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Original Article

BIRC7 promotes epithelial-mesenchymal transition and metastasis in papillary thyroid carcinoma through restraining autophagy

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Abstract: Papillary thyroid carcinoma (PTC) is the most common cancer of the endocrine system, which is usually associated with a favorable therapeutic response and prognosis. However, metastatic spreading occurs in around 5% of the PTC patients. Identification of molecular markers could early predict the metastatic potential, which is essential for reducing the patient's overtreatment. Baculoviral IAP Repeat Containing 7 (BIRC7) is an inhibitor of apoptosis protein (IAP) family gene that is known to be linked to tumor progression, but its role in the setting of PTC metastasis remains unknown. This study, therefore, aims to explore the role of BIRC7 in the metastasis and autophagy of PTC and elucidate its underlying molecular mechanisms. BIRC7 expression was assessed in fresh samples of human PTC and normal tissues via qRT-PCR and immunohistochemistry. In addition, BIRC7 was overexpressed and silenced in PTC cell lines followed by transmission electron microscopy, western blotting, immunofluorescence microscopy, wound healing and invasion assays. We further explored the relevance of BIRC7 *in vivo* using a tumor xenograft model. Our results demonstrated that BIRC7 plays a pro-invasive role in PTC. BIRC7 expression is significantly upregulated in PTC compared with matched thyroid normal tissues. In addition, we found that BIRC7 knockdown induced a significant reduction in PTC cell EMT and metastasis *in vitro* and *in vivo*, while overexpression of BIRC7 markedly enhanced PTC cell migration and invasion. Moreover, our data showed that BIRC7 was able to suppress autophagy through modulating the expression of ATG5 and BECN1, and that this suppression is responsible for BIRC7 silence induced suppression of EMT and metastasis of PTC cell. We further found that targeting both BIRC7 and mTOR enhances autophagy in PTC cells and to achieve synergistic antimetastatic efficacy *in vitro* and *in vivo*. These findings indicate that the suppression of autophagy by BIRC7 drives the invasion and metastasis of PTC cells, thus suggesting that the activation of autophagy may inhibit metastasis of PTC with high BIRC7 expression.

Keywords: BIRC7, papillary thyroid carcinoma, autophagy, EMT, metastasis

Introduction

Papillary thyroid carcinomas (PTC) is the most common form of endocrine malignancy, making up the most common malignancies derived from thyrocytes [1-3]. After the effective and reasonable treatment, the 5-year survival rate of PTC is over 90% [4]. The previous study showed that PTC manifested regional cervical lymphatic metastases in as many as 30% to 80% of cases at initial diagnosis [5, 6]. However, around 15% of cases with lymph node metastasis manifest aggressive behavior, characterized by local invasion, distant metastasis, treatment resistance, and increased recurrence rate and mortality [7]. Moreover, PTC can become highly invasive and can readily de-differentiate, thereby leading to the development of poorly- or undifferentiated thyroid cancer, which has a much lower survival rate. Identifying markers that are characteristic of such aggressive forms of PTC is vital, as a better understanding of what drives highly metastatic PTC will have important therapeutic implications.

Inhibitor of apoptosis protein (IAP) is often highlighted as possible targets of therapeutic treatment in cancer, with certain IAP inhibitors having been tested in preclinical settings [8-11]. Baculoviral IAP Repeat Containing 7 (BIRC7) is

a recently discovered IAP family member that is largely absent in normal tissues, whereas it is expressed at high levels in a range of tumor types [12]. BIRC7 is known to be closely related to apoptosis, and overexpression of BIRC7 in tumors has been linked with increased chemo- and radio-resistance, recurrence, and decreased patient survival [12, 13]. Therefore, BIRC7 is strongly believed to be a potential new target in cancer therapy. However, the impact of tumor microenvironment on BIRC7 expression and its regulatory mechanisms in PTC remains unclear. Metastasis relies upon the progression through a series of steps wherein tumors acquire invasive properties allowing them to readily survive in an extra-tumoral environment. The early stages of this process involve cancer cells progressing through the epithelial-to-mesenchymal transition (EMT), which is a process wherein epithelial cells lose their characteristic morphological and functional properties so as to more closely resemble mesenchymal cells, with altered adhesive properties and motility [14-16]. This thereby confers increased migratory potential to tumor cells, thereby increasing their ability to invade surrounding tissues [17]. Nevertheless, it is unknown whether BIRC7 can promote EMT and metastasis in PTC.

Autophagy is a conserved process and mechanism wherein cellular organelles and proteins are metabolically degraded, which is essential for regulating cell growth and homeostasis [18]. Autophagy has been implicated in cancer progression, both with a tumor-promoting or tumor-restraining function. It can also impact tumor cell phenotypes to modulate cellular migration and invasion [17, 19, 20]. Silencing of BIRC7 has been shown to increase autophagic activity, thereby sensitizing colon cancer and renal carcinoma cells to chemotherapy [21, 22]. However, the role and mechanism of BIRC7 in regulating autophagy during PTC metastasis are completely unclear.

In the present report, we examined the functional relevance of BIRC7-associated autophagy in PTC, with a focus on EMT and tumor metastasis. Our results revealed that the expression of BIRC7 was substantially elevated in primary PTC tissues and that downregulation of BIRC7 was sufficient to induce autophagy, and thereby interfere with EMT and metastatic progression. Our data demonstrate for the first time that BIRC7-suppressed autophagy promotes tumor cell migration, invasion and metastasis in the PTC.

Materials and methods

Cell lines and cell culture

PTC cell lines (BCPAP, TPC-1, K1 and IHH4) were from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, Shanghai Institute of Cell Biology (Shanghai, China). Human anaplastic thyroid carcinoma (ATC) cell line 8505C was kindly provided by Dr. Qingan Yu (The First Affiliated Hospital of Harbin Medical University, Harbin, China). Human normal thyroid epithelial cell line Nthy-ori-3-1 were purchased from the European Collection of Cell Culture (ECACC, Salisbury, UK). BCPAP, IHH4, 8505C, K1 and TPC-1 cells were grown in RPMI-1640 (Thermo, NH, USA) containing 10% FBS (Thermo, NH, USA), whereas Nthy-ori-3-1 were grown in F12K medium (Thermo, NH, USA) containing 10% FBS. In addition, all cell media was supplemented with penicillin (100 U/mL) and streptomycin (100 U/mL) at 37°C in a 5% CO_2 incubator.

Patients and tissue specimens

A total of 48 pairs of PTC tumor samples and corresponding adjacent normal tissue were collected from Department of Thyroid Surgery, the First Affiliated Hospital Harbin Medical University (China) between October 2017 and June 2018. Of the 48 PTC patients, 30 had PTC tissue with lymph node metastasis and 18 had PTC without lymph node metastasis. Samples were snap-frozen and stored at -80°C until use in qRT-PCR experiments. In addition, we conducted immunohistochemical staining of formalin-fixed paraffin-embedded PTC patient samples and normal control samples. Two endocrine pathologists diagnosed all patient samples, and only representative tumor sections were utilized in analyses. The Research Ethics Committee of the First Affiliated Hospital Harbin Medical University approved this study, which was consistent with the Declaration of Helsinki. All patients provided written informed consent.

Reagents

Antibodies specific for P62 (ab207305) and BECN1 (ab62557) were from Abcam, while those specific for BIRC7 (#5471), ATG5 (#2630), LC3 (#4108), E-cadherin (#3195), N-cadherin (#13116), Vimentin (#5741), and Snail (#3879) were from Cell Signaling Technology (CST). Anti-

β-actin and anti-GAPDH were from Bosterbio. Rapamycin and 3-Methyladenine were from Sigma-Aldric, while ATG5 and BECN1-specific siRNAs were from Santa Cruz, as was a control siRNA construct.

qRT-PCR

TRIzol (Invitrogen) was used to isolate total RNA based on provided directions, with agarose gel electrophoresis used to assess RNA integrity. cDNA was synthesized from 1 μg total RNA via the Superscript III First-Strand Synthesis System (Toyobo, Osaka, Japan) in a 10 μL volume with the following thermocycler settings: 37°C for 15 min, 50°C for 5 min, and 98°C for 5 min. The Fast SYBR Green Master Mix was used for qRT-PCR (Applied Biosystems Inc., CA, USA), with β-actin utilized to normalize data. The BIRC7 primers were (forward) 5'-GGTGAGGTGCTTCTTCTGCTATGG-3' and (reverse) 5'-GCTGCGTCTTCCGGTTCTTCC-3'. The β-actin primers were (forward) 5'-CATGTACGTTGCTATCCAGGC-3' and (reverse) 5'-CTCCTTAATGTCACGCACGAT-3'.

