were performed according to the manufacturer's instructions for the Immunol Fluorescence Staining Kit (P0179, Beyotime, China). The IHC staining scores were calculated by multiplying the positive staining region (ranging from 0 to 4) and the positive levels (ranging from 0 to 4). Scores > 9 were considered as the highly expressed group, whereas scores < 9 were considered as the lowly expressed group.

Electron microscopy

Electron microscopy experiments were performed as previously described [27]. In a typical procedure, cells were fixed with 2% glutaraldehyde fixative solution for 2 h, washed, and used for electron microscopy analysis. A Philips CM120 electron microscopy device was used.

CCK-8 analysis

The CCK-8 analyses were performed according to the manufacturers' instructions (CK04-500, Dojindo, Japan). OD values were determined with a Multiskan™ FC microplate photometer (Thermo Fisher) at 450 nm.

Wound-healing assays

Wound-healing assays were performed as previously described [28]. In a typical procedure, 143B cells were cultured in a 6-well plate in DMEM supplemented with 10% FBS. siRNAs targeting Beclin1, LC3B, or nontargeting siRNA were transfected into 143B cells with Lipofectamine 2000 (Invitrogen). The cells were scratched with a pipette tip after 18 h of transfection. Cell migration into the gap was imaged 24 h after scratching.

Transwell assays

Transwell assays were performed as previously described [28]. siRNA targeting Beclin1, LC3B, or nontargeting siRNA-transduced 143B cells was used for the Transwell assays. Matrigel (50 μL, BD) was added to the upper chamber of the Transwell plate before cell seeding. Cells (1×10^5) cultured in DMEM supplemented with 2% FBS were seeded in the top chamber. DMEM supplemented with 10% FBS was added to the bottom chamber. The metastasized cells across the Matrigel were stained with crystal violet.

Xenograft experiment

The animal experiments were approved by the Institutional Animal Care and Use Committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine and were conducted in accordance with institutional animal protocols. NW5 cells were stably transfected with luciferase expression plasmid by lentiviral transfection, and Luc-NW5 cells were obtained. Luc-NW5 cells were injected to NOD-SCID mice paratibially. Twenty days after xenografting, nice tumor-burdened mice were randomly divided into two groups and treated with 3-methyladenine (3-MA) or placebo. Tumor length and width were examined every five days post-injection by using a caliper. Tumor volume was calculated by length $\times$ (width)^2/2.

Reanalysis of H3K27ac ChIP-seq in osteosarcoma cells

H3K27ac ChIP-seq data sets in osteosarcoma cells, including metastatic (LM7, MNNG, 143B, and MG63.3) and nonmetastatic (MG63, Saos-2, Hu09, and HOS) cells, were downloaded from the GEO database (GSE74230) [29]. Raw reads were mapped to the human genome (hg38) with bowtie2 [30]. H3K27ac peaks were called by MACS2 [31] and merged by bedtools [32]. H3K27ac signals of each cell under distinct peaks were calculated by homer2 [33]. Differential peaks were identified by fold-change and *P* value (*t*-test). Representative H3K27ac signals among the regulatory regions were visualized using the WashU browser.

Statistical analysis

All statistical analyses were performed with RStudio. For the survival analysis between two groups, log-rank tests were used. The Kaplan–Meier plot was generated with the Plot function in R. To compare differences between two groups such as autophagosome numbers, cell growth, and tumor sizes, two-tailed and unpaired Student's *t*-tests were performed.

Results

Autophagy-related genes were highly expressed in metastatic osteosarcoma

To determine the autophagic status of osteosarcoma and its relationship with malignancies, we first performed electron microscopy analysis, the gold standard for determining autophagosomes, in metastatic and nonmetastatic osteosarcoma cells. We observed that NW5 and 143B cells (identified as metastatic osteosarcoma in previous studies [29,34]) had more autophagosomes and more autophagolysosomes than U2OS cells (identified as nonmetastatic osteosarcoma in previous studies [29,34]) under steady status (Fig. 1A–1D). We also found that

