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Running title: Role of miR-5681b/Beclin-1 in PCa

The miR-5681b/Beclin-1 axis regulates tumor autophagy to affect the proliferation and apoptosis of prostate cancer cells

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Prostate cancer (PCa) is a leading cause of cancer-related mortality among men. This study aims to investigate the regulatory effect of microRNA (miR)-5681b, a potential upstream miR of Beclin-1, on PCa cell proliferation and apoptosis. Two PCa cell lines (PC3 and LnCaP cells) with relatively low miR-5681b expression were treated with miR-5681b mimic, pcDNA3.1-Beclin-1, or an autophagy activator Rapamycin. A xenograft tumor model was established in nude mice, and the tumor-bearing mice were treated with agomir miR-5681b. The levels of miR-5681b, Beclin-1 mRNA, and apoptosis- and autophagy-associated proteins were evaluated using western blot and RT-qPCR. The binding between Beclin-1 and miR-5681b was testified by dual-luciferase reporter gene assay. Cell biological behaviors, as well as Ki-67-positive cells and apoptosis in mouse tumor tissues, were examined. The results showed that miR-5681b was downregulated in PCa cells and targeted Beclin-1. miR-5681b overexpression in PCa cells significantly suppressed cell proliferation and B-cell lymphoma 2 (Bcl-2) levels while augmenting cell apoptosis and the levels of Bcl-2-associated X (Bax) and cleaved caspase-3. Importantly, miR-5681b inhibited PCa cell proliferation and autophagy but promoted PCa cell apoptosis, whereas Beclin-1 upregulation reversed these effects. Activating autophagy also reversed miR-5681b-regulated proliferation and apoptosis of PCa cells. *In vivo*, miR-5681b overexpression inhibited PCa tumor growth by modulating the Beclin-1-mediated autophagy pathway. Collectively, these findings suggested that miR-5681b was lowly expressed in PCa cells, and miR-5681b overexpression inhibited autophagy by targeting Beclin-1, thereby suppressing the growth of PCa.

Key words: miR-5681b; Beclin-1; prostate cancer; autophagy; proliferation; apoptosis; autophagy agonists; rapamycin

Prostate cancer (PCa) is one of the most common non-cutaneous malignancies in men, and its incidence has been rising significantly in the Asian population [1, 2]. The prevalence of PCa increases with age, and the risk of developing the disease becomes substantially higher in older individuals [3]. The progression of prostate malignancy involves multiple stages, beginning with prostate intraepithelial neoplasia (PIN), advancing to localized PCa, subsequently evolving into advanced prostate adenocarcinoma with local invasion, and ultimately inducing metastasis [4]. PCa can be classified as androgen-sensitive or androgen-insensitive based on its response to testosterone stimulation, which directly influences therapeutic strategies [5]. Current treatment options include active surveillance, radiotherapy, chemotherapy, hormone therapy, cryotherapy, and surgery [6]. Despite advances in clinical management, PCa continues to pose major challenges, including overtreatment of essentially benign lesions and limited therapeutic options for metastatic disease [7]. Therefore, further research into the mechanisms underlying PCa is essential for identifying new therapeutic targets.

Autophagy is a key intracellular process responsible for the degradation and recycling of damaged molecules [8]. It occurs frequently during tumorigenesis and cancer chemotherapy. Functional autophagy can protect cancer cells from chemotherapy-induced cytotoxicity, contributing to treatment resistance and refractory diseases [9]. Beclin-1, the first essential autophagy-related protein discovered, is critically involved in the development of several cancers when deleted or impaired [10]. Studies have demonstrated that Beclin-1 regulates multiple pathways, including mTOR, PI3K/AKT, and p53, thereby influencing cell development and viability [11-13]. Given the close relationship between autophagy and apoptosis, autophagy can influence the sensitivity of PCa cells to chemotherapy and radiation [14]. In addition, Beclin-1 can interact with microRNAs (miRNAs) to modulate autophagy in tumor cells. For example, miR-93 interacts with Beclin-1 to regulate autophagy in glioblastoma, affecting tumorigenicity and treatment resistance [15]. Knockdown of Beclin-1 inhibits autophagy and induces apoptosis in BPH-1 cells [16]. However, the relevant upstream mechanisms of Beclin-1 involving miRNAs in PCa have not been fully elucidated.

miRNAs are defined as a type of non-coding RNA molecule with a length of roughly 21-25 nucleotides, which are widely present in eukaryotic cells [17]. miRNAs play a critical regulatory

function in cellular processes and exert influence over the translation efficiency and stability of mRNA molecules through their interaction with the 3'-untranslated region (3'-UTR) of the target mRNA, thereby governing gene expression [18]. miRNAs are pivotal regulators in a wide range of biological processes, including cell apoptosis, growth, and metabolism [17]. Notably, the significance of miRNAs in the occurrence and progression of PCa has been extensively investigated and acknowledged in academic research. For instance, miR-21 is overexpressed in PCa and is recognized as a promoting factor that enhances cellular viability and proliferation while suppressing programmed cell death [19]. Conversely, several miRNAs, such as miR-34a and miR-129-5p, have been observed to impede the growth and spread of PCa cells while promoting apoptosis [20, 21]. Accordingly, a thorough analysis of PCa-associated miRNAs is necessary to identify novel PCa-related miRNAs and their corresponding target genes, which is instrumental in elucidating new molecular targets and devising individualized treatment strategies. In this study, we obtained downregulated miRNAs in PCa patients from the dbDEMC database, PCa-related miRNAs from the GeneCards database, and upstream miRNAs of Beclin-1 from the miRanda database. The intersection of the results of these three datasets yielded the unique candidate, miR-5681b, which was selected as the research subject. Nonetheless, to date, there have been no literature reports on the expression pattern and mechanism of miR-5681b in malignant tumors. Surprisingly, preliminary evidence suggests that miR-5681b can inhibit the proliferation of vascular smooth muscle cells [22], suggesting that miR-5681b may influence cellular phenotypes by regulating cell proliferation. Uncontrolled and excessive cell division and proliferation can lead to the formation of malignant tumors [23]. Therefore, we hypothesized that miR-5681b may influence PCa cell autophagy by targeting and inhibiting Beclin-1 expression, thereby affecting PCa cell proliferation and apoptosis. A detailed investigation into the relationship between miR-5681b, Beclin-1, and autophagy in PCa cells may offer new insights for developing targeted therapeutic strategies. This study may help clarify the molecular mechanisms underlying autophagy regulation in PCa progression and provide a theoretical foundation for novel anticancer treatments.

Materials and methods

Ethics statement. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Xi'an JiaoTong University Yulin Hospital. Significant efforts were made in order to minimize both the number of animals used and their respective suffering.

Bioinformatics analysis. LogFC > 2 and FDR < 0.05 were introduced as filter criteria to choose the differential miRNAs in PCa patients downloaded from the dbDMEC 3.0 database (https://www.biosino.org/dbDEMC/index) (GSE112264, EXP00622; serum samples of 809 patients with PCa, 241 negative prostate biopsies, 500 patients with other cancer types were obtained from the National Cancer Center in Japan, and serum samples of 41 healthy control samples were acquired from two other hospitals in Japan). The differential gene volcano map was plotted by the R package "ggplot2". The miRanda database (https://mirdb.org/) and the Genecards database (https://www.genecards.org/) were employed to identify potential upstream miRNAs of Beclin-1 and PCa-related miRNAs, respectively. The intersection of these three databases identified miR-5681b as the sole candidate for further investigation.