Wound-healing and invasion assays

Cell migration was assessed using the wound healing assay. For these experiments, cells were grown until 80-90% confluent in 6-well plates, at which time a 10 uL sterile pipette tip was used to create a scratch wound in the cell monolayer. Media containing 2% FBS was then added to cells for 24 h, after which a phase-contrast microscope (Leica) was used to image cells. To assess cell invasion, 8 μm pore size inserts were used for invasion assay, with the upper surface of the membrane being coated using Matrigel (BD Biosciences, CA, USA) based on provided directions. After coating, 2×10^5 PTC cells were added to the upper chamber for 24 h at 37°C, and then those cells that had not invaded were removed using a cotton swab. Methanol was used to fix invasive cells at the bottom of the Matrigel, followed by leucocrystal violet staining and counting of cells via microscopy.

Immunohistochemistry (IHC)

BIRC7 was analyzed via IHC as in previous reports [23]. For IHC staining, 4-μm-thick sections were mounted onto glass slides, dewaxed with xylene, dehydrated using a gradient of ethanol concentrations, and treated for 10 min with 3% hydrogen peroxide. Slides were then warmed for 10 min to 95°C in citrate buffer (pH = 6.0) for antigen retrieval, and 10% goat serum albumin was used to block sections at room temperature for 2 h with gentle shaking. Samples were then probed overnight with anti-BIRC7 at 4°C overnight in a humid environment, washed thrice using PBS, probed for 1 h at room temperature with an appropriate secondary antibody, and then developed using diaminobenzidine (DAB) as a chromogen. Hematoxylin was used to counterstain sections. As a control, samples were treated with PBS instead of the primary antibody. Brown regions within the nucleus or cytoplasm were considered to be positive signals.

Western blotting

RIPA cell lysis reagent containing proteinase and phosphatase inhibitors (Solarbio, Beijing, China) was used to isolate protein samples, with the BCA assay used for protein quantification. Protein was then separated via SDS-PAGE, transferred to a PVDF membrane (Millpore, MA, USA), and probed overnight with primary antibodies at 4°C, followed by a 1 h room temperature incubation with HRP-conjugated secondary antibodies (Pierce, PA, USA). Enhanced chemiluminescence (Millpore, MA, USA) was then used to visualize protein, with the Molecular Imager System (BIO-RAD, Hercules, USA) used for image capture. β-Actin or GAPDH served as loading controls.

Transmission electron microscopy (TEM)

For the TEM studies, the PTC cells were fixed with 2.5% glutaraldehyde followed by postfixed in1% osmium tetroxide buffer for 2-3 h, washed three times with PBS, dehydrated, embedded in paraffin, cut into 70-nm-thick sections using an Ultrathin slicing machine, and stained with uranyl acetate-lead citrate. The cells were then observed using a transmission electron microscope (JEM1230, JEOL Ltd., Tokyo, Japan) for the detection of autophagic vacuoles.

In vivo models of metastasis

Nude male BALB/c mice (nu/nu; 4 weeks old) were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animal experiments were conducted in accordance with standard protocols of the Institutional Animal Care and Use Committee of Harbin Medical

University. Animals received a tail vein injection of 1×10^6 tumor cells per animal. Mice were treated i.p. daily × 5 days for 5 consecutive weeks with 5 mg/kg rapamycin or vehicle alone. Mice were euthanized at 5 weeks post-implantation, lungs were dissected and stored in liquid nitrogen or fixed in formalin for further analysis, and hematoxylin and eosin (HE) stained for metastatic nodules.

Autophagic flux assay

The mRFP-GFP-LC3B reporter (Biosmedi Co., Shanghai, China) was used to assess autophagic flux via plating 2×10^4 cells overnight into Lab-TekII Chamber Slides (Nalge Nunc). Cells were then treated as appropriate, fixed using 4% paraformaldehyde, mounted using SlowFade Gold Reagent (Thermo, NH, USA), and imaged via a C1 confocal microscope with a Plan APO 60X/1.45 oil immersion objective (Nikon). EZ-C1 v3.00 (Nikon) was used for image acquisition, with Photoshop CS4 (Adobe) used to adjust whole-image contrast and brightness. A Zeiss Axioplan 2 microscope was used to capture images for quantifying red and green punctae numbers with the Axio Vision v4.8.2.0 software, with red puncta being subtracted from green manually to yield a net result.

BIRC7 knockdown and overexpression

We purchased lentiviral vectors that encoding BIRC7 cDNA and vectors expressed either control shRNA or shRNA specific for ATG5 or BIRC7 from Biosmedi Co. (Shanghai, China), and confirmed their identities via sequencing. The BIRC7 shRNA sequence was 5'-GAAGAGACTTTGTCCACAGTGTGCA-3', while that for ATG5 was 5'-CTTGTTTCACGCTATATCA-3'. Western blotting was used to validate knockdown and overexpression. PTC cells were infected with these lentiviruses, and then subjected to puromycin selection for 14 days.

Statistical analysis

Data are means ± SEM, with GraphPad Prism 6.0 used for statistical analyses. results were compared via unpaired t-tests (two-tailed), ANOVAs, or repeated-measures ANOVAs with Bonferroni post hoc tests for multiple comparisons. $P<0.05$ was the significance threshold.

Results

PTC tissue samples and PTC cell lines exhibit elevated BIRC7 expression

We first assessed BIRC7 expression with immunochemistry in samples from primary PTC patients (n = 48), revealing significantly increased BIRC7 expression in PTC tissues relative to paired control tissues (**Figure 1A**). Consistent with this, BIRC7 expression was also elevated at the mRNA level in PTC tissues relative to controls (**Figure 1B**), and western blotting confirmed higher protein expression in PTC tissues (**Figure 1D**). In addition, we found that higher BIRC7 expression was associated with increased lymph node metastasis (**Figure 1C**). We further examined BIRC7 expression in the BCPAP, TPC-1, K1, and IHH4 PTC cell lines and anaplastic thyroid carcinoma (ATC) cell line 8505C as well as normal thyroid epithelial cell line Nthy-ori-3-1. The results revealed that there is no detectable BIRC7 expression in Nthy-ori-3-1 cells, whereas other lines exhibit significantly higher expression (**Figure 1E**). These results indicated that BIRC7 was barely expressed in normal tissues but increased in PTC tissue, and implies that BIRC7 participate in the PTC progression.

BIRC7 enhances in vitro PTC cells invasion and migration

To directly assess the ability of BIRC7 to influence the metastasis of PTC cells, we transfected BCPAP and K1 cell lines with BIRC7-overexpressing constructs and shRNA to knockdown BIRC7 (OE and KD, respectively). Western blotting confirmed the successful modulation of BIRC7 expression levels in these cells (**Figure 2A**). We then used these cells for *in vitro* analyses of PTC cell migration and invasion, revealing that this invasive and migratory activity was significantly increased in cells overexpressing BIRC7, and was markedly decreased upon BIRC7 knockdown with matrigel invasion assay (**Figure 2B**) and wound-healing assay (**Figure 2C**). These results thus indicate a direct role for BIRC7 in promoting the migratory and invasive behaviors of PTC cells.