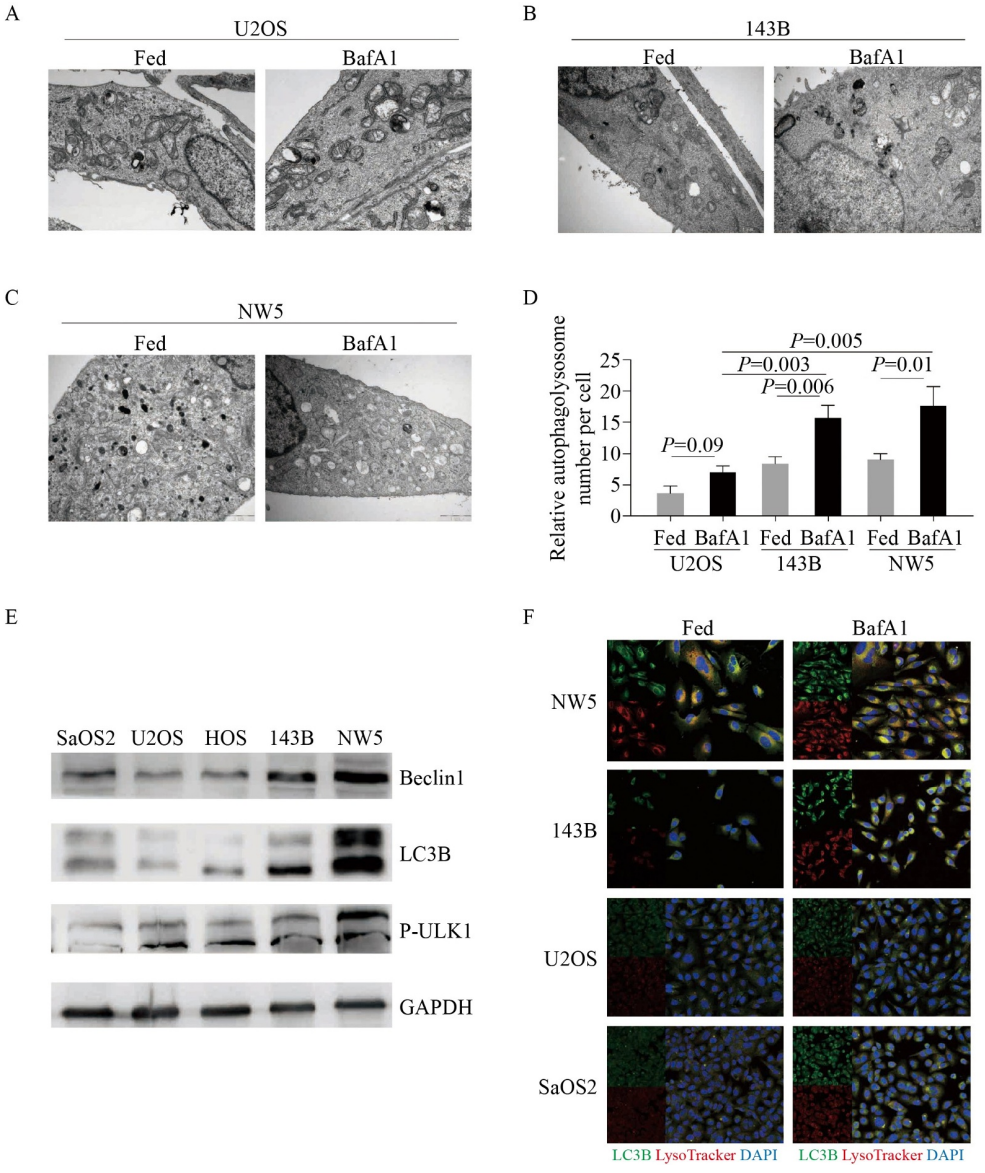

Fig. 1 Autophagic flux in metastatic and nonmetastatic osteosarcoma cells. (A–D) Illustration of autophagosomes and autophagolysosomes in NW5, 143B, and U2OS cells before and after BafA1 treatment. The autophagosomes were determined by electron microscopy. (E) Expression of LC3B, Beclin1, and P-ULK1 in osteosarcoma cells. The expression of LC3B, Beclin1, and P-ULK1 was determined in SaOS2, U2OS, HOS, 143B, and NW5 cells by Western blot. GAPDH served as the internal control. (F) Determination of autophagic flux in osteosarcoma cells. The expression and distribution of LC3B in NW5, 143B, U2OS, and SaOS2 cells were determined before and after BafA1 treatment. Green represents LC3B, red represents lysosomes, and blue represents DAPI. The yellow color in the merged image shows the colocalization of LC3 with lysosomes.

autophagosomes increased and autophagolysosomes decreased upon bafilomycin A1 (BafA1) treatment in 143B and NW5 cells. This finding indicated that BafA1 could indeed block autophagosome-lysosome fusion, as well as support high levels of autophagy and autophagic flux in metastatic osteosarcoma.

We subsequently examined the expression of two well-established autophagy-related genes, LC3B and Beclin1, in metastatic (143B and NW5) [29,34] and nonmetastatic (SaOS2, U2OS, and HOS) osteosarcoma cell lines [29,34]. LC3B and Beclin1 expression was much higher in metastatic osteosarcoma cells than in nonmetastatic ones (Figs. 1E and S1). Considering that lower levels of autophagic markers may also be observed in the rapid degradation of the autolysosome, we further examined autophagic flux by blocking autophagosome–lysosome fusion by using BafA1. Consistent with the results of Western blot experiments, the basal expression of LC3B was much higher in metastatic (143B and NW5) than in nonmetastatic (U2OS and SaOS2) osteosarcoma cells (Fig. 1F). Moreover, upon treatment with the autophagosome-lysosome fusion inhibitor BafA1, stronger

expression and colocalization of LC3B with lysosomes were observed in 143B and NW5 cells but not in U2OS cells or SaOS2 cells (Fig. 1F). This finding indicated strong autophagic flux and autophagy in metastatic osteosarcoma cells.

High levels of Beclin1 and LC3B were related to poor prognosis of osteosarcoma

To further investigate the correlation between autophagy and the clinical outcome of osteosarcoma, we performed IHC staining with antibodies against Beclin1 and LC3B in 82 osteosarcoma samples. We found that Beclin1 and LC3B were highly expressed in the majority of osteosarcoma cells, especially osteosarcomas with adverse outcomes (Fig. 2A and 2B), suggesting that activated autophagy may be a malignancy factor of osteosarcoma.

The 82 osteosarcomas were further divided into groups according to their Beclin1 or LC3B expression level based on the IHC staining score, and overall survival and metastasis-free survival were examined. As shown in Fig. 2C and 2D, high levels of LC3B ($P < 0.0001$, hazard ratio = 12.53, by log-rank test) and Beclin1 ($P < 0.0001$, hazard ratio = 6.471, by log-rank test) were significantly related to poor metastasis-free survival. This finding suggested that activated autophagy pathways may cause the metastasis of osteosarcoma cells. Additionally, high levels of LC3B ($P < 0.0001$, hazard ratio = 6.936, by log-rank test) and Beclin1 ($P < 0.0001$, hazard ratio = 3.130, by log-rank test) were also significantly related to poor overall survival in osteosarcoma (Fig. 2E and 2F). Additionally, these observations strongly suggested that the autophagic factors Beclin1 and LC3B could be used as prognostic markers for osteosarcoma.

Knockdown of Beclin1 and LC3B or inhibition of autophagy could suppress cell growth and the invasion of osteosarcoma cells

To further investigate the function of autophagic factors in osteosarcoma metastasis, we knocked down the expression of LC3B and Beclin1 in 143B cells (a metastatic osteosarcoma cell line) and detected their effects on cell growth, cell migration, and metastasis. Cell-proliferation assays, wound-healing assays, and Transwell assays showed that LC3B and Beclin1 knockdown in 143B cells reduced cell growth, migration, and metastasis (Fig. 3A–3D).

We also used 3-MA, a type III phosphatidylinositol 3-kinase inhibitor that blocks autophagosome formation, to inhibit autophagy in osteosarcoma cells. Similar results were observed with LC3B and Beclin1 knockdown (Fig. 3E and 3F).

We also analyzed the effects of autophagy inhibition on tumor growth of osteosarcoma *in vivo*. We established a xenograft model of osteosarcoma using Luc-NW5 cells in NOD-SCID mice via paratibial injection. We found that treatment with the autophagy inhibitor 3-MA significantly reduced the tumor sizes (Fig. 4A and 4B).

Targeting autophagy-sensitized osteosarcoma cells to chemotherapy

We then investigated autophagy in osteosarcoma and its correlation with chemotherapeutic outcome. We first determined the autophagy status post chemotherapy of osteosarcoma by electron microscopy. Results showed that chemotherapy with cisplatin and doxorubicin further increased autophagolysosomes in NW5 cells (Fig. 5A and 5B), indicating that the autophagy level further increased upon chemotherapy.

We next knocked down LC3B and Beclin1 in NW5 cells with or without doxorubicin. We found that LC3B and Beclin1 knockdown enhanced the cell growth inhibition of doxorubicin (Fig. 5C and 5D), indicating that autophagy inhibition could sensitize osteosarcoma to chemotherapy. Additionally, we treated NW5 cells with 3-MA, an autophagy inhibitor. We found that 3-MA treatment resulted in decreased IC50 of doxorubicin on NW5 cells, indicating that inhibition of autophagy could be synergistic with chemotherapy on the inhibition of osteosarcoma cell growth (Fig. 5E).

To further confirm whether the synergistic effects of 3-MA were attributed to autophagic regulation, we detected the expression of LC3B upon treatment with 3-MA and doxorubicin. Doxorubicin increased the expression of LC3B, whereas 3-MA decreased the expression of LC3B (Figs. 5F and S2), supporting the notion that chemotherapy increased autophagy in osteosarcoma. We also found that the blockade of autophagosome-lysosome fusion by using BafA1 cannot reverse the 3-MA-induced decrease in LC3B, indicating that autophagic flux was blocked. Thus, the 3-MA-induced sensitization to chemotherapy was attributed to its regulation of autophagy. Together, these results indicated that autophagy inhibition partially inhibited the metastatic ability and enhanced the chemotherapeutic effect in osteosarcoma.