Cell culture. The human normal prostate epithelial cell line RWPE-1 and PCa cell lines (DU145, PC3, LnCaP, and 22RV1) were obtained from BeNa Culture Collection (Beijing, China). RWPE-1 cells (2.5×10^5) were seeded in Dulbecco's modified Eagle medium (Sigma, St. Louis, MO, USA) using 24-well plates, while PCa cell lines (2.5×10^5) were seeded in Roswell Park Memorial Institute 1640 medium (Sigma) comprising 10% fetal bovine serum (Sigma), followed by cell culture in a humidified incubator at 37 °C with 5% CO_2.

Cell transfection and grouping. Mimic negative control (NC), miR-5681b mimic, pcDNA3.1 empty vector, and pcDNA3.1-Beclin-1 were transfected into PC3 and LnCaP cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The final concentrations were 50 nM for mimic NC and miR-5681b mimic and 100 nM for the pcDNA3.1 empty vector and pcDNA3.1-Beclin-1. All transfected materials were obtained from GenePharma (Shanghai, China). Subsequent experiments were performed 48 h following cell transfection.

Cells were grouped as follows: 1) Blank group; 2) mimic NC group: PC3 and LnCaP cells were introduced with mimic NC; 3) miR-5681b mimic group: PC3 and LnCaP cells were delivered with

miR-5681b mimic; 4) miR-5681b mimic+pcDNA group: PC3 and LnCaP cells were co-transfected with miR-5681b mimic and pcDNA3.1 empty vector; 5) miR-5681b mimic+pcDNA-Beclin-1 group: miR-5681b mimic and pcDNA3.1-Beclin-1 were co-transfected into PC3 and LnCaP cells; 6) miR-5681b mimic+Rapamycin group: PC3 and LnCaP cells were delivered with miR-5681b mimic and concomitantly treated with 50 mM of an autophagy activator Rapamycin (Sigma) for a duration of 24 hours [24]; 7) miR-5681b mimic+DMSO group: PC3 and LnCaP cells were treated with miR-5681b mimic and while being treated with 50 mM dimethyl sulfoxide (DMSO; Sigma) for 24 h.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from samples using the TRIzol reagent (Invitrogen). RNA concentration and quality were assessed with a NanoDrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA), and complementary DNA was then synthesized. mRNA was reverse-transcribed using the SuperScriptTM III Reverse Transcriptase Kit (Invitrogen), and miRNA was reverse-transcribed using the miRCURY LNA RT Kit (Qiagen, Duesseldorf, Germany). RT-qPCR was carried out using THUNDERBIRD SYBR® qPCR Mix (Toyobo, Osaka, Japan). RNA relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the mRNA internal reference gene, and U6 served as the internal reference gene for miRNA [25]. The amplified primer sequences of each gene and its primer are detailed in F1.

Dual-luciferase reporter gene assay. The targeted binding sites between miR-5681b and Beclin-1 were predicted by the MiRanda database (https://mirdb.org/). Complementary binding sequences and mutant sequences of miR-5681b and Beclin-1 were amplified and cloned into a pmiR-GLO luciferase vector (Promega, Madison, WI, USA) to construct wild-type (Beclin-1-WT) and mutant (Beclin-1-MUT) reporter plasmids. Thereafter, Beclin-1-WT or Beclin-1-MUT was then co-transfected with mimic NC and miR-5681b mimic (GenePharma) into PC3 and LnCaP cells using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions. Luciferase activity was evaluated 48 h after transfection.

Cell counting kit-8 (CCK-8) assay. Cell viability was assessed using a CCK-8 kit (Solarbio, Beijing, China). Briefly, cells were seeded in 96-well plates (5×10^4 cells/well) and incubated overnight with 5% CO_2 at 37 °C. Subsequently, 10 µl of CCK-8 reagent was added to each well and

incubated for 2 h. Absorbance was measured at 450 nm under a microplate reader (BioTek, Winooski, VT, USA).

5-ethynyl-2'-deoxyuridine (EdU) staining. Cell proliferation was evaluated using the EdU Stain Proliferation Kit (Abcam, Cambridge, UK). Cells were incubated with EdU solution under optimal growth conditions for 24 h, washed, and then fixed with the fixative solution for 15 min. After fixation, cells were permeabilized with the permeation buffer solution for 15 min and washed again. A reaction mixture was applied to label incorporated EdU, and cells were incubated for 30 min. Lastly, fluorescent signals were visualized with a DSY2000X inverted fluorescence microscope (DP80, Olympus, Tokyo, Japan).

Flow cytometry (FCM) assay. Apoptosis was assessed using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining. Cells were collected, centrifuged at 800 × g, and the supernatant was removed. Afterward, the cell pellet was resuspended using the Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA) based on the provided protocols, followed by the addition of 5 µl FITC and 5 µl PI in the dark. Finally, apoptotic cells were quantified using a BD FACSCalibur flow cytometer (MoFloAstrios EQ, Beckman Coulter, CA, USA).

Experimental animals. BALB/c nude mice (equal numbers of males and females; weighing 18-20 g) were purchased from Shaanxi Shanyao Medical Biotechnology Co., Ltd. (Xi'an, China). The mice were housed under a sterile environment at 26-28 °C with a relative humidity of 40-60%, *ad libitum* access to food and water, and a 12 h light/dark cycle.

Animal treatment and grouping. To induce *in vivo* tumor formation, 2×10^6 PC3 cells were subcutaneously injected into the right axillary region of the forelimb of nude mice. Tumor volume was measured with a vernier caliper, and grouping treatment was initiated when tumors reached 50 mm^3. After tumor establishment *in vivo*, the nude mice were randomly assigned to three groups (n=6 mice/group): 1) the PCa group: tumorigenesis was induced in nude mice by injection of PC3 cells, and an equal dose of phosphate-buffered saline (PBS) was injected into mice without miRNA treatment; 2) the agomir miR-5681b group: tumor-bearing mice were injected with agomir miR-5681b; and 3) the agomir NC group: tumor-bearing mice were injected with negative control agomir NC. Each nude mouse received intratumoral multipoint injection of 3 nmol of agomir

miR-5681b (HY-R01760A, MCE) or agomir NC (HY-R04602A, MCE) every two days over a two-week period, with a total of 7 injections (21 nmol). Twenty-four hours after completion of all interventions, tumor tissues were harvested. A portion of the tumor tissues from each group was fixed in 4% paraformaldehyde (Solarbio) for 24 h, dehydrated in ethanol, cleared in xylene, embedded in paraffin, and sectioned at 5 μm thickness. Sections were deparaffinized and hydrated for immunofluorescence. The remaining tumor tissues were prepared into tissue homogenates for western blot, RT-qPCR, and kit detection.

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Xi'an JiaoTong University Yulin Hospital (Approval No. 2025-085). Significant efforts were made in order to minimize both the number of animals used and their respective suffering.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining. Tumor tissue sections were incubated with Protease K working solution (Sigma-Aldrich, St. Louis, MO, USA) for 22 min at 37 °C before three PBS washes. Tissue paraffin sections were treated with 0.1% triton for 20 min. Subsequent to PBS washing, the sections were added dropwise with buffer and incubated for 10 min at ambient temperature. The appropriate amounts of TDTase, dUTP, and buffer were mixed at a ratio of 1:5:50 following the instructions of the TUNEL staining kit (T2196, Solarbio) and incubated with the sections at 37 °C for 2 h, followed by PBS washing. DAPI staining solution was added dropwise for 10 min section incubation at room temperature in the dark. After being blocked with anti-fluorescence quenching agents, the sections were observed and photographed under a confocal fluorescence microscope (DMI8, Leica, Weztlar, Germany).