BIRC7 suppresses PTC cell autophagy

BIRC7 has previously been shown to regulate chemotherapeutic sensitivity via modulating autophagy [21]. The specific role of BIRC7 in

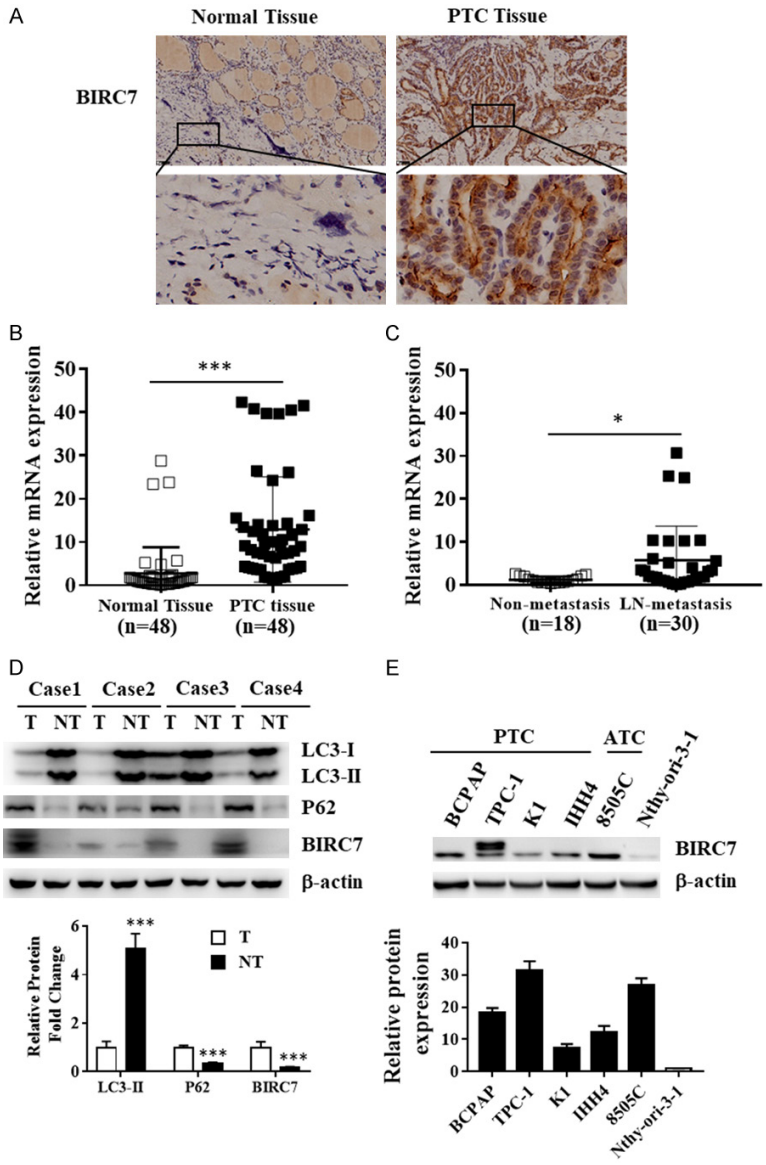


Figure 1. BIRC7 expression is increased in PTC. A. Assessment of BIRC7 levels via IHC in paired normal and PTC tissues. B. Assessment of BIRC7 expression via qRT-PCR in 48 pairs of normal and PTC patient samples. C. Relative expression of BIRC7 in non-metastasis group (n = 18) and lymph node metastasis group (n = 30). D. Elevated BIRC7 and P62 protein levels as well as decreased LC3-II level in PTC tissue samples (T) relative to normal tissue (NT). E. Assessment of BIRC7 protein levels in PTC cell lines and in a normal thyroid epithelial cell line (Nthy-ori 3-1). Data are means ± S.E.M. $^{***}P<0.001$.

regulating PTC cell autophagy, however, has not been assessed, particularly in the metastatic progression of PTC. The western blotting analysis showed that there are reduced levels of LC3II and increased P62 levels in PTC tissues (**Figure 1C**). We, therefore, assessed the autophagic activity of PTC cells via western blotting in BCPAP cells in which BIRC7 was overexpressed or silenced. Our data show that BIRC7 knockdown led to increased levels of LC3II and reduced P62 levels while overexpressing BIRC7 had the opposite effects (**Figure 3A**), indicating that BIRC7 significantly suppressed autophagy in PTC cells.

We further sought to confirm these findings via the transfection of the adenoviral mRFP-GFP-LC3 reporter vector into BIRC7 KD and BIRC7 OE BCPAP cells. BIRC7 knockdown showed increased RFP-positive red puncta compared with control cells, indicating that BIRC7 knockdown induced autophagic flux, while BIRC7 OE inhibited autophagic activity (**Figure 3B**). Additionally, as observed by TEM, autophagosome formation was significantly induced in the BIRC7 KD BCPAP cells, whereas the overexpression of BIRC7 expression in BCPAP cells resulted in a decreased formation of autophagosomes (**Figure 3C**). Together, these findings thus strongly indicate a role for BIRC7 in suppressing autophagy in PTC cells.

BIRC7 regulates autophagy in PTC cells through a pathway dependent upon BECN1 and ATG5

One main autophagic pathway is known to be BECN1- and ATG5-dependent, and so we sought to examine the relevance of BIRC7 to this pathway via assessing changes in ATG5 and BECN1 expression in BIRC7 KD or OE cells. We found that knocking down BIRC7 increased BECN1 and ATG5 expression, whereas overexpressing it had the opposite effect (**Figure 4A**). We then employed siRNAs specific for BECN1 or ATG5 (**Figure 4A**) and transfected these into BIRC7 KD cells prior

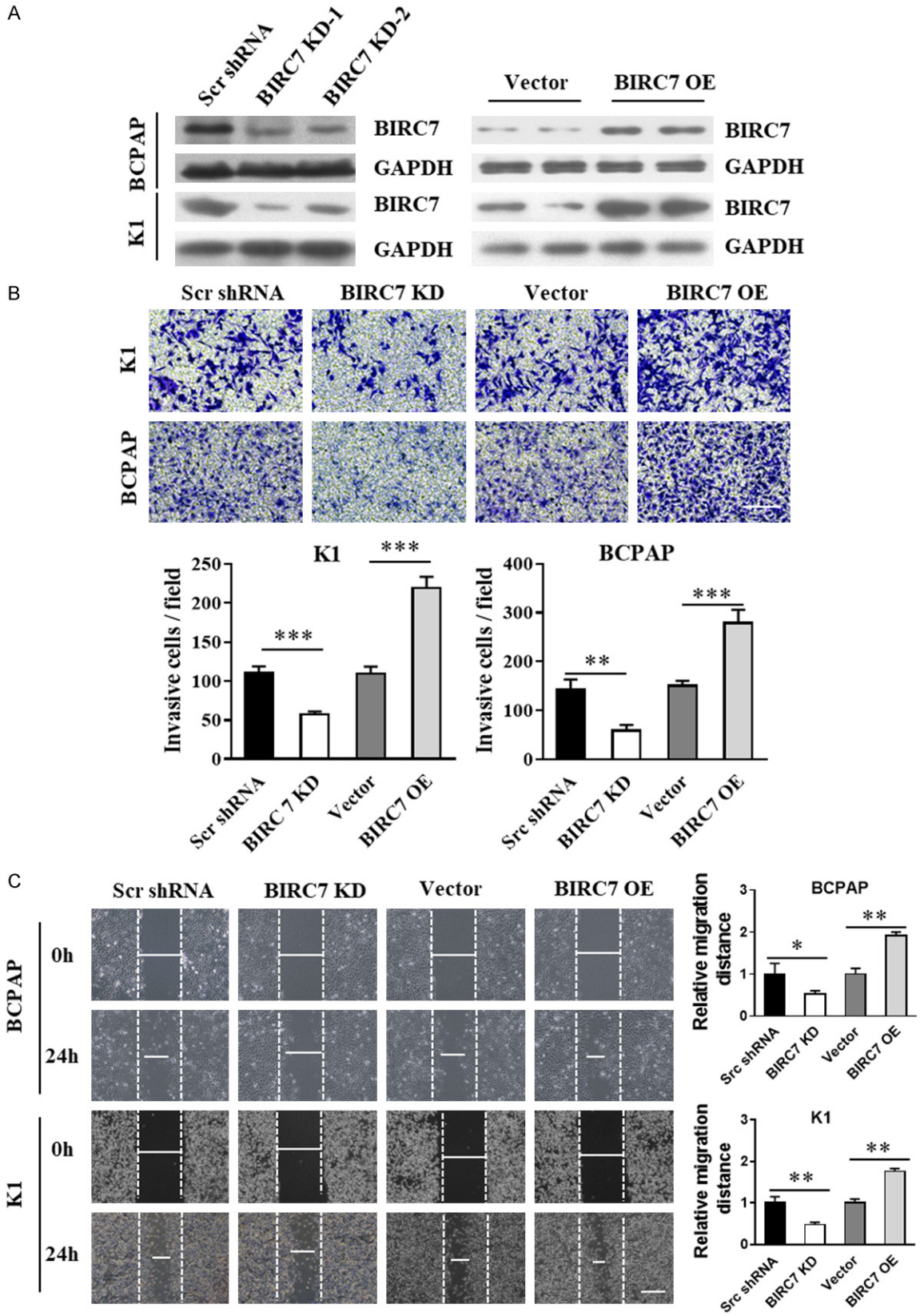


Figure 2. BIRC7 induces migratory and invasive activity in PTC cells. A. BIRC7 knockdown and overexpression as confirmed via western blotting relative to control cells. B. BIRC7 KD and OE cells and appropriate controls were used in a matrigel invasion assay. Scale bar = 100 μm. C. BIRC7 KD and OE cells and appropriate controls were used in a wound-healing assay. Scale bar = 100 μm. (n = 3 each). Data are means ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