Enhancers of autophagy-related genes were highly activated in metastatic osteosarcoma

Next, we investigated the mechanisms underlying the activation of potential factors that contributed to osteosarcoma metastasis. We first analyzed the transcription features of these autophagy-related genes, such as BECN1, MAP1LC3B (LC3B), MAP1LC3A (LC3A), and ATG5. We found that H3K27ac signals were abundantly enriched at the regulatory regions of these cells (Fig. 6). Given that abundant H3K27ac signals

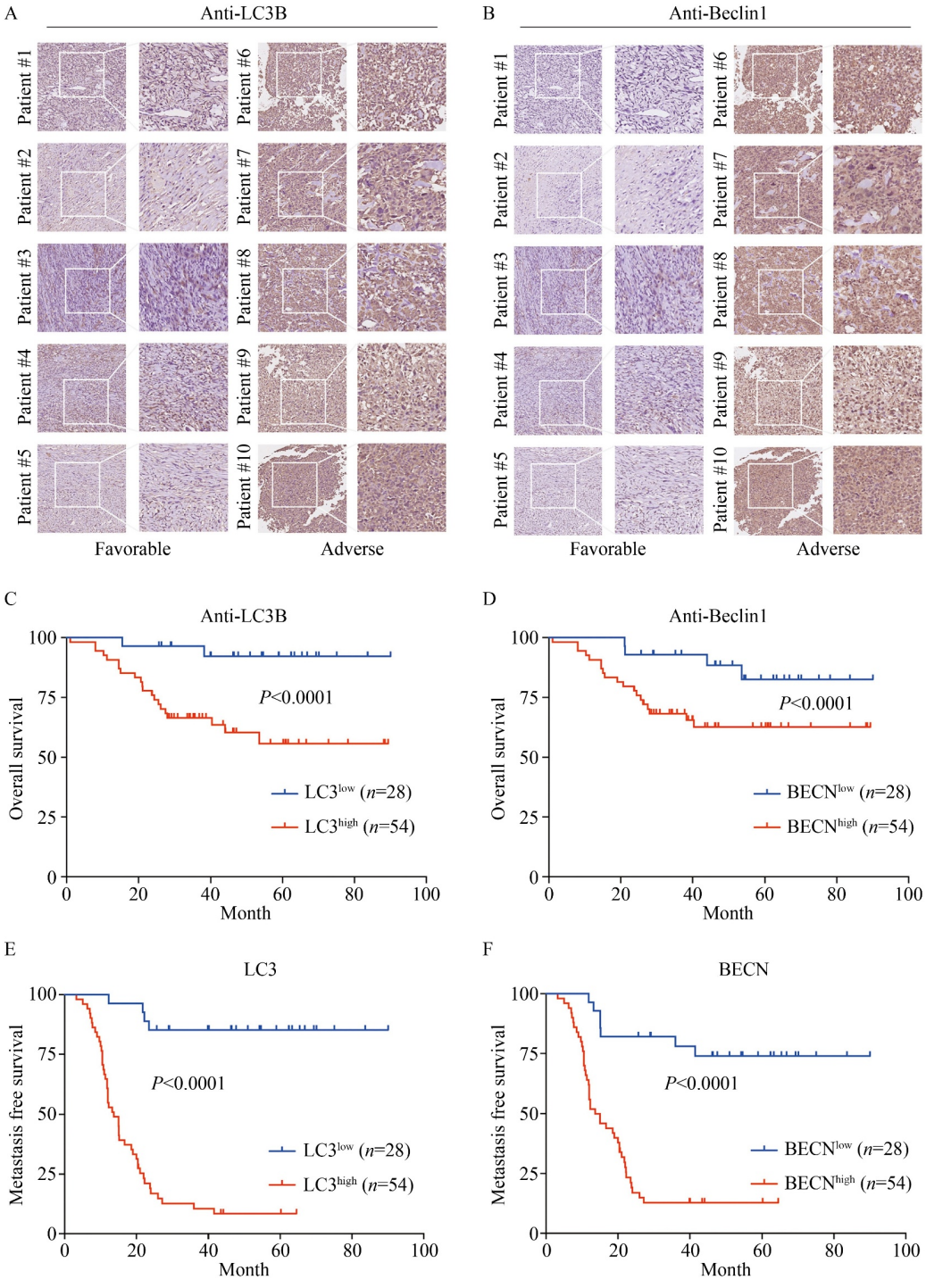

Fig. 2 Expression of the autophagy-related genes Beclin1 and LC3B in primary tissues of osteosarcoma. (A, B) Representative illustration of Beclin1 and LC3B expression in the tumor tissue of osteosarcoma with favorable or adverse outcomes. Beclin1 and LC3B expression in osteosarcoma tumor tissues was detected by immunochemistry with antibodies against Beclin1 and LC3B. Three tumor tissues from patients with poor prognosis and three from patients with favorable prognosis are illustrated. (C, D) High levels of Beclin1 and LC3B were related to poor overall survival of osteosarcoma. (E, F) High levels of Beclin1 and LC3B were related to poor metastasis-free survival of osteosarcoma.

are reportedly associated with superenhancer regulation, superenhancers have been reported to be involved in the malignant transformation of many cancers. Thus, we further analyzed the superenhancer effect in metastatic and nonmetastatic osteosarcoma cells, including MG63, SaOS-2, Hu09, and HOS cells. Interestingly, we found that BECN1, MAP1LC3B, MAP1LC3A and ATG5 were all regulated by superenhancer in metastatic osteosarcoma