Immunohistochemistry (IHC). Tumor tissue sections were subjected to antigen retrieval through boiling in 0.01 M sodium citrate buffer (Sigma-Aldrich), followed by incubation with 3% H_2O_2 at room temperature for 15 min. Sections were then blocked with 5% goat serum (Solarbio) for 30 min and incubated overnight at 4 °C with anti-Ki-67 primary antibodies (0.5 μg/ml, #ab15580, Abcam). Afterward, the sections were incubated with horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) H&L secondary antibodies (1:1000, #ab6721, Abcam) at room temperature for 2 h. Visualization was performed using 3,3-diaminobenzidine (Solarbio), followed by section counterstaining and mounting. After that, the sections were examined and photographed

under a light microscope, and the percentage of positively stained cells was quantified using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Western blot. Total proteins were extracted from cells using radio-immunoprecipitation assay cell lysis buffer (Beyotime Biotechnology, Shanghai, China). Protein concentrations were determined using a bicinchoninic acid protein detection kit (Rockford, IL, USA). Afterward, 30 μg/well of the protein sample was loaded onto a 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. After being blockaded with 5% skim milk for 2 h, the PVDF membrane was cultured in the presence of primary antibodies against rabbit anti-Beclin-1 (1/2000, #ab207612, Abcam) [26], rabbit anti-Cleaved Caspase-1 (1/500, #ab32042, Abcam) [27], rabbit anti-B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) (1/1000, #ab32503, Abcam) [28], rabbit anti-Bcl-2 (1/2000, #ab182858, Abcam) [29], rabbit anti-light chain-3B (LC3B) (1/3000, #ab51520, Abcam) [30], rabbit anti-P62 (1/10000, #ab109012, Abcam) [31], and rabbit anti-β-actin (1/1000, #ab8226, Abcam) [32] overnight at 4 °C. After being rinsed thrice with Tris-buffered saline with Tween-20, the membranes were incubated with HRP-labeled goat anti-rabbit IgG H&L (1:5000, #ab6721, Abcam) [33] for 1 h at room temperature, followed by visualization using enhanced chemiluminescence working solution (EMD Millipore, Billerica, MA, USA). The quantification of proteins was conducted utilizing Image Pro Plus 6.0 software (Media Cybernetics). β-actin served as an internal reference. Additionally, the linear range of β-actin was assessed using different total protein contents, following the methodology described in a previous study [34]. The results showed that β-actin levels exhibited no significant variation when the protein content exceeded 4 μg, indicating protein overload. Therefore, in this study, 3 μg of protein samples were loaded for western blot to ensure that the internal reference protein signal was maintained within the effective linear range (Supplementary Figure S1).

Statistical analysis. Statistical analyses and plotting were performed using GraphPad Prism 9.5.0 software (GraphPad Software Inc., San Diego, CA, USA). The Shapiro-Wilk test was employed to assess the normal distribution of data. Normally distributed measurement data were presented as mean±standard deviation and compared between two groups using the independent sample *t* test.

One-way analysis of variance (ANOVA) was applied for multi-group comparisons, followed by Tukey's test. $P < 0.05$ was indicative of significant differences.

Results

miR-5681b expression is decreased in PCa cell lines and can target Beclin-1. The differential miRNAs of PCa patients were screened from the dbDMEC database using the filter criteria of LogFC < 2 and FDR < 0.05. A volcano plot (Figure 1A) was generated to visualize downregulated miRNAs in PCa (Supplementary Table S1). PCa-related miRNAs were obtained from the Genecards database (Supplementary Table S3). Previous studies have implicated the autophagy-related gene Beclin-1 in PCa pathogenesis and unveiled its role in inhibiting tumor cell apoptosis [16, 35]. Subsequently, the upstream miRNA of Beclin-1 was predicted using the miRanda database (Supplementary Table S2). Finally, the intersection of these results was determined using Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/index.html), identifying miR-5681b as an upstream miRNA of Beclin-1, which was significantly downregulated in PCa (Figure 1B).

To investigate miR-5681b expression in PCa cells and its regulatory relationship with Beclin-1, we cultured RWPE-1 (normal prostate epithelial) and PCa cell lines (LnCaP, DU145, PC3, and 22RV1) *in vitro*. RT-qPCR data revealed significantly lower miR-5681b expression in PCa cell lines compared to RWPE-1 (all $p < 0.05$, Figure 1C). Accordingly, PC3 and LnCaP cells with relatively low expression of miR-5681b were selected for the subsequent experiments. The binding sites between miR-5681b and Beclin-1 were identified using the miRanda database (Figure 1D). Dual-luciferase assay demonstrated that co-transfection of Beclin-1 wild-type (Beclin-1-WT) and miR-5681b mimic significantly reduced luciferase activity (all $p < 0.05$, Figure 1E), whereas no significant changes were observed in the luciferase activity of cells treated with Beclin-1-WT and mimic NC (all $p > 0.05$, Figure 1E). Additionally, miR-5681b was overexpressed in PC3 and LnCaP cells (all $p < 0.05$, Figure 1F), which resulted in decreased Beclin-1 mRNA and protein levels in PC3 and LnCaP cells (all $p < 0.05$, Figures 1G, 1H). These findings indicate that miR-5681b is downregulated in PC3 cells and miR-5681b targets Beclin-1.

miR-5681b inhibits proliferation and facilitates apoptosis in PCa cells. To explore the role of miR-5681b in PCa cells, we evaluated cell viability using CCK-8 assay. miR-5681b mimic significantly reduced cell viability ($p < 0.05$, Figure 2A). EdU staining confirmed that miR-5681b mimic inhibited PCa cell proliferation ($p < 0.05$, Figure 2B). Additionally, apoptosis was enhanced following miR-5681b mimic transfection (all $p < 0.05$, Figure 2C). miR-5681b mimic elevated the expression of cleaved caspase-3 and Bax and lowered Bcl-2 expression (all $p < 0.05$, Figure 2D). These results indicate that miR-5681b suppresses PCa cell proliferation and promotes apoptosis.

Beclin-1 upregulation partially reverses the effects of miR-5681b on PCa cell proliferation and apoptosis. To further explore whether miR-5681b regulates proliferation and apoptosis in PCa cells by targeting Beclin-1, PC3 and LNCaP cells were co-transfected with miR-5681b mimic and either pcDNA3.1-Beclin-1 or the empty pcDNA3.1 vector. Compared to the miR-5681b mimic+pcDNA group, the mRNA and protein expression of Beclin-1 were increased in the miR-5681b mimic+pcDNA-Beclin-1 group (all $p < 0.05$, Figures 3A, 3B), accompanied by boosted cell viability (Figure 3C) and proliferation (Figure 3D) ($p < 0.05$), and reduced apoptosis ($p < 0.05$, Figure 3E). Meanwhile, the expression of Bax and cleaved caspase-3 in the miR-5681b mimic+pcDNA-Beclin-1 group decreased as compared to that in the miR-5681b mimic+pcDNA group, with elevated Bcl-2 expression ($p < 0.05$, Figure 3F). These results illustrate that miR-5681b targets Beclin-1 to suppress proliferation and boost apoptosis in PCa cells, and Beclin-1 overexpression partially reverses these effects.

miR-5681b regulates PCa cell autophagy by targeting Beclin-1. Beclin-1 is a well-established gene associated with the process of autophagy [36]. Western blot demonstrated that LC3-I and p62 protein expression was augmented, and LC3-II/LC3-I and LC3-II levels declined after miR-5681b mimic transfection, while these effects were reversed by Beclin-1 overexpression ($p < 0.05$, Figure 4A). In accordance, miR-5681b limits autophagy in PCa cells through the inhibition of Beclin-1 expression.