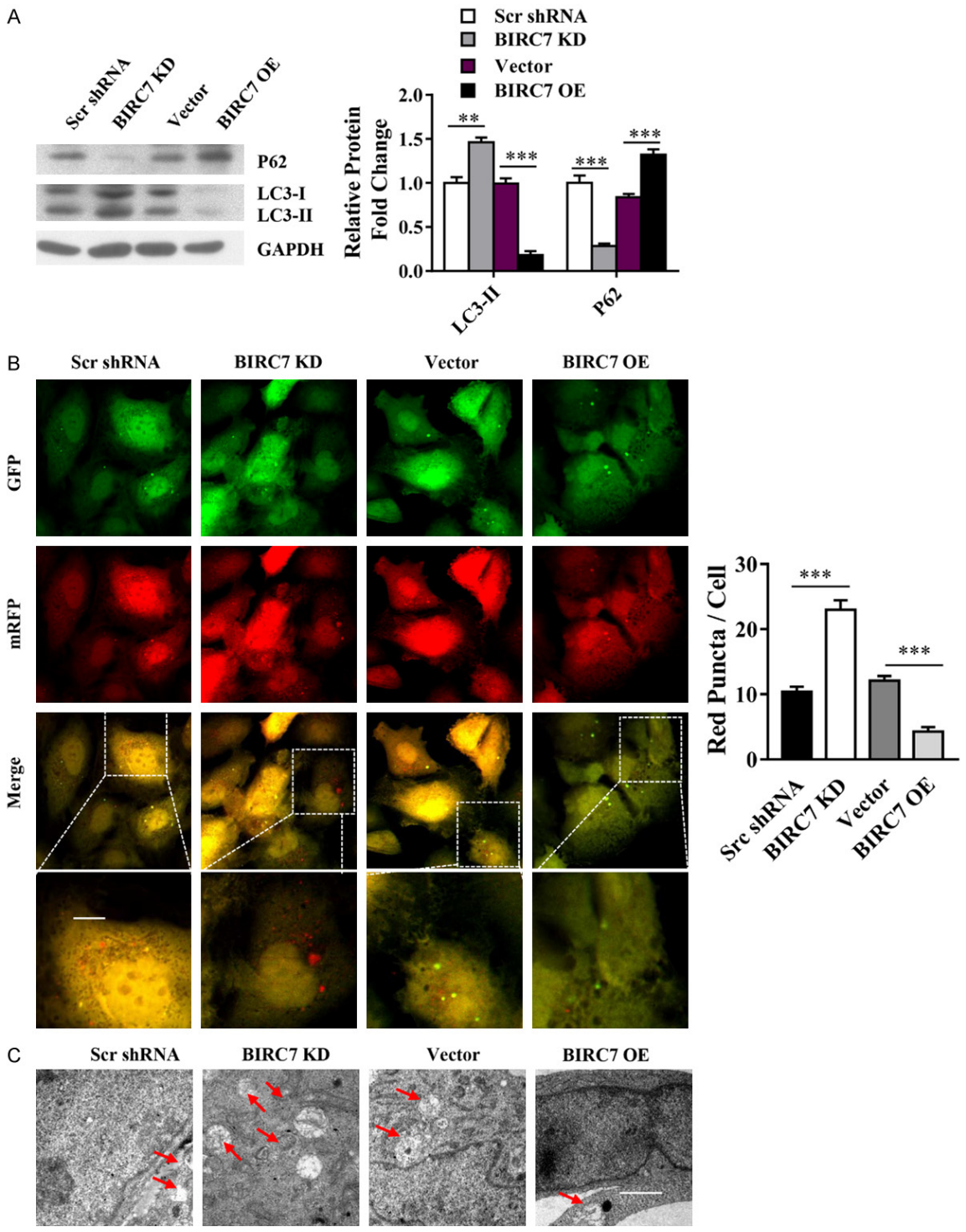


Figure 3. BIRC7 suppresses autophagy in PTC cells. A. Levels of P62 and LC3II were assessed by western blotting in BCPAP BIRC7 KD or OE cells. B. BCPAP cells expressing the mRFP-GFP-LC3 adenoviral reporter vector were used in immunofluorescence analyses, with RFP-only puncta numbers quantified and graphed for 3 independent replicate experiments (Scale bar = 10 μm; n = 3 each). C. Electron microscopy showed the different autophagic activation in BCPAP BIRC7 KD or OE cells. Scale bar = 10 μm. Data are means ± S.E.M. $^{**}P<0.01$, $^{***}P<0.001$.

to assessing LC3II and LC3 puncta levels in these cells. We found that downregulating either ATG5 or BECN1 led to a significant reduction in LC3II levels and LC3 puncta relative to cells