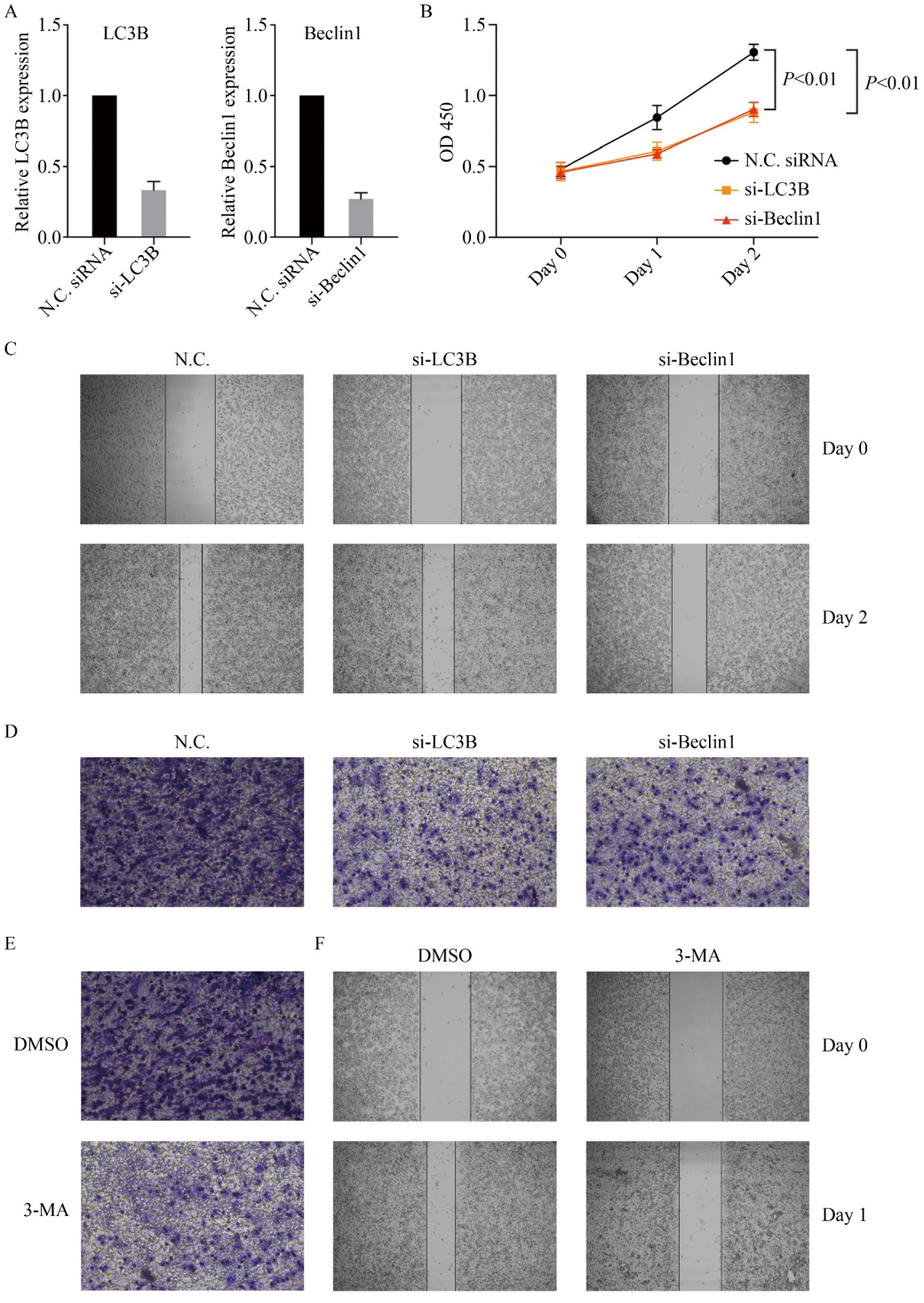

Fig. 3 Knockdown of Beclin1 or LC3B inhibited the proliferation, invasion, and metastasis of osteosarcoma cells. (A) Validation of knockdown efficiency. (B–D) LC3B and Beclin1 knockdown inhibited the proliferation, invasion, and metastasis of 143B cells. Cell-viability (B), wound-healing (C), and Transwell (D) experiments were performed in 143 cells transfected with siRNA targeting LC3B, Beclin1, or nonspecific siRNA (N. C.). (E, F) Inhibition of autophagy by 3-MA inhibited the invasion and metastasis of 143B cells. Wound-healing (E) and Transwell (F) experiments were performed in 143 cells treated with DMSO or 3-MA.

cells but not in nonmetastatic osteosarcoma cells (Fig. 6). These results indicated that transcriptional activation by superenhancer may be involved in overactivated autophagic features in osteosarcoma.

Inhibition of the superenhancer-inhibited cell invasion of osteosarcoma

Finally, to determine whether autophagy could be

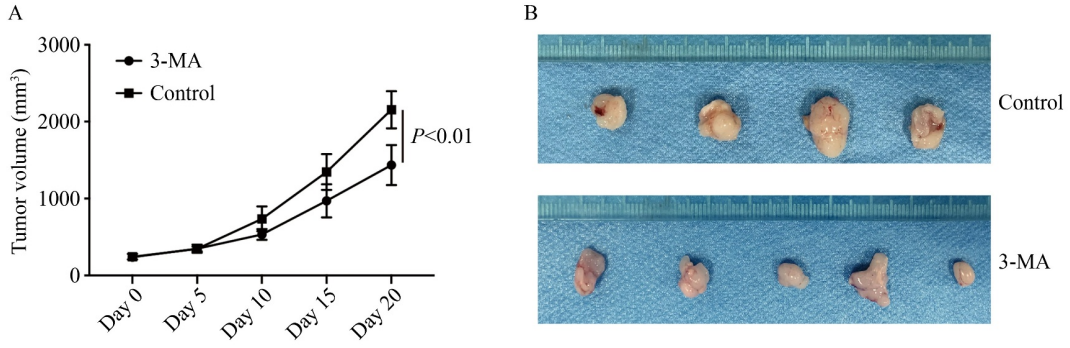

Fig. 4 Inhibition of autophagy inhibited the tumor growth and metastasis of osteosarcoma *in vivo*. (A, B) 3-MA inhibited the tumor growth of osteosarcoma. The tumor volumes (A) and tumor sizes (B) of 3-MA- or placebo-treated Luc-NW5 xenograft mice are shown. NW5 cells were xenografted in NOD-SCID mice via paratibial injection. The tumor-burdened mice were treated with 3-MA or placebo 20 days after xenografting.