Activation of autophagy partially abrogates the effect of miR-5681b on PCa cell apoptosis and proliferation. We further treated PC3 and LNCaP cells with the autophagy inhibitor 3-MA to suppress cell autophagy. The results showed that 3-MA markedly reduced the levels of the autophagy-related protein LC3-II and the LC3-II/LC3-I ratio, while increasing LC3-I and P62

protein expression in PC3 and LNCaP cells ($p < 0.05$, Figure 5A). In addition, 3-MA treatment obviously decreased PC3 and LNCaP cell viability ($p < 0.05$, Figure 5B) and proliferation ($p < 0.05$, Figure 5C), while accelerating their apoptosis ($p < 0.05$, Figure 6B). Meanwhile, the expression of Bax and cleaved caspase-3 was prominently augmented, and Bcl-2 expression was lowered in PC3 and LNCaP cells upon 3-MA treatment (all $p < 0.05$, Figure 6A). PC3 and LnCaP cells were subjected to treatment with the autophagy activator Rapamycin, concomitant with the overexpression of miR-5681b. Compared to the miR-5681b mimic group, the miR-5681b mimic+Rapamycin group showed elevations in the level of LC3-II and the ratio of LC3-II/LC3-I and declines in the levels of LC3-I and P62 proteins ($p < 0.05$, Figure 5A). Correspondingly, cell viability ($p < 0.05$, Figure 5B) and proliferation ($p < 0.05$, Figure 5C) were increased and apoptosis was attenuated ($p < 0.05$, Figure 6B) in the miR-5681b mimic+Rapamycin group when compared with the miR-5681b mimic group, accompanied by lowered Cleaved caspase-3 and Bax levels and augmented Bcl-2 levels (all $p < 0.05$, Figure 6A). Collectively, these findings highlight that inhibition of autophagy depresses PCa cell proliferation and potentiates apoptosis, whereas activation of autophagy partially nullifies the regulatory effects of miR-5681b on proliferation and apoptosis in PCa cells.

miR-5681b suppresses Beclin-1 to mediate autophagy, thereby inhibiting *in vivo* tumor growth of PCa. An *in vivo* PCa model was first established by subcutaneously implanting 2×10^6 PC3 cells into the right axillary region of nude mice, followed by intratumoral injections of agomir miR-5681b or agomir NC. The results revealed that agomir miR-5681b treatment significantly increased miR-5681b expression in tumor tissues ($p < 0.05$, Figure 7A), while reducing Beclin-1 expression ($p < 0.05$, Figures 7B, 7C). Moreover, agomir miR-5681b markedly enhanced apoptosis ($p < 0.05$, Figure 7D), decreased proliferation ($p < 0.05$, Figure 7E) and autophagy ($p < 0.05$, Figure 7F), and obviously reduced tumor volume and weight ($p < 0.05$, Figure 7G). Taken together, these findings demonstrate that miR-5681b inhibits Beclin-1 mediated autophagy, thereby suppressing *in vivo* growth of PCa.

Discussion

PCa remains the most commonly diagnosed non-cutaneous malignancy in men and is a major cause of cancer-related mortality worldwide [37]. Although localized PCa has a relatively high long-term survival rate, metastatic PCa remains largely incurable despite multimodal treatment approaches [7]. Emerging evidence highlights the role of miRNAs in regulating key genes involved in drug resistance and aggressiveness of PCa [38, 39]. Notably, deletion of a single copy of Beclin-1 has been reported in 40-75% of PCa cases [40]. Herein, this study aims to elucidate the mechanism by which miR-5681b regulates the autophagy-related protein Beclin-1, thereby modulating the proliferation and apoptosis of PCa cells.

miRNAs modulate post-transcriptional gene expression by binding to complementary sequences within the 3′-UTR of target mRNAs, leading to mRNA degradation or translational repression [41]. Approximately 60% of human genes are modulated by miRNAs [42]. Beclin-1 has been implicated in the initiation and progression of PCa [35]. Not surprisingly, Beclin-1 is also modulated by its upstream miRNAs, including miR-30a, as previously reported [43]. In this study, bioinformatics analysis identified miR-5681b as an upstream regulator of Beclin-1, which was downregulated in PCa. In androgen-independent PCa, several tumor-suppressive miRNAs (like miR-31, miR-148a, and miR-200b-3p) are downregulated, contributing to tumor aggressiveness, progression, and resistance to androgen deprivation [39]. Furthermore, we confirmed reduced miR-5681b expression in PCa cells and validated its targeting on Beclin-1 by RT-qPCR and dual-luciferase assays. Overexpression of miR-5681b significantly decreased Beclin-1 mRNA and protein expression. Likewise, miR-30a may target Beclin-1 and suppress autophagy by decreasing Beclin-1 expression [43]. In conclusion, this study demonstrated for the first time that miR-5681b expression was reduced in PCa cell lines and that it directly suppressed Beclin-1 expression.

PCa is characterized by the dysregulation of multiple miRNAs, which function as either tumor suppressors or oncogenes [44]. Beclin-1 is a vital autophagy protein, and yeast two-hybrid screening revealed that it interacts effectively with Bcl-2 [45]. Numerous studies highlight the critical roles of miRNAs in PCa progression, including effects on proliferation, cell cycle, invasion, metastasis, and apoptosis, functioning as tumor repressors or oncogenes depending on their target genes [46-48]. Our results showed that miR-5681b upregulation inhibited viability, proliferation, and Bcl-2 expression, while promoting apoptosis and increasing cleaved caspase-3 and Bax levels

in PCa cells; these effects were partially reversed by Beclin-1 overexpression. Similarly, miR-92a overexpression suppresses PCa cell invasion, viability, and migration [49]. miR-340 also reduces invasiveness, proliferation, and migration, while driving apoptosis in PCa cells [50]. Additionally, overexpressed miR-106a promotes apoptosis and inhibits proliferation, migration, and invasion by directly targeting IL-8 in PCa cells [51]. Zhang et al. have reported that elevated miR-216a expression suppresses PCa cell proliferation and accelerates cell apoptosis, whereas Beclin-1 overexpression negates the effects of miR-216a [52]. To our knowledge, our study is the first study to describe the role of miR-5681b on PCa progression. Briefly, miR-5681b suppresses PCa cell proliferation and induces apoptosis, which is partially abrogated by Beclin-1 upregulation.

Autophagy plays a critical role in promoting the survival and proliferation of PCa cells [14]. LC3 is commonly utilized as a biomarker for autophagy because of its strong correlation with autophagosome abundance, marked by the conversion of LC3-I to LC3-II [53]. p62 is a selective autophagy receptor involved in multiple pathways and is ubiquitously expressed in cells [54]. Interestingly, p62/SQSTM1 accumulation, an autophagy receptor, is positively correlated with LC3-II elevation [55]. In our study, co-overexpression of miR-5681b and Beclin-1 diminished LC3-I and p62 protein expression and elevated LC3-II/LC3-I and LC3-II levels. Consistently, miR-146b has been reported to inhibit autophagy in PCa via the PTEN/Akt/mTOR pathway [56]. Furthermore, miR-30a is well documented to directly target Beclin-1 in cancer cells [43]. As reported, the PI3K/Akt/mTOR pathway can be activated by downregulating Beclin-1 in lung cancer cells [57]. However, mTOR inhibitors may prevent the reduction of Beclin-1 [58], and artesunate induces Beclin-1-mediated autophagy through the suppression of mTOR signaling [59]. Overall, miR-5681b can suppress PCa cell autophagy through targeted repression of Beclin-1.