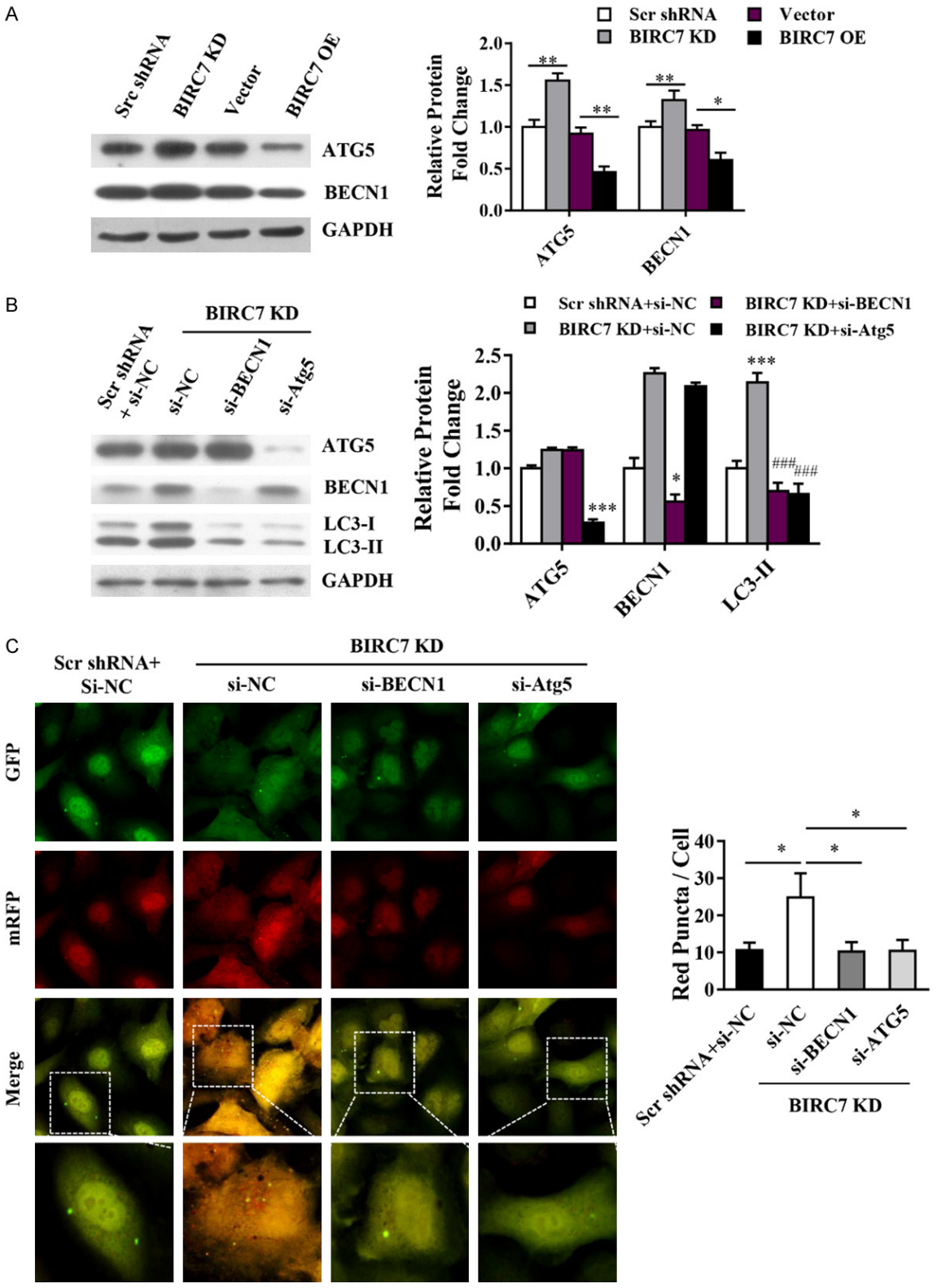


Figure 4. BIRC7 controls the autophagy of PTC cells via BECN1 and ATG5. A. Measurement of BECN1 and ATG5 expression in BIRC7 KD and OE BCPAP cells as assessed via western blotting. B. Measurement of LC3II protein levels in BIRC7 KD or control shRNA cells in which siRNA specific for ATG5 or BECN1 were transfected, as measured by western blotting. $^{*}P<0.05$, $^{***}P<0.001$ vs. Scr shRNA + si-NC group. $^{\#\#\#}P<0.001$ vs. BIRC7 KD + si-NC group. C. BIRC7 KD or control BCPAP cells expressing the mRFP-GFP-LC3 adenoviral reporter vector and transfected with

siRNAs specific for ATG5 or BECN1 were used in immunofluorescence analyses, with RFP-only puncta numbers quantified and graphed for 3 independent replicate experiments (Scale bar = 10 μm; n = 3 each). Data are means ± S.E.M. $^{*}P<0.05$.

transfected with a control siRNA (**Figure 4B** and **4C**). This thus suggested that BIRC7 knockdown leads to the induction of autophagy through a mechanism that depends upon BECN1 and ATG5.

BECN1 and ATG5 impact the ability of BIRC7 to control PTC cell migration and invasion

Autophagic processes in cancer cells are known to play context-dependent functions that can promote or inhibit metastasis. Our results showed that BIRC7 inhibited PTC cell autophagy and promoted PTC cell migration, invasion, and metastasis, suggesting that BIRC7-suppressed autophagy is associated with its metastasis promotion in PTC cells. We next directly assessed this putative link in BIRC7 KD cells in which ATG5 or BECN1 was knocked down with specific siRNA. We found that knocking down both ATG5 as well as BECN1 was associated with a marked increase in BIRC7 KD cell migration and invasion, whereas this knockdown did not substantially alter these parameters in control cells (**Figure 5A**, **5B**). We further found that treated cells using 3-methyladenine (3-MA), which is a class III phosphatidylinositol 3-kinase (PtdIns3K) inhibitor, led to enhanced BIRC7 KD but not control cell invasion and migration (**Figure 5C** and **5D**). Those data suggested that the ability of BIRC7 to suppress autophagy is responsible for the enhanced migration and invasion of PTC cells *in vitro*.

BIRC7-mediated suppression of autophagy induces EMT-like alterations of PTC cells

The epithelial-mesenchymal transition (EMT) is a key step in tumor metastatic progression. We, therefore, explored the relevance of BIRC7 expression to PTC cells EMT via western blot, revealing that knocking down BIRC7 led to decreased expression of markers of mesenchymal cells (Vimentin and N-cadherin) as well as dramatically downregulated Snail expression, and increased expression of E-cadherin, which marks epithelial cells, whereas overexpressing BIRC7 had the opposite effect (**Figure 6A**). This suggested that BIRC7 induces EMT-like alterations in the PTC cells.

We further assessed the expression of EMT marker levels in BIRC7 KD and control cells in which ATG5 or BECN1 was silenced or which had been treated with 3-MA, revealing that inhibiting autophagy was associated with decreased E-cadherin levels and increased levels of Snail, Vimentin and N-cadherin in BIRC7 KD cells, while only a slight impact on these same markers in control cells (**Figure 6B**, **6C**). This thus indicated that the ability of BIRC7 to suppress autophagy directly leads to EMT-like changes in PTC cells.

Inhibiting both BIRC7 and mTOR leads to enhanced autophagy and anti-tumor activity in PTC

Our results demonstrated that BIRC7 inhibition-induced autophagy impaired cell invasion. However, whether co-targeting BIRC7 and autophagy could lead to a more deleterious effect on PTC cell migration and invasion needs to be further investigated. As the mammalian target of rapamycin (mTOR) is known to be a key regulator of autophagy, we chose to use rapamycin treatment in order to induce autophagy in treated cells. Using the mRFP-GFP-LC3 reporter, we assessed the effects of combined BIRC7 knockdown and rapamycin treatment on PTC cells, revealing significantly and synergistically increased autophagic flux in co-treated cells (**Figure 7A**), as confirmed based upon LC3-I/LC3-II conversion (**Figure 7D**). We then explored the effect of this combination treatment on PTC cell migration and invasion, revealing that whereas rapamycin or BIRC7 knockdown alone had a limited impact on these activities, the effect was more pronounced following combination treatment (**Figure 7B**, **7C**). In addition, combination treatment was linked to significantly higher levels of E-cadherin and decreased N-cadherin, Snail, and Vimentin relative to either treatment alone (**Figure 7D**). Using a model of experimental lung metastasis, we found that the nodule formation was significantly reduced upon the combined treatment compared with BIRC7 knockdown alone (**Figure 8A**). Together, these findings thus indicate that simultaneous targeting of both BIRC7 and mTOR offers a means of significantly enhancing