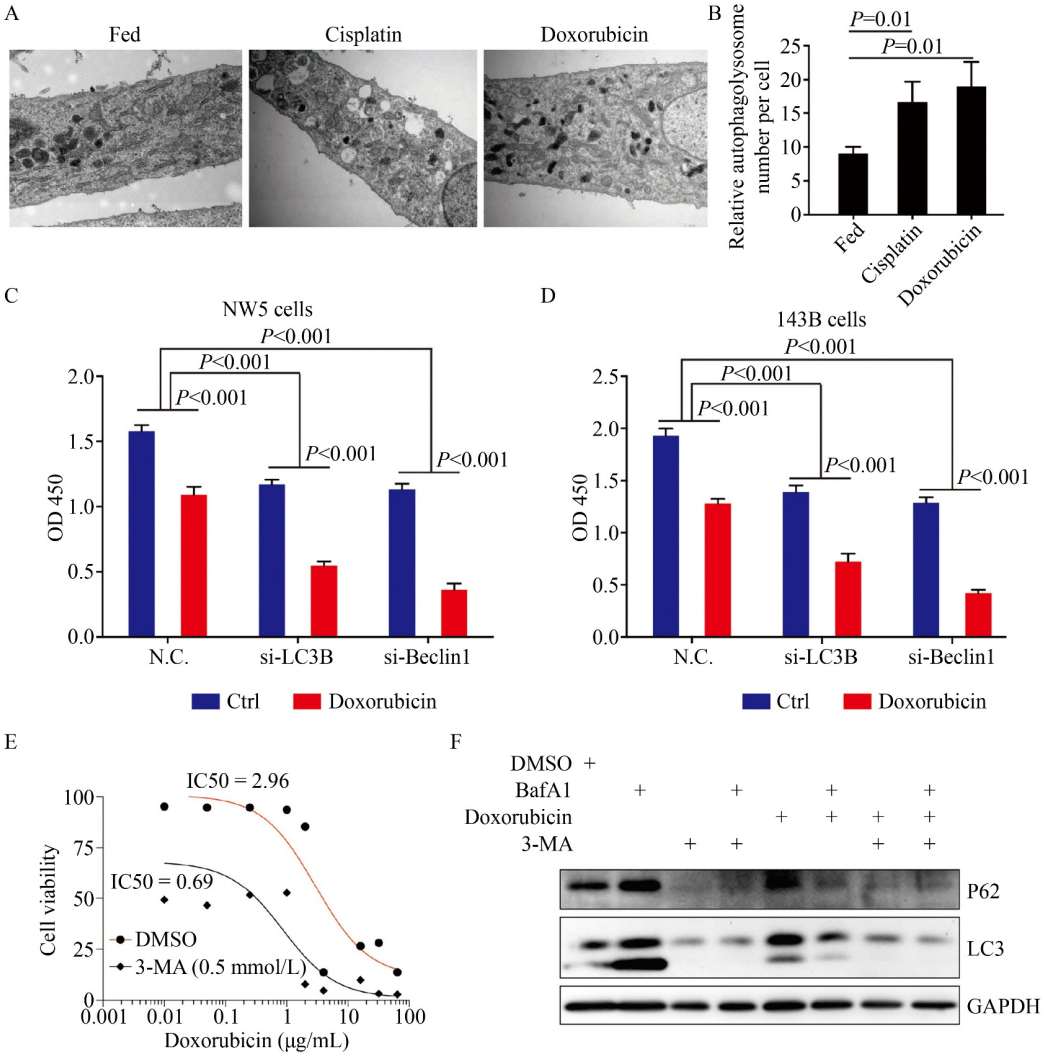

Fig. 5 Targeting LC3B- and Beclin1-sensitized osteosarcoma cells to chemotherapy. (A, B) Chemotherapy enhanced autophagy in NW5 cells. Autophagosomes and autophagolysosomes detected by electron microscopy (A) in NW5 cells treated with cisplatin and doxorubicin are shown. The statistical analysis is shown (B). (C, D) Knockdown of LC3B- and Beclin1-sensitized NW5 and 143B cells to doxorubicin chemotherapy. The NW5 (C) or 143B (D) cells transfected with N.C. siRNA or siRNA targeting LC3B or Beclin1 was treated with doxorubicin for 24 h. Cell viability was detected by CCK-8 assays. (E) Autophagy inhibition by 3-MA synergistically inhibited the cell growth of osteosarcoma cells. The IC50 of doxorubicin in DMSO- or 3-MA-treated NW cells was detected by CCK8. (F) 3-MA inhibited doxorubicin induced autophagy. The protein levels of LC3B were detected in 3-MA-, BafA1-, or doxorubicin-treated osteosarcoma cells.

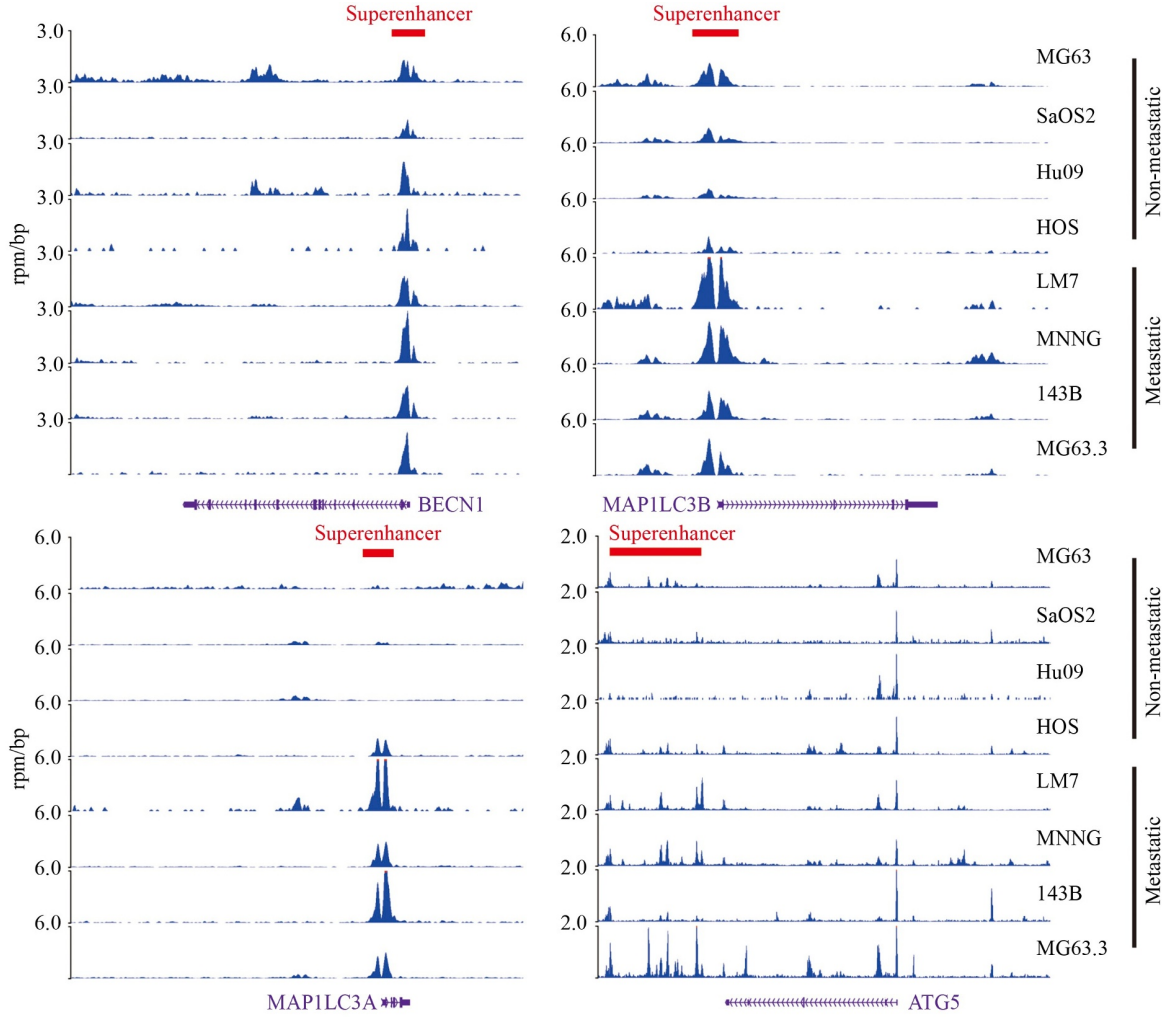

Fig. 6 H3K27ac signals at the regulatory regions of autophagy-related genes in metastatic and nonmetastatic osteosarcoma cells. Schematic of H3K27ac signals among the regulatory regions of Beclin1 (BECN1), MAP1LC3B (LC3B), MAP1LC3A (LC3A), and ATG5 in metastatic (LM7, MNNG, 143B, and MG63.3) and nonmetastatic (MG63, SaOS2, Hu09, and HOS) cells.

inhibited by superenhancers, we treated 143B and NW5 cells with JQ-1 (a BRD4 inhibitor) and found that the expression of BECN1, MAP1LC3B, MAP1LC3A, and ATG5 significantly decreased post-treatment (Fig. 7A). Invasion ability was also significantly reduced with JQ-1 treatment (Fig. 7B), further supporting the notion that autophagy played a vital role in osteosarcoma.

Discussion

Metastatic osteosarcoma is a life-threatening malignant disease in children and adolescents. Unveiling the factors contributing to metastatic and malignant transformation is required to improve the diagnosis and prediction of disease progression. Here, we reported that highly activated autophagy was the hallmark of metastatic osteosarcoma. We showed that higher levels of autophagic flux were associated with poor prognosis and metastasis of osteosarcoma. Targeting autophagy sensitized osteosarcoma to chemotherapy.