Tumor growth can be promoted or inhibited through the regulation of immune cell homeostasis, activation, proliferation, and differentiation by activating autophagy [60]. The autophagy activator Rapamycin was employed in the treatment of PC3 cells with simultaneous overexpression of miR-5681b. Our results demonstrated that autophagy activation stimulated PCa cell proliferation and inhibited apoptosis. Consistent with these findings, Rapamycin has been shown to induce autophagy in human neuroblastoma cells, as evidenced by elevated levels of LC3-II, LC3-II/LC3-I, and Beclin-1 and diminished levels of p62 [61]. PCa cell proliferation can be increased by

activating autophagy before treatment with olaparib [62]. In addition, treatment with Rapamycin restores autophagy and suppresses mTORC1-mediated apoptosis in cancer cells, with its anti-apoptotic effects linked to reduced p62 levels [63]. Taken together, the effects of miR-5681b on PCa cell apoptosis and proliferation can be partially reversed by autophagy activation. Our xenograft tumor experiments in nude mice also confirmed that miR-5681b inhibited Beclin-1 to regulate autophagy, thereby affecting the *in vivo* growth of PCa.

In conclusion, this study elucidates the mechanism of miR-5681b in regulating autophagy-related protein Beclin-1 and then affecting PCa cell proliferation and apoptosis. Bioinformatics and literature analyses identified miR-5681b as a significantly downregulated upstream regulator of Beclin-1 in PCa. Overexpression of miR-5681b suppressed autophagy by targeting Beclin-1, thereby inhibiting proliferation and accelerating apoptosis in PCa cells. Nevertheless, several limitations of this study should be acknowledged. On the one hand, currently, no studies have reported an association between miR-5681b and PCa. However, this study for the first time demonstrated that miR-5681b expression was significantly downregulated in PCa cells. Overexpression of miR-5681b repressed cell autophagy by targeting and suppressing Beclin-1, thereby protecting against the growth of PCa. Additionally, previous studies have unveiled that miR-21 serves as a potential biomarker for PCa and plays a critical role in PCa cell migration and proliferation [64, 65]. In addition to miR-21, miR-1246 also functions as a biomarker for PCa and is closely associated with higher pathological grade, positive metastasis, and poor prognosis of PCa [66]. These results imply that low miR-5681b expression may be related to the occurrence, progression, and prognosis of PCa. Future studies will further explore whether miR-5681b could serve as a potential biomarker for the occurrence and prognosis of PCa at the clinical level. On the other hand, additional investigation is required to analyze the upstream targets of miR-5681b. In the future, more comprehensive studies will be conducted to elucidate the regulatory mechanism underlying PCa involving miR-5681b.

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Supplementary data are available in the online version of the paper.

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Figure Legends

Figure 1. miR-5681b is lowly expressed in PCa cell lines and targets Beclin-1. A-B) miR-5681b was significantly under-expressed in PCa and showed targeted binding to Beclin-1, with the results obtained based on bioinformatics informatics combined with literature analysis; C, F) RT-qPCR to determine the level of miR-5681b in cells; D) The targeted binding sites between Beclin-1 and miR-5681b were obtained from the miRanda database; E) Dual-luciferase assay; G) RT-qPCR to measure the mRNA level of Beclin-1 in cells; H) Western blot to determine the level of Beclin-1 protein. All cell experiments were biologically repeated three times (n=3), and all technical tests were repeated three times. The data were represented by mean±standard deviation, and independent sample *t*-tests were used for comparisons between the two groups of data in panel E). One-way

ANOVA was implemented for inter-group comparisons of data in panels C, F-H), followed by Tukey's multiple comparison test. $p < 0.05$ indicated a significant difference

Figure 2. miR-5681b inhibits the proliferation and facilitates apoptosis of PCa cells. A) CCK-8 assay to assess cell viability; B) EdU staining to evaluate cell proliferation; C) FCM assay to assess cell apoptosis; D) Western blot to determine the levels of cleaved caspase-3, Bax, and Bcl-2 proteins. All cell experiments were biologically repeated three times (n=3), and all technical tests were repeated three times. Data were expressed as mean±standard deviation. One-way ANOVA was used for inter-group comparisons, followed by Tukey's multiple comparison test. $p < 0.05$ indicated a significant difference

Figure 3. Beclin-1 upregulation partially reverses the effects of miR-5681b on PCa cell proliferation and apoptosis. A) Beclin-1 mRNA level was determined by RT-qPCR; B, F) The levels of Beclin-1, Bax, Bcl-2, and cleaved caspase-3 proteins were measured by western blot; C) Cell viability was assessed by CCK8 assay; D) Cell proliferation was evaluated by EdU staining; E) Cell apoptosis was assessed by FCM assay. All cell experiments were biologically repeated three times (n=3), and all technical tests were repeated three times. Data were presented as mean±standard deviation. One-way ANOVA was implemented for data comparisons among multiple groups, and Tukey's multiple comparison test was used for the post-hoc test. $p < 0.05$ indicated a significant difference

Figure 4. miR-5681b regulates PCa cell autophagy by targeting Beclin-1. Western blot to measure the levels of autophagy-related proteins LC3-II, LC3-I, and p62. The repetitive cell experiments were conducted three times. Data were presented as mean±standard deviation. One-way ANOVA was implemented for data comparisons among multiple groups, and Tukey's multiple comparison test was used for the post-hoc test. $p < 0.05$ indicated a significant difference.

Figure 5. Activation of autophagy partially averted the regulatory effects of miR-5681b on PCa cell apoptosis and proliferation. A) The levels of autophagy-related proteins LC3-II, LC3-I, p62

were determined by western blot; B) Cell viability was evaluated by CCK-8 assay; C) Cell proliferation was evaluated by EdU staining; Data were presented as mean±standard deviation. One-way ANOVA was implemented for data comparisons among multiple groups, followed by Tukey's multiple comparison test. $p < 0.05$ indicated a significant difference.

Figure 6. Activation of autophagy partially averted the regulatory effects of miR-5681b on PCa cell apoptosis and proliferation. A) The levels of apoptosis-related proteins Bax and Bcl-2 were determined by western blot; B) FCM to assess cell apoptosis. All cell experiments were biologically repeated three times (n=3), and all technical tests were repeated three times. Data were presented as mean±standard deviation. One-way ANOVA was implemented for data comparisons among multiple groups, followed by Tukey's multiple comparison test. $p < 0.05$ indicated a significant difference

Figure 7. miR-5681b/Beclin-1 regulates autophagy to affects the *in vivo* growth of PCa. A) RT-qPCR detection of miR-5681b levels in tumor tissues from nude mice; B-C) RT-qPCR and western blot to measure Beclin-1 levels in tumor tissues from nude mice; D) TUNEL assay for detecting apoptosis in tumor tissues; E) IHC detection of Ki-67-positive cells to cell proliferation in tumor tissues; F) Western blot measurement of autophagy-related proteins (LC3-II, LC3-I, and p62) in tumor tissues; G) Tumor images and weight quantification map. All animal experiments were performed with six biological replicates (n=6), and each technical test was repeated three times. Data are presented as mean ± standard deviation. Intergroup comparisons were analyzed with one-way ANOVA, followed by Tukey's multiple comparisons test. $p < 0.05$ indicated a significant difference.

674 **Table 1.** Primer sequences.