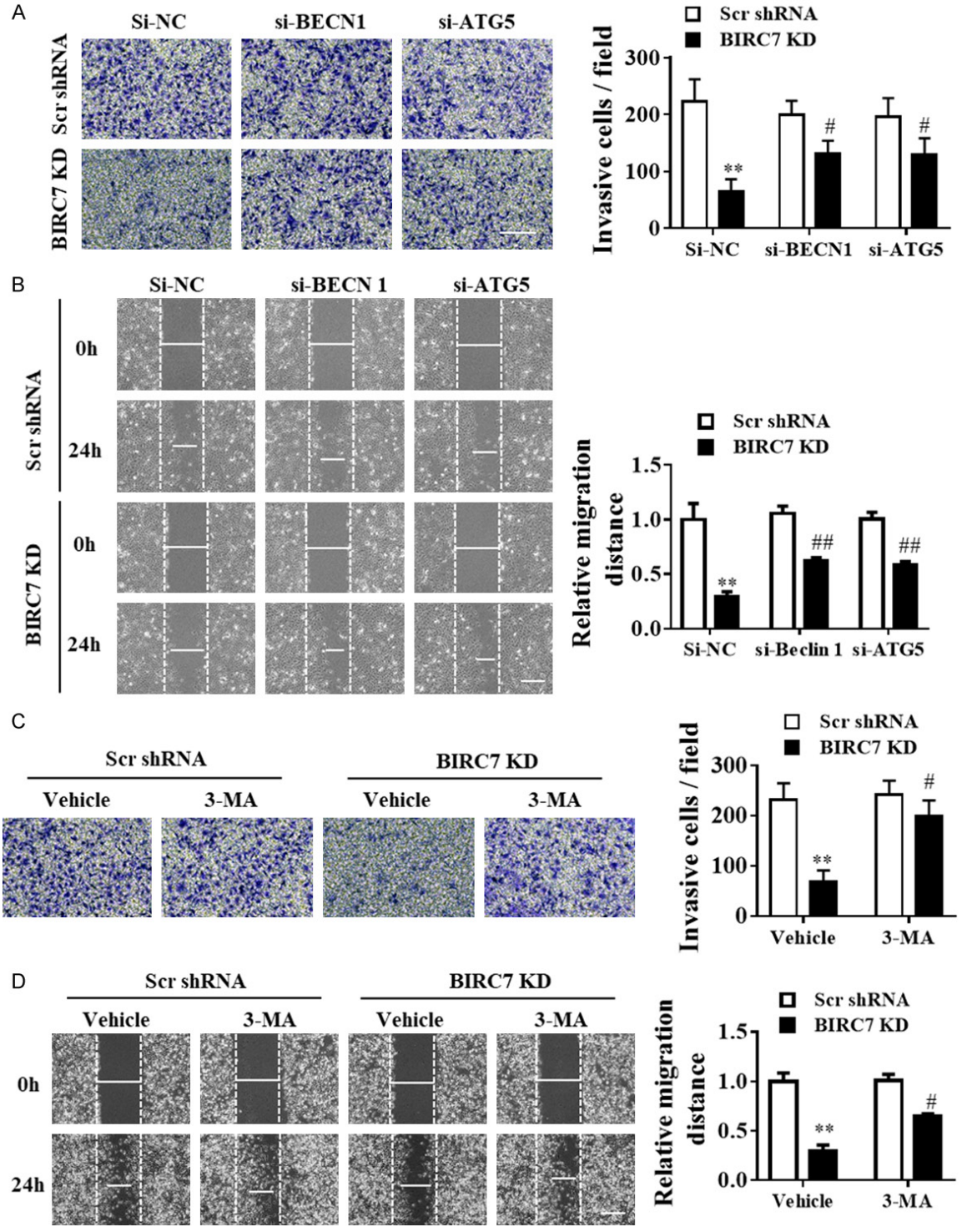


Figure 5. Suppression of autophagy by BIRC7 enhances PTC cell migration and invasion. (A, B) BIRC7 KD or shRNA-transduced BCPAP cell invasion (A) and migration (B) after transfection with siRNAs specific for BECN1, ATG5, or control. $^{**}P<0.01$ vs. Scr shRNA + si-NC group. $^{\#}P<0.05$ vs. BIRC7 KD + si-NC group. (C, D) BIRC7 KD or shRNA-transduced BCPAP cell invasion (C) and migration (D) after 3-MA treatment. Data are means ± S.E.M. $^{**}P<0.01$ versus Scr shRNA + Vehicle group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. BIRC7 KD + Vehicle group. Scale bar = 100 μm. All assays were conducted in triplicate.

autophagy within PTC cells, thereby disrupting their metastatic activities, highlighting this as a possible therapeutic strategy for treating those with metastatic or pre-metastatic PTC.

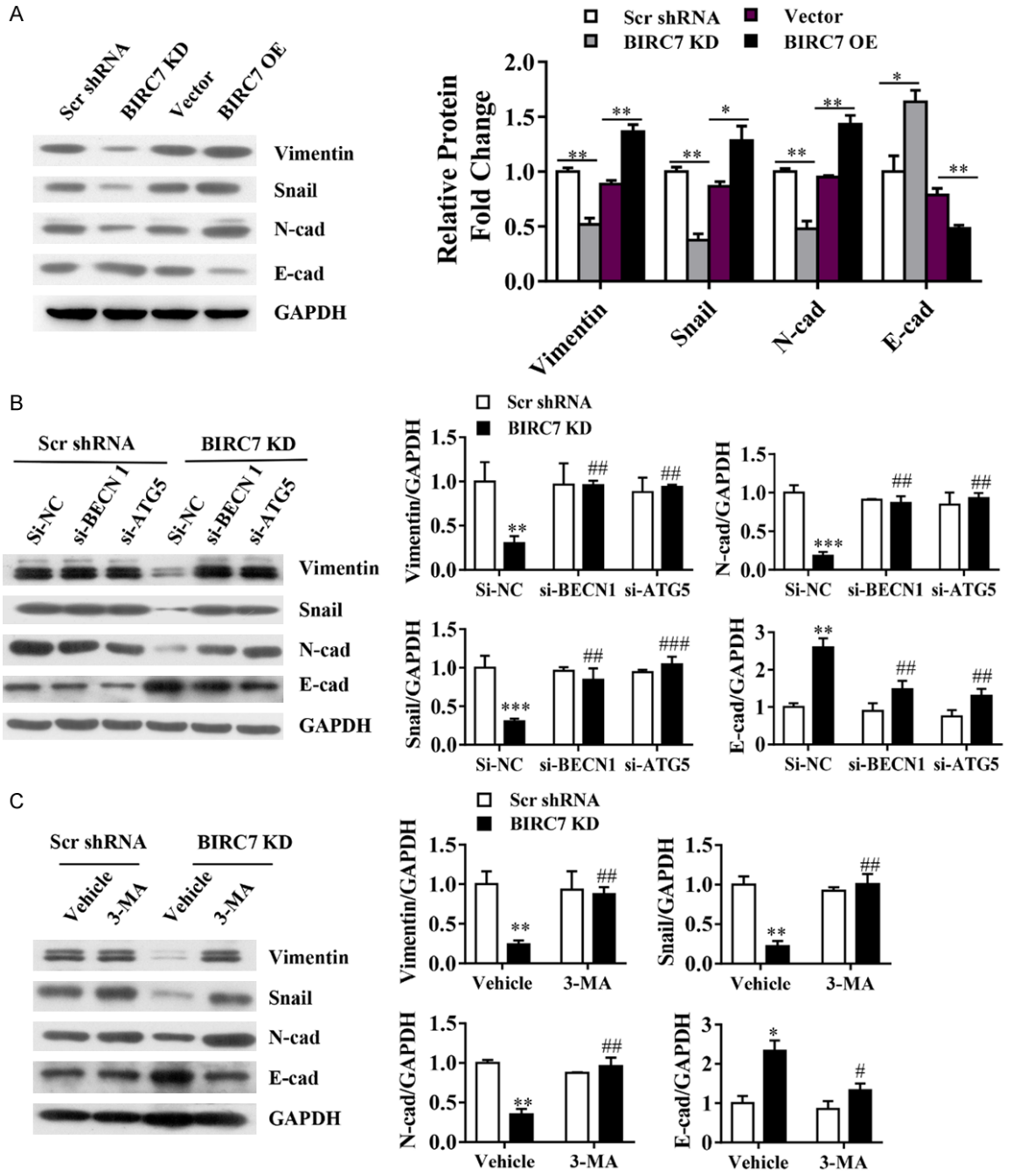
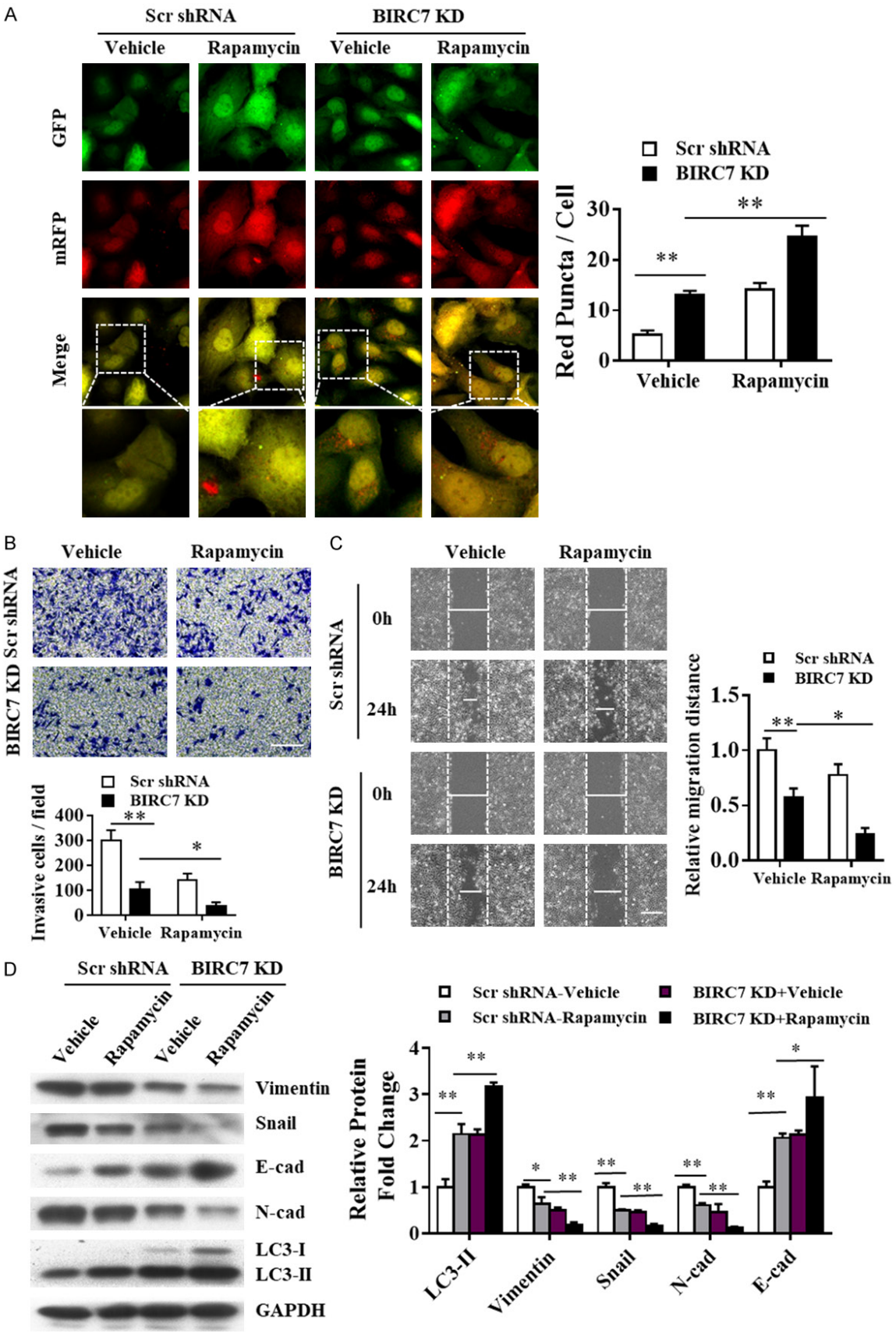