Lines of evidence have demonstrated that targeting autophagy inhibits the cell growth of osteosarcoma, but little is reported on the relationship between autophagy and metastasis of osteosarcoma. Previous studies testing the effects of autophagy inhibitors on osteosarcoma cells have demonstrated that targeting autophagy with spautin-1 (Beclin1 inhibitor) [35], geldanamycin (Hsp90 inhibitor) [36], and rapamycin (mTOR inhibitor) [24] inhibits the growth of osteosarcoma cells. Additionally, factors contributing to drug resistance in osteosarcomas such as HSP90AA1 and GFRA1 regulate resistance by promoting autophagy. Thus, enhanced autophagy is a negative signal for osteosarcoma therapy and a positive factor for osteosarcoma progression. Herein, we found that targeting autophagy by 3-MA could suppress tumor growth *in vivo*, although some limitations of this approach remain, including the following: (1) the method for tracking tumor size may be inaccurate due to the

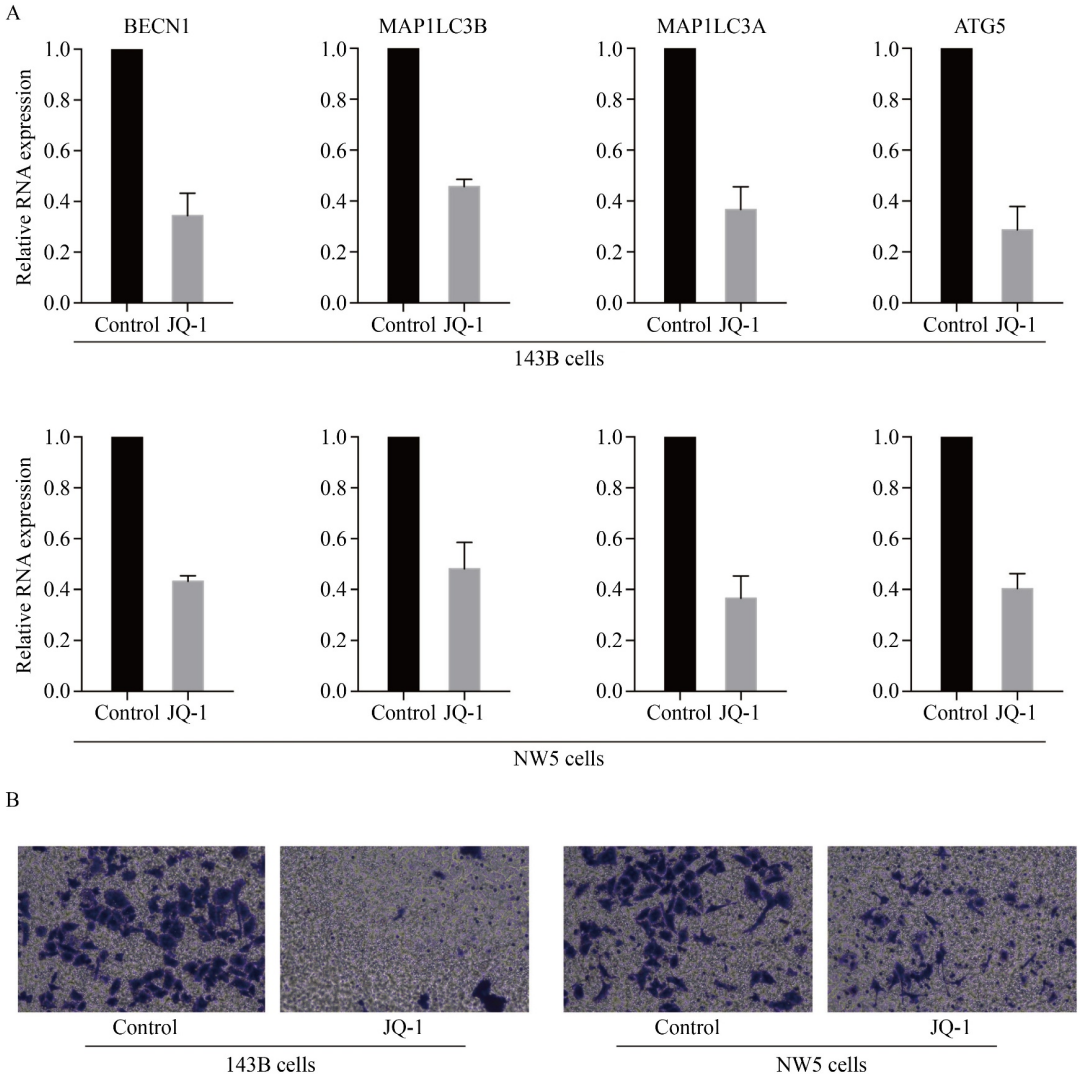

Fig. 7 Inhibition of SEs inhibited autophagy and metastasis in osteosarcoma. (A) JQ-1 inhibited the expression of Beclin1 (BECN1), MAP1LC3B (LC3B), MAP1LC3A (LC3A), and ATG5 in 143B and NW5 cells. (B) JQ-1 inhibited the metastasis of 143B and NW5 cells. 143 B and NW5 cells were treated with 1 μmol/L JQ-1 for 48 h, and the expression of BECN1, MAP1LC3B, and MAP1LC3A were examined by qRT-PCR experiments. GAPDH served as internal control. Cell invasion and metastasis were examined by the Transwell assays.

depth of the tumor site and the implications of nontumor tissues (such as skin, muscle, and bone), which should be improved in the future; and (2) the effect of 3-MA on metastasis must be explored. We further reported that targeting autophagy by 3-MA enhanced the effects of chemotherapy in metastatic osteosarcoma cells. Thus, targeting autophagy can also be effective in metastatic osteosarcoma.

Beclin1 is an autophagy-related gene homologous to ATG6 [10,37]. It is a subunit of the phosphatidylinositol-3-kinase complex and is responsible for the formation of phosphatidylinositol 3-phosphate [10]. It has been intensively studied as a key factor in autophagy and the development of tumors [38]. The induction of Beclin1 can reportedly inhibit tumorigenesis via autophagy [39]. Recently, higher levels of Beclin1 have been shown to contribute to drug resistance in malignant diseases through its regulation of autophagy. Studies on osteosarcoma have also demonstrated that increased levels of Beclin1 contribute to protective autophagy in response to paclitaxel-mediated chemotherapy [40]. Downregulation of Beclin1 has further been shown to increase the sensitivity of gemcitabine-induced cell death [41]. Lines of evidence have demonstrated that a high level of Beclin1 is correlated with favorable prognosis in breast [42] and gastric [43] cancers, whereas a higher level of LC3 is correlated with poorer prognosis in breast cancer [44]. Herein, we reported that Beclin1 and LC3 were highly expressed in osteosarcoma with poor prognosis, indicating that the functions of Beclin1 and LC3 could be versatile. Increased levels of Beclin1 and LC3 resulting in autophagy should be one of the key

factors determining the metastasis and poor prognosis of osteosarcoma.

Superenhancers are cis-regulatory elements and regulators of cell identity. They are characterized by abundant BRD4 and MED1 binding and H3K27ac signals. Lines of evidence have unveiled the contribution of superenhancer to malignant transformations. A superenhancer drives the high expression of oncogenes such as MYC [45–47], HOXB8 [48], and CCAT1 [49] in malignancies. Small chemical compounds targeting BRD4 such as JQ-1 can decrease BRD4 activity and are recognized as inhibitors of superenhancers [50,51]. Previous studies have reported that JQ-1 can suppress the proliferation of U2OS and 143B cells by inhibiting the MYC oncogene [52]. Herein, we reported that autophagy-related genes were regulated and activated by superenhancer, and that JQ-1 decreased the expression of these genes. Additionally, JQ-1 inhibited osteosarcoma metastasis. To date, targeting superenhancers in osteosarcoma is rarely reported. Accordingly, we suggested that the inhibition of superenhancers may decrease osteosarcoma metastasis by reducing the expression of autophagy-related gene.