Gene	Forward 5'-3'	Reverse 5'-3'
Beclin1	GGCGGCTCCTATTCCATCAA	TGACCAGGTAAAGCTTAGATGTCA
miR-5681b	GTATTGCCACCCTTTCTAGTC	GTCCAGTTTTTTTTTTTTTTGAAGAG
U6	CTCGCTTCGGCAGCACA	TTCTTGGGTAGTTTGCAGTT
GAPDH	GATTGTTGCCATCAACGACC	GTGCAGGATGCATTGCTGAC

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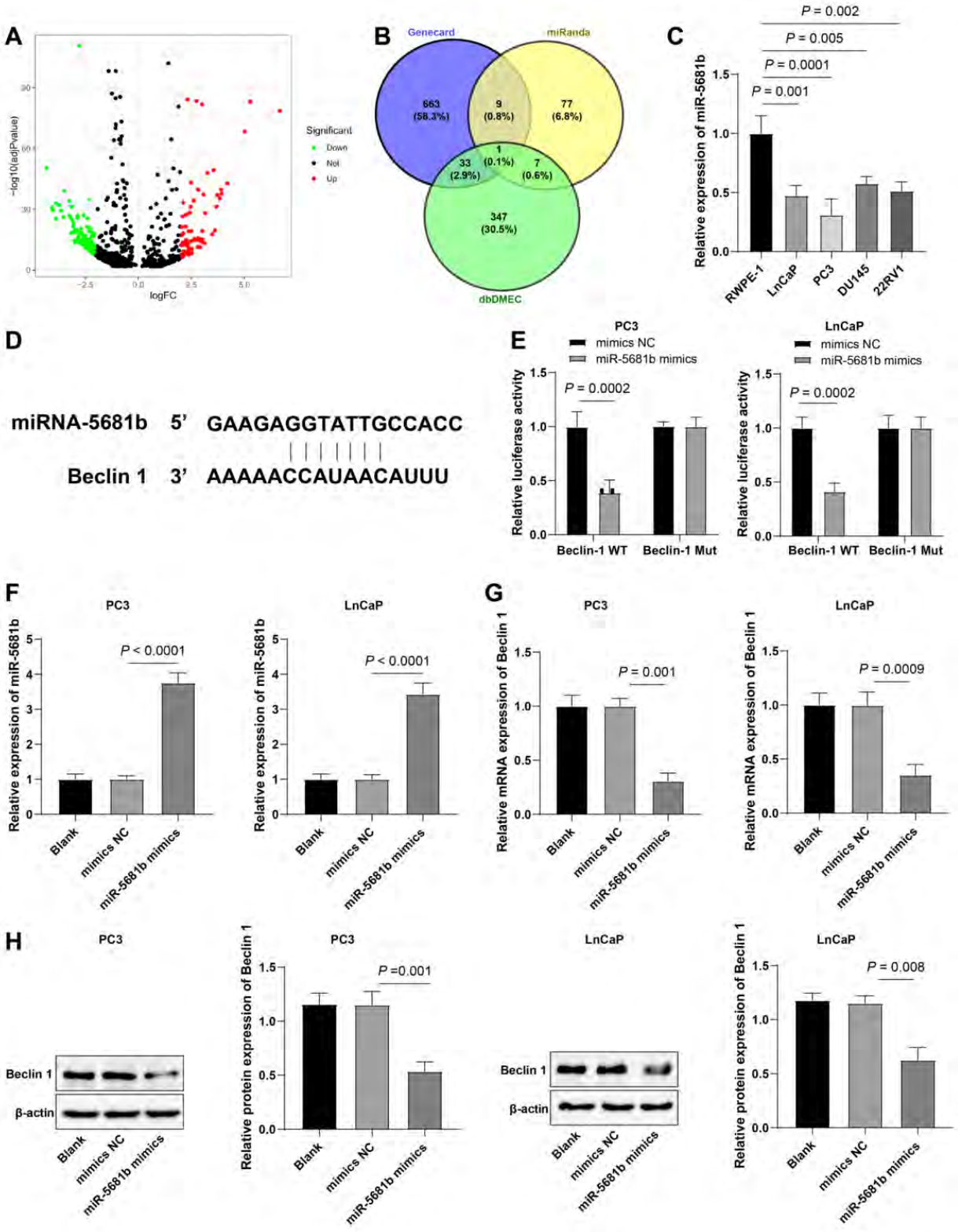
Fig. 1 [Download full resolution image]



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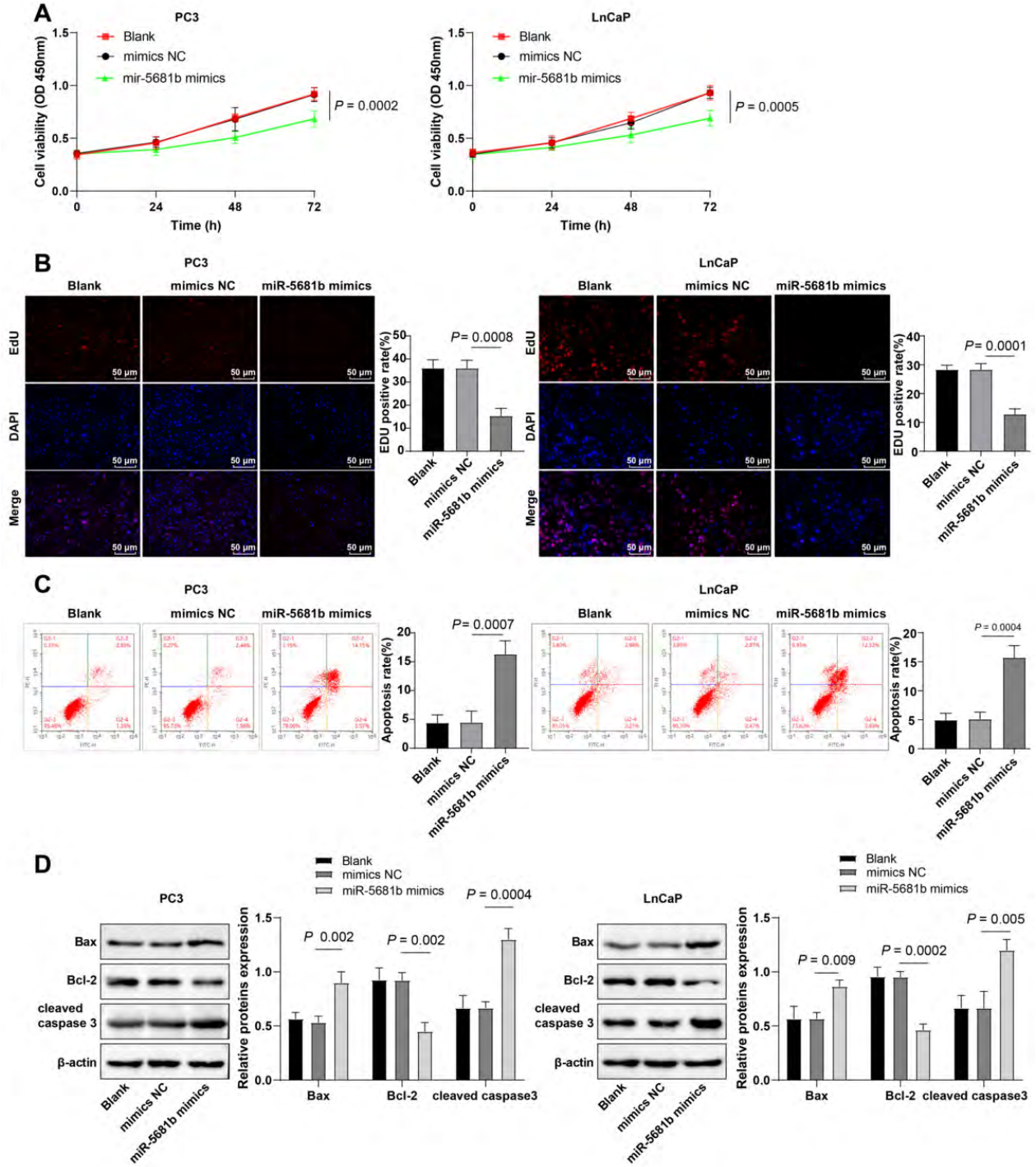