Figure 6. Suppression of autophagy by BIRC7 leads to EMT-like changes in PCT cells. (A) Levels of E-cadherin, N-cadherin, Vimentin, and Snail were assessed in BIRC7 OE and KD cells and controls via western blotting. $^{*}P<0.05$, $^{**}P<0.01$ versus indicated group. (B, C) Levels of E-cadherin, N-cadherin, Vimentin, and Snail were assessed in BIRC7 KD cells and controls via western blotting in cells transfected with siRNAs specific for ATG5 and BECN1 (B), or in cells treated using 3-MA (C). Data are means ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. corresponding Scr shRNA group. $^{\#}P<0.05$ vs. corresponding BIRC7 KD group. All assays were conducted in triplicate.

Downregulating BIRC7 enhances autophagy and impairs in vivo lung colonization of PTC cell

The *in vivo* antimetastatic effects of BIRC7 inhibition in PTC was determined in an experimental lung metastasis model. As shown in **Figure 8B**, BIRC7 KD significantly decreased the nodule formation of PTC cells as shown the decreased percentage of lung areas occupied by tumors in cells infected with the BIRC7 KD virus vector compared to the cells infected with the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GAPDH
Scr shRNA-Vehicle
Scr shRNA-Rapamycin
BIRC7 KD+Vehicle
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Relative Protein Fold Change
4
3
2
1
0
LC3-II
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Snail
N-cad
E-cad

Figure 7. Inhibiting both mTOR and BIRC7 can enhance autophagy and mediated synergistic anti-tumor efficacy in PTC. BIRC7 KD and control BCPAP cells were treated for 24 h using Rapamycin (100 nM) or DMSO control, and then the mRFP-GFP-LC3 adenoviral reporter was used to assess autophagic flux (Scale bar = 10 μm) (A). Cell invasion (B) and migration (C) assays were performed (Scale bar = 100 μm). (D) Western blotting was used to assess the expression of the indicated proteins in treated BCPAP cells. Data are means ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. All assays were conducted in triplicate.

empty virus vector. What's more, HE staining showed that tail vein injection of BIRC7 KD cells into nude mice led to significantly less and smaller nodules in the lung (**Figure 8B**). We further explored the implications of the inhibition of autophagy in this model system via stably knocking down ATG5 in BIRC7 KD cells, revealing a significant increase in the formation of BIRC7 KD cell colonization following ATG5 knockdown, whereas no significant effect was evident in ATG5 knockdown cells in which BIRC7 expression was normal (**Figure 8B**), thus suggesting that the ability of BIRC7 to suppress autophagy increased the colonization of PTC cells *in vivo*.

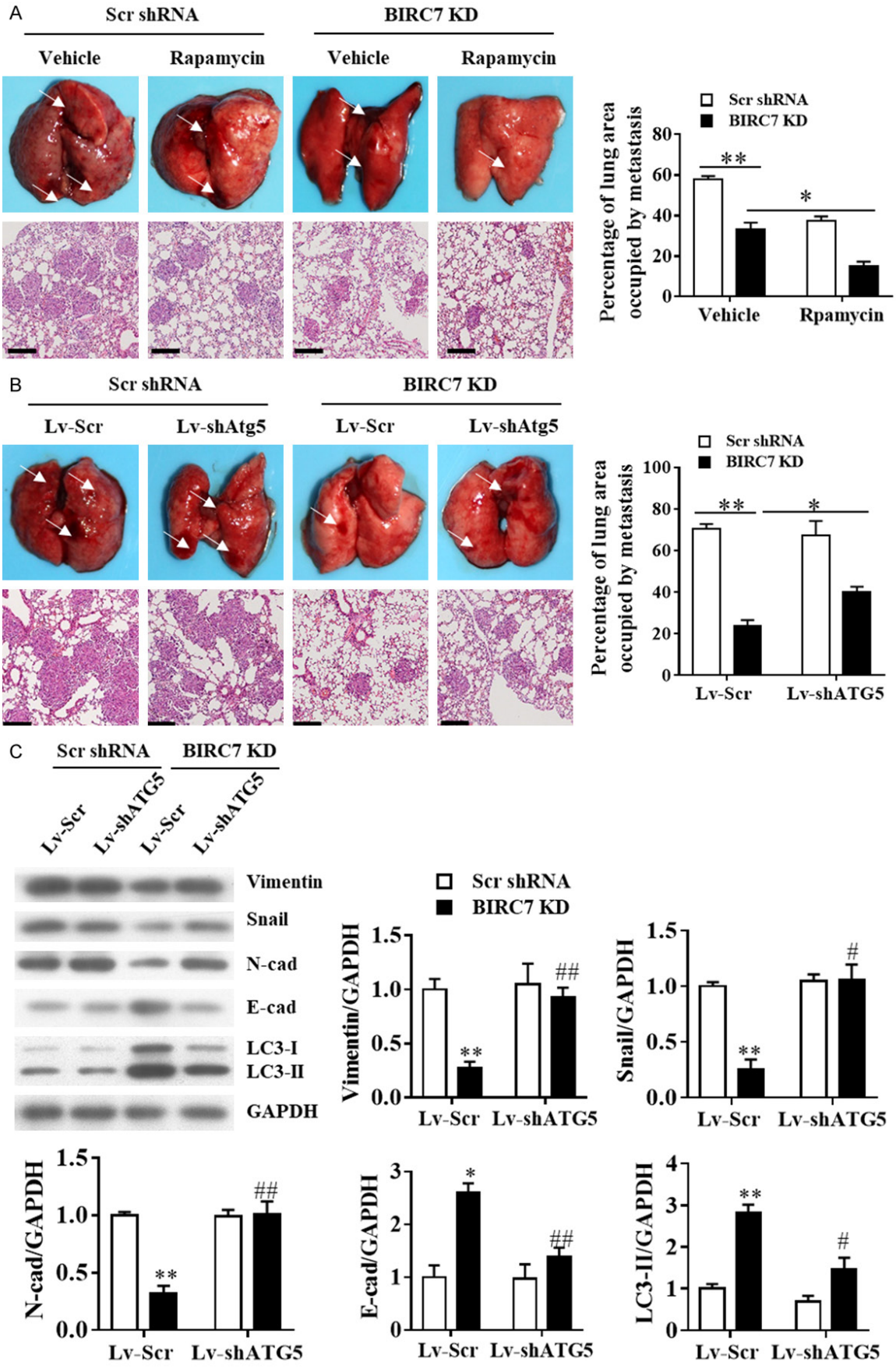
We further found that knockdown of BIRC7 was associated with higher levels of *in vivo* LC3-I/LC3-II conversion and E-cadherin expression, as well as reduced expression of N-cadherin, Vimentin, and Snail, with ATG5 knockdown reversing these phenotypes (**Figure 8C**). Our study demonstrated that inhibiting BIRC7 impairs the invasion of PTC cells at least in part via inducing autophagy and suppressing the EMT.