Our study highlighted that autophagy contributed to metastasis and poor prognosis of osteosarcoma. Functional studies indicated that targeting autophagy may also be a potential approach to metastatic-osteosarcoma therapy, and more evidence is needed in future studies.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 81602069). We thank the National Research Center for Translational Medicine (Shanghai) for supporting this study.

Compliance with ethics guidelines

Hongyi Wang, Zhuochao Liu, Jun Wang, Fangqiong Hu, Qi Zhou, Li Wei, Qiyuan Bao, Jizhuang Wang, Jing Liang, Zhihong Liu, and Weibin Zhang declare that they have no conflicts of interest. This study was approved by the Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine and conducted according to the *Declaration of Helsinki* and its amendments. Informed consent was acquired.

Electronic Supplementary Material Supplementary material is available in the online version of this article at https://doi.org/10.1007/s11684-022-0919-0 and is accessible for authorized users.

References

1. Kelley LM, Schlegel M, Hecker-Nolting S, Kevric M, Haller B, Rössig C, Reichardt P, Kager L, Kühne T, Gosheger G, Windhager R, Specht K, Rechl H, Tunn PU, Baumhoer D, Wirth T, Werner M, von Kalle T, Nathrath M, Burdach S, Bielack S, von Lüttichau I. Pathological fracture and prognosis of high-grade osteosarcoma of the extremities: an analysis of 2,847 Consecutive Cooperative Osteosarcoma Study Group (COSS) patients. J Clin Oncol 2020; 38(8): 823–833
2. Duffaud F, Mir O, Boudou-Rouquette P, Piperno-Neumann S, Penel N, Bompas E, Delcambre C, Kalbacher E, Italiano A, Collard O, Chevreau C, Saada E, Isambert N, Delaye J, Schiffler C, Bouvier C, Vidal V, Chabaud S, Blay JY; French Sarcoma Group. Efficacy and safety of regorafenib in adult patients with metastatic osteosarcoma: a non-comparative, randomised, double-blind, placebo-controlled, phase 2 study. Lancet Oncol 2019; 20(1): 120–133
3. Zhao J, Dean DC, Hornicek FJ, Yu X, Duan Z. Emerging next-generation sequencing-based discoveries for targeted osteosarcoma therapy. Cancer Lett 2020; 474: 158–167
4. Anwar MA, El-Baba C, Elnaggar MH, Elkholy YO, Mottawea M, Johar D, Al Shehabi TS, Kobeissy F, Moussalem C, Massaad E, Omeis I, Darwiche N, Eid AH. Novel therapeutic strategies for spinal osteosarcomas. Semin Cancer Biol 2020; 64: 83–92
5. Heymann D. Metastatic osteosarcoma challenged by regorafenib. Lancet Oncol 2019; 20(1): 12–14
6. Italiano A, Mir O, Mathoulin-Pelissier S, Penel N, Piperno-Neumann S, Bompas E, Chevreau C, Duffaud F, Entz-Werlé N, Saada E, Ray-Coquard I, Lervat C, Gaspar N, Marec-Berard P, Pacquement H, Wright J, Toulmonde M, Bessede A, Crombe A, Kind M, Bellera C, Blay JY. Cabozantinib in patients with advanced Ewing sarcoma or osteosarcoma (CABONE): a multicentre, single-arm, phase 2 trial. Lancet Oncol 2020; 21(3): 446–455
7. Lamb CA, Yoshimori T, Tooze SA. The autophagosome: origins unknown, biogenesis complex. Nat Rev Mol Cell Biol 2013; 14(12): 759–774
8. Kim KH, Lee MS. Autophagy—a key player in cellular and body metabolism. Nat Rev Endocrinol 2014; 10(6): 322–337
9. Heras-Sandoval D, Pérez-Rojas JM, Hernández-Damián J, Pedraza-Chaverri J. The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. Cell Signal 2014; 26(12): 2694–2701
10. Kang R, Zeh HJ, Lotze MT, Tang D. The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ 2011; 18(4): 571–580
11. Janku F, McConkey DJ, Hong DS, Kurzrock R. Autophagy as a target for anticancer therapy. Nat Rev Clin Oncol 2011; 8(9): 528–539
12. Cicchini M, Karantza V, Xia B. Molecular pathways: autophagy in cancer—a matter of timing and context. Clin Cancer Res 2015; 21(3): 498–504
13. White E. Deconvoluting the context-dependent role for autophagy in cancer. Nat Rev Cancer 2012; 12(6): 401–410
14. Levy JMM, Towers CG, Thorburn A. Targeting autophagy in cancer. Nat Rev Cancer 2017; 17(9): 528–542
15. Galluzzi L, Bravo-San Pedro JM, Demaria S, Formenti SC, Kroemer G. Activating autophagy to potentiate immunogenic chemotherapy and radiation therapy. Nat Rev Clin Oncol 2017; 14(4): 247–258
16. Mathew R, Karantza-Wadsworth V, White E. Role of autophagy in