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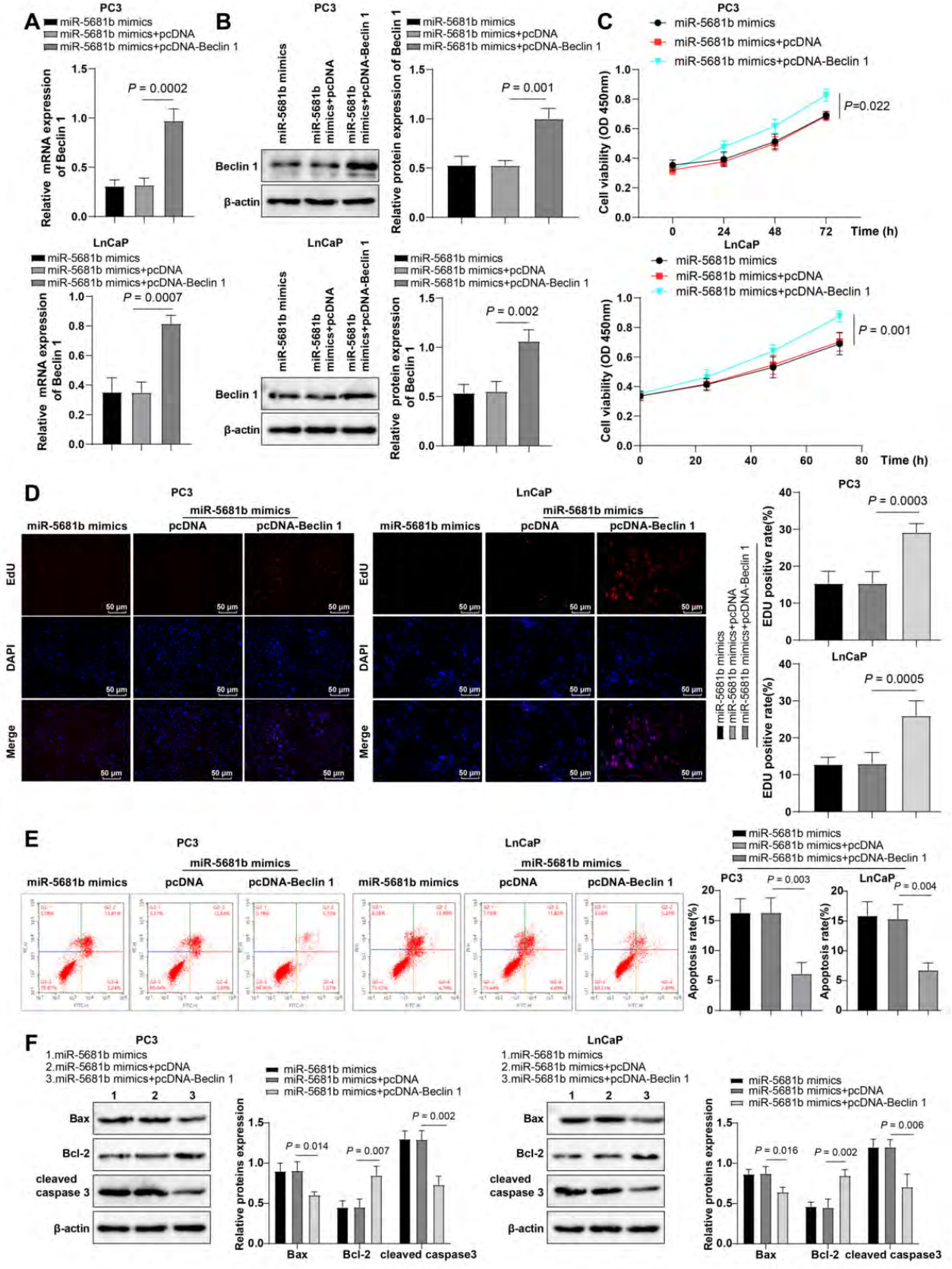

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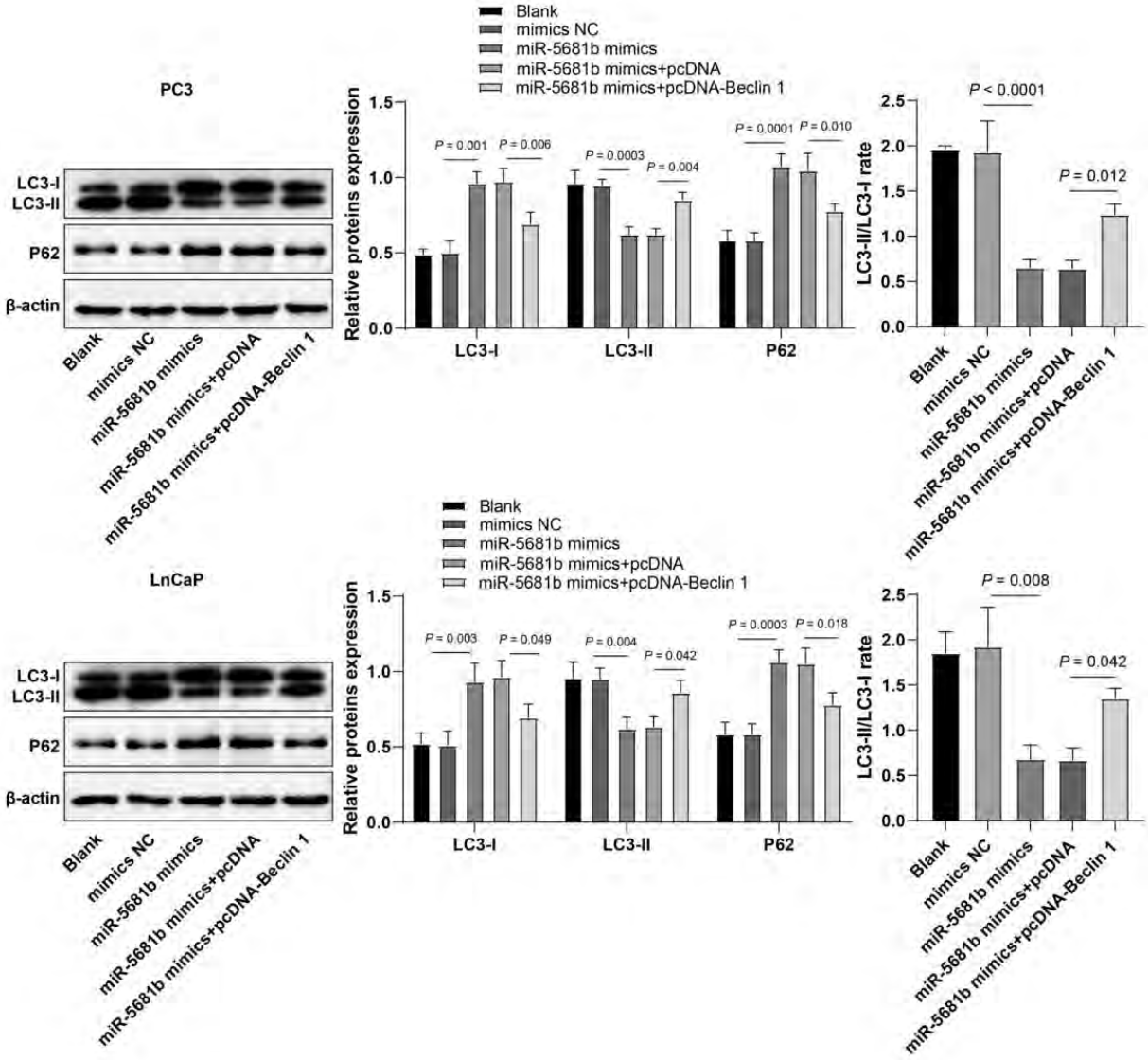