Discussion

BIRC7 has been shown to play a key role in controlling the sensitivity of multiple cancer types to chemotherapy, in addition to regulating tumor progression [21, 22, 24-26], but its specific relevance in the context of PTC has not previously been explored. Herein we specifically assessed the role of BIRC7 in PTC metastasis and investigated the underlying molecular mechanisms. We found that BIRC7 plays a pro-invasive role in PTC, as it was expressed at higher levels in primary patient PTC tissue samples relative to control samples. In addition, knocking down BIRC7 inhibited its ability to promote invasion in an EMT-dependent manner through a mechanism at least partially dependent upon the induction of autophagy, with BIRC7 overexpression having the opposite effect. To our knowledge, this is the first report specifically report that BIRC7-mediated regulation of autophagy plays a role in regulating PTC progression.

BIRC7, as a recently identified IAP family member, has been shown to be essential in several tumor types. The majority of adult tissues, with the exception of the placenta, do not express BIRC7, and yet it is expressed at high levels in multiple cancer cell lines [27], as well as in bladder cancer [28], lymphoma [8], lung cancer [29], hepatocellular carcinoma [30], and renal carcinoma [31, 32]. As such, BIRC7 represents an attractive therapeutic target. Its roles in other cancer types have been studied extensively, little is known about the potential role of BIRC7 in the development, especially in the metastasis of PTC. The purpose of this article is to investigate the effects and underlying mechanisms of BIRC7 on the metastasis of PTC. We assessed the expression of BIRC7 in PTC cell lines relative to nonmalignant thyroid cell line Nthy-ori-3-1 cells, and in primary PTC tissues and normal tissue, our data indicate that BIRC7 was overexpressed in invasive PTC cells. Moreover, our results showed that downregulation of BIRC7 could significantly impair PTC invasion and migration capability in vitro and in vivo, whereas its overexpression could dramatically promote PTC invasion and migration in vitro. Together these results thus identify a central role for BIRC7 in PTC metastasis. Biomarkers of PTC metastasis can accurately identify metastatic cells and aggressive tumor behavior. Through comparing the expression of BIRC7 in matched normal thyroid tissue, primary PTC, and metastasis samples via qRT-PCR, we found that BIRC7 is upregulated in primary PTC and further induced in PTC with lymph node metastasis. Moreover, our *in vitro* and *in vivo* findings suggest that BIRC7 is associated with invasive process of PTC. Our data suggest that BIRC7 is associated with PTC lymph node metastasis and may as a potential biomarker to predict PTC lymph node metastasis.

EMT is a multi-stage process whereby cells lose their epithelial characteristics and exhi-


A
Scr shRNA
BIRC7 KD
Vehicle
Rapamycin
Vehicle
Rapamycin
Scr shRNA
BIRC7 KD
Percentage of lung area occupied by metastasis
Vehicle
Rpamycin
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BIRC7 KD
Lv-Scr
Lv-shAtg5
Lv-Scr
Lv-shAtg5
Scr shRNA
BIRC7 KD
Percentage of lung area occupied by metastasis
Lv-Scr
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Lv-shATG5
Vimentin
Snail
N-cad
E-cad
LC3-I
LC3-II
GAPDH
Scr shRNA
BIRC7 KD
Vimentin/GAPDH
Lv-Scr
Lv-shATG5
Snail/GAPDH
Lv-Scr
Lv-shATG5
N-cad/GAPDH
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Lv-shATG5
E-cad/GAPDH
Lv-Scr
Lv-shATG5
LC3-II/GAPDH
Lv-Scr
Lv-shATG5

Figure 8. Suppression of autophagy by BIRC7 is associated with enhanced PTC lung colonization *in vivo*. A. Lung colonization of BIRC7 KD PTC cells was assessed using a lung metastasis model *in vivo* were untreated, treated with rapamycin (5 mg/kg/day) (n = 5 mice/group). Representative lung images (upper) and H&E-stained sections (lower) are shown, with lung colonization indicated by white arrows. The percentage of lung areas occupied by tumors is additionally quantified. Scale bar = 100 μm. B. Lung colonization of BIRC7 KD PTC cells expressing a stable ATG5-specific shRNA or control cells was assessed using a lung metastasis model *in vivo* (n = 5 mice/group). Representative lung images (upper) and H&E-stained sections (lower) are shown, with lung colonization indicated by white arrows. The percentage of lung areas occupied by tumors is additionally quantified. Scale bar = 100 μm. C. Western blotting results indicating LC3-I/LC3-II conversion and EMT marker levels *in vivo* in different groups. Data are means ± S.E.M. $^{*}P<0.05$, $^{**}P<0.01$ vs. Scr shRNA + Lv-Scr group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. BIRC7 KD + Lv-Scr group.

bit major alterations with respect to their morphology, adhesion, and migratory capabilities [14, 16, 33]. EMT arises early during tumor metastasis, playing a key role in mediating the development of an aggressive and invasive tumor phenotype. Central EMT features include reduced adhesion and enhanced motility [34]. E-cadherin is the best-studied marker of epithelial cells, and its expression can be suppressed by Slug and Snail, which are transcriptional repressors. In contrast, vimentin is a marker of mesenchymal cells during the EMT [16]. In this report, we found that overexpressing BIRC7 led to reduced E-cadherin expression and increased Vimentin, N-cadherin, and Snail expression in PTC cells, while the BIRC7 knockdown confers the opposite effects. This thus indicated that BIRC7 induced the EMT in PTC cells, highlight a potential direct link between BIRC7 and the metastasis of PTC.

Autophagy plays a key role in controlling the metastasis of cancers in a context-dependent fashion such that it can promote metastasis of certain tumors while inhibiting such progression in others [35]. There also remains a controversial association between autophagy and the EMT, with the EMT promoting autophagy so as to enhance metastatic cancer cell survival, and with autophagy negatively regulating the EMT such that activating autophagy can reduce EMT-associated protein stability and lead to EMT phenotypic reversion [36-38]. We found that inducing autophagy via knocking down BIRC7 was linked to a partial EMT phenotypic reversion within PTC cells, and we further found that impairing autophagy in these cells reversed the observed defects in migration and invasion upon BIRC7 knockdown both *in vitro* and *in vivo*. Most importantly, knocking down ATG5 in BIRC7 KD cells eliminated the anti-metastatic effects of knocking down BIRC7 *in vivo*. As such, this suggests that the ability of BIRC7 to suppress autophagy enhances the migration and metastasis of PTC and other cancer cell types. In line with this, inhibiting SGK1 to induce autophagy has been shown to repress the EMT and mediate anti-metastatic effects in prostate cancer [39]. In contrast, human astrocytes have been shown to upregulate autophagy, thereby promoting circulating breast cancer cell brain invasion [40]. More interestingly, we found that rapamycin and BIRC7 knockdown synergistically enhanced the anti-invasive effect of PTC cells. In addition, combination therapy significantly impaired EMT compared to treatment alone. Therefore, we hypothesized that the combined targeting of mTOR and BIRC7 could reverse EMT and significantly prevent PTC cell invasion due to its enhanced anti-invasive effect. As such, there remains much that is not known regarding the role played by autophagy in the context of tumor cell EMT and metastasis, making a more comprehensive understanding of the contextual inputs in individual scenarios important [41]. Further investigation is thus essential in order to better clarify the specific role of autophagy in metastasis to develop novel therapeutic strategies to inhibit metastasis.

In conclusion, these results suggest a potential model whereby BIRC7 represses PTC cell autophagy, thereby enhancing the migration and invasion of these cells. In this model, BIRC7 represses BECN1 and ATG5-dependent autophagy, autophagic activity inhibition induces EMT-like alterations and then promotes PTC cell migration and invasion. As such, targeting of BIRC7 may be a clinically beneficial strategy in PTC patients, and thus this study provides a basis for future investigations into this and other strategies for PTC treatment.

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Disclosure of conflict of interest

None.

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