cancer. Nat Rev Cancer 2007; 7(12): 961–967
17. Li X, He S, Ma B. Autophagy and autophagy-related proteins in cancer. Mol Cancer 2020; 19(1): 12
18. Bursch W. The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ 2001; 8(6): 569–581
19. Chen S, Rehman SK, Zhang W, Wen A, Yao L, Zhang J. Autophagy is a therapeutic target in anticancer drug resistance. Biochim Biophys Acta 2010; 1806(2): 220–229
20. Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, Han W, Lou F, Yang J, Zhang Q, Wang X, He C, Pan H. Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis 2013; 4(10): e838
21. Dalby KN, Tekedereli I, Lopez-Berestein G, Ozpolat B. Targeting the prodeath and prosurvival functions of autophagy as novel therapeutic strategies in cancer. Autophagy 2010; 6(3): 322–329
22. Kim M, Jung JY, Choi S, Lee H, Morales LD, Koh JT, Kim SH, Choi YD, Choi C, Slaga TJ, Kim WJ, Kim DJ. GFRA1 promotes cisplatin-induced chemoresistance in osteosarcoma by inducing autophagy. Autophagy 2017; 13(1): 149–168
23. Huang J, Liu K, Yu Y, Xie M, Kang R, Vernon P, Cao L, Tang D, Ni J. Targeting HMGB1-mediated autophagy as a novel therapeutic strategy for osteosarcoma. Autophagy 2012; 8(2): 275–277
24. Horie R, Nakamura O, Yamagami Y, Mori M, Nishimura H, Fukuoka N, Yamamoto T. Apoptosis and antitumor effects induced by the combination of an mTOR inhibitor and an autophagy inhibitor in human osteosarcoma MG63 cells. Int J Oncol 2016; 48(1): 37–44
25. Liu K, Ren T, Huang Y, Sun K, Bao X, Wang S, Zheng B, Guo W. Apatinib promotes autophagy and apoptosis through VEGFR2/STAT3/BCL-2 signaling in osteosarcoma. Cell Death Dis 2017; 8(8): e3015
26. Lin C, He H, Liu H, Li R, Chen Y, Qi Y, Jiang Q, Chen L, Zhang P, Zhang H, Li H, Zhang W, Sun Y, Xu J. Tumour-associated macrophages-derived CXCL8 determines immune evasion through autonomous PD-L1 expression in gastric cancer. Gut 2019; 68(10): 1764–1773
27. Wang W, Wang X, Fujioka H, Hoppel C, Whone AL, Caldwell MA, Cullen PJ, Liu J, Zhu X. Parkinson's disease-associated mutant VPS35 causes mitochondrial dysfunction by recycling DLP1 complexes. Nat Med 2016; 22(1): 54–63
28. Ren T, Zheng B, Huang Y, Wang S, Bao X, Liu K, Guo W. Osteosarcoma cell intrinsic PD-L2 signals promote invasion and metastasis via the RhoA-ROCK-LIMK2 and autophagy pathways. Cell Death Dis 2019; 10(4): 261
29. Morrow JJ, Bayles I, Funnell APW, Miller TE, Saiakhova A, Lizardo MM, Bartels CF, Kapteijn MY, Hung S, Mendoza A, Dhillon G, Chee DR, Myers JT, Allen F, Gambarotti M, Righi A, DiFeo A, Rubin BP, Huang AY, Meltzer PS, Helman LJ, Picci P, Versteeg HH, Stamatoyannopoulos JA, Khanna C, Scacheri PC. Positively selected enhancer elements endow osteosarcoma cells with metastatic competence. Nat Med 2018; 24(2): 176–185
30. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012; 9(4): 357–359
31. Feng J, Liu T, Qin B, Zhang Y, Liu XS. Identifying ChIP-seq enrichment using MACS. Nat Protoc 2012; 7(9): 1728–1740
32. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 2010; 26(6): 841–842
33. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, Cheng JX, Murre C, Singh H, Glass CK. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 2010; 38(4): 576–589
34. Patil SL, Palat A, Pan Y, Rajapakshe K, Mirchandani R, Bondesson M, Yustein JT, Coarfa C, Gunaratne PH. MicroRNA-509-3p inhibits cellular migration, invasion, and proliferation, and sensitizes osteosarcoma to cisplatin. Sci Rep 2019; 9(1): 19089
35. Schott CR, Ludwig L, Mutsaers AJ, Foster RA, Wood GA. The autophagy inhibitor spautin-1, either alone or combined with doxorubicin, decreases cell survival and colony formation in canine appendicular osteosarcoma cells. PLoS One 2018; 13(10): e0206427
36. Mori M, Hitora T, Nakamura O, Yamagami Y, Horie R, Nishimura H, Yamamoto T. Hsp90 inhibitor induces autophagy and apoptosis in osteosarcoma cells. Int J Oncol 2015; 46(1): 47–54
37. Salminen A, Kaarniranta K, Kauppinen A, Ojala J, Haapasalo A, Soininen H, Hiltunen M. Impaired autophagy and APP processing in Alzheimer's disease: the potential role of Beclin 1 interactome. Prog Neurobiol 2013; 106-107: 33–54
38. Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 2003; 112(12): 1809–1820
39. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999; 402(6762): 672–676
40. Guo Y, Huang C, Li G, Chen T, Li J, Huang Z. Paxilitaxel induces apoptosis accompanied by protective autophagy in osteosarcoma cells through hypoxia-inducible factor-1α pathway. Mol Med Rep 2015; 12(3): 3681–3687
41. Tao H, Chen F, Liu H, Hu Y, Wang Y, Li H. Wnt/β-catenin signaling pathway activation reverses gemcitabine resistance by attenuating Beclin1-mediated autophagy in the MG63 human osteosarcoma cell line. Mol Med Rep 2017; 16(2): 1701–1706
42. Dong M, Wan XB, Yuan ZY, Wei L, Fan XJ, Wang TT, Lv YC, Li X, Chen ZH, Chen J, Lin Q, Wen JY, Ma XK, Liu Q, Wu XY. Low expression of Beclin 1 and elevated expression of HIF-1α refine distant metastasis risk and predict poor prognosis of ER-positive, HER2-negative breast cancer. Med Oncol 2013; 30(1): 355
43. Chen YB, Hou JH, Feng XY, Chen S, Zhou ZW, Zhang XS, Cai MY. Decreased expression of Beclin 1 correlates with a metastatic phenotypic feature and adverse prognosis of gastric carcinomas. J Surg Oncol 2012; 105(6): 542–547
44. Zhao H, Yang M, Zhao J, Wang J, Zhang Y, Zhang Q. High expression of LC3B is associated with progression and poor outcome in triple-negative breast cancer. Med Oncol 2013; 30(1): 475
45. Scholz BA, Sumida N, de Lima CDM, Chachoua I, Martino M, Tzelepis I, Nikoshkov A, Zhao H, Mehmood R, Sifakis EG, Bhartiya D, Göndör A, Ohlsson R. WNT signaling and AHCTF1 promote oncogenic MYC expression through super-enhancer-mediated gene gating. Nat Genet 2019; 51(12): 1723–1731
46. Bahr C, von Paleske L, Uslu VV, Remeseiro S, Takayama N, Ng SW, Murison A, Langenfeld K, Petretich M, Scognamiglio R, Zeisberger P, Benk AS, Amit I, Zandstra PW, Lupien M, Dick JE,

Trumpp A, Spitz F. A Myc enhancer cluster regulates normal and leukaemic haematopoietic stem cell hierarchies. Nature 2018; 553(7689): 515–520

47. Chipumuro E, Marco E, Christensen CL, Kwiatkowski N, Zhang T, Hatheway CM, Abraham BJ, Sharma B, Yeung C, Altabef A, Perez-Atayde A, Wong KK, Yuan GC, Gray NS, Young RA, George RE. CDK7 inhibition suppresses super-enhancer-linked oncogenic transcription in MYCN-driven cancer. Cell 2014; 159(5): 1126–1139
48. Ying Y, Wang Y, Huang X, Sun Y, Zhang J, Li M, Zeng J, Wang M, Xiao W, Zhong L, Xu B, Li L, Tao Q, Wang X, Shu XS. Oncogenic HOXB8 is driven by MYC-regulated super-enhancer and potentiates colorectal cancer invasiveness via BACH1. Oncogene 2020; 39(5): 1004–1017
49. Jiang Y, Jiang YY, Xie JJ, Mayakonda A, Hazawa M, Chen L, Xiao JF, Li CQ, Huang ML, Ding LW, Sun QY, Xu L, Kanojia D, Jeitany M, Deng JW, Liao LD, Soukiasian HJ, Berman BP, Hao JJ, Xu LY, Li EM, Wang MR, Bi XG, Lin DC, Koeffler HP. Co-activation of super-enhancer-driven CCAT1 by TP63 and SOX2 promotes squamous cancer progression. Nat Commun 2018; 9(1): 3619
50. Borck PC, Guo LW, Plutzky J. BET epigenetic reader proteins in cardiovascular transcriptional programs. Circ Res 2020; 126(9): 1190–1208
51. Chapuy B, McKeown MR, Lin CY, Monti S, Roemer MG, Qi J, Rahl PB, Sun HH, Yeda KT, Doench JG, Reichert E, Kung AL, Rodig SJ, Young RA, Shipp MA, Bradner JE. Discovery and characterization of super-enhancer-associated dependencies in diffuse large B cell lymphoma. Cancer Cell 2013; 24(6): 777–790
52. Chen D, Zhao Z, Huang Z, Chen DC, Zhu XX, Wang YZ, Yan YW, Tang S, Madhavan S, Ni W, Huang ZP, Li W, Ji W, Shen H, Lin S, Jiang YZ. Super enhancer inhibitors suppress MYC driven transcriptional amplification and tumor progression in osteosarcoma. Bone Res 2018; 6(1): 11