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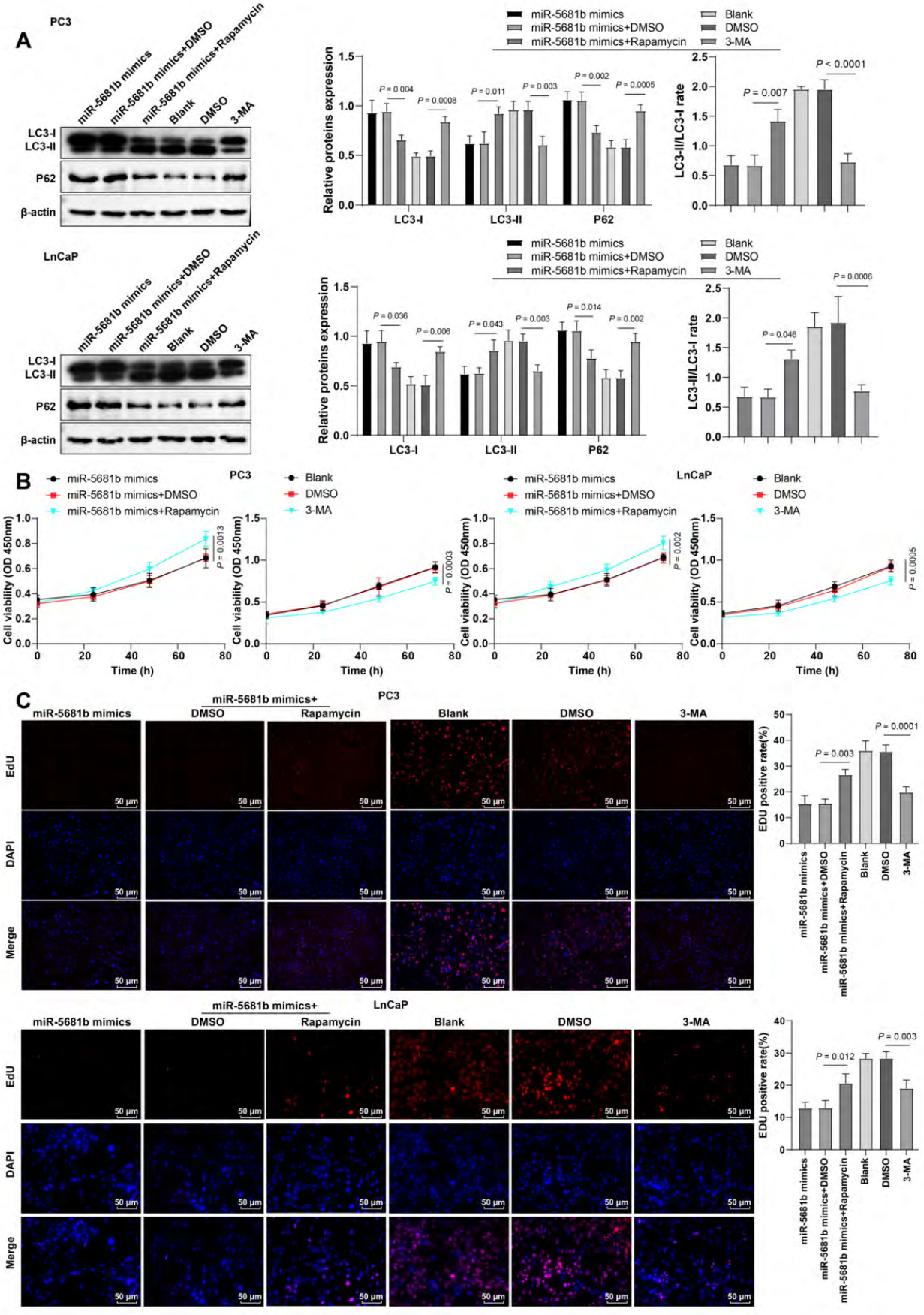

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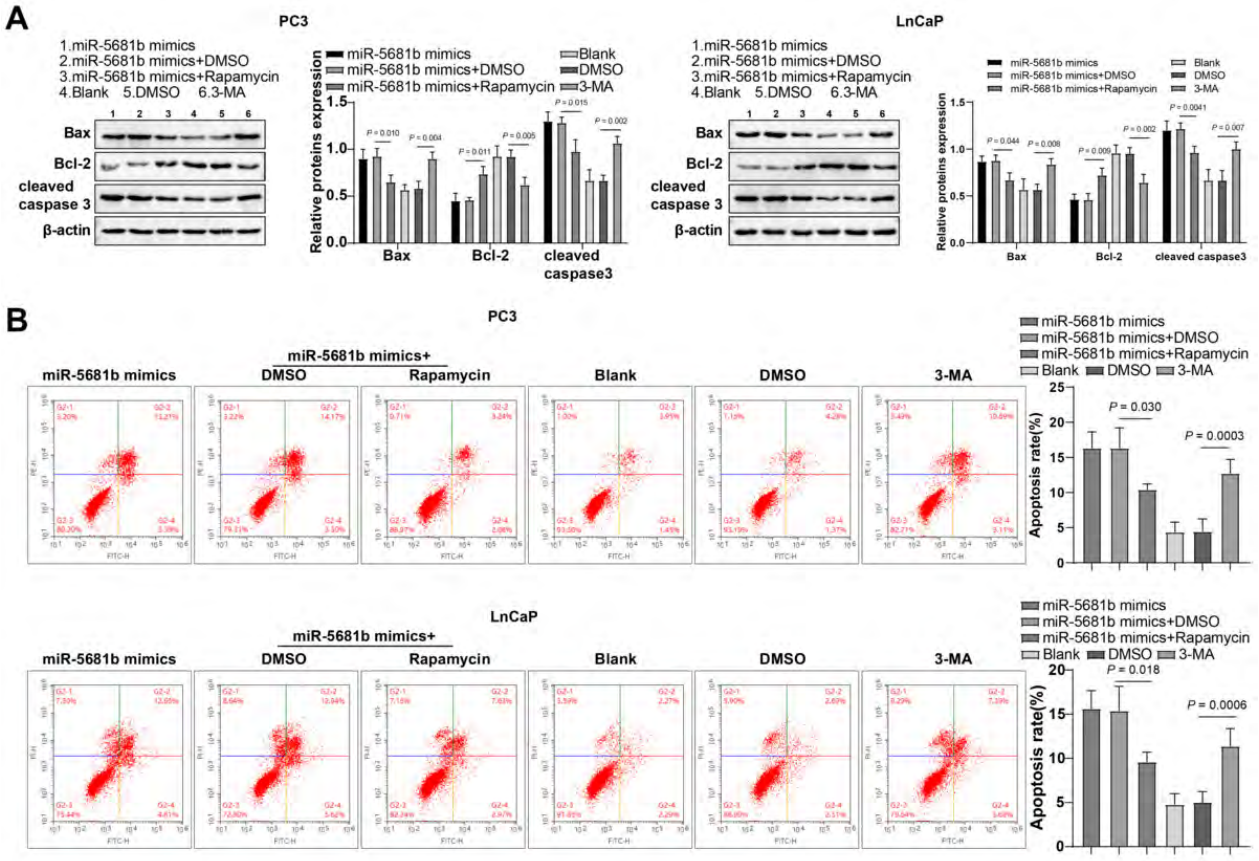

Fig. 7 [Download full resolution image]