Rb deficiency accelerates progression of carcinoma of the urinary bladder *in vivo* and *in vitro* through inhibiting autophagy and apoptosis

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Abstract. Urinary bladder cancer is known as a common cancer diagnosed across the world and results in significant mortality and morbidity rates among patients. The retinoblastoma (Rb) protein, as a main tumor suppressor, controls cellular responses to potentially oncogenic stimulation. Rb phosphorylation could disrupt E2F complex formation, resulting in diverse transcription factor dysfunction. In our study, we investigated how Rb is involved in controlling urinary bladder cancer progression. The results indicate that Rb expression is reduced in mice with urinary bladder tumor, and its suppression leads to urinary bladder cancer progression *in vivo* and *in vitro*. Rb mutation directly results in tumor size with lower survival rate *in vivo*. Rb knockdown *in vitro* promoted bladder tumor cell proliferation, migration and invasion. Interestingly, Rb knockout and knockdown result in autophagy and apoptosis inhibition via suppressing p53 and caspase-3 signaling pathways, enhancing bladder cancer development *in vitro* and *in vivo*. These findings reveal that Rb deficiency accelerated urinary bladder cancer progression, exposing an important role of Rb in suppressing urinary bladder cancer for treatment in the future.

Introduction

Bladder cancer is one of the most common genitourinary cancers (1). More than 90% of bladder cancer cases are diagnosed as bladder urothelial carcinoma (2). Approximately 75-85% of the patients harbored superficial bladder cancer (3). Despite transurethral resection of bladder tumor and intravesical therapy, 1-45% of cases progress to invasive bladder cancer, known as muscle-invasive bladder cancer, within 5 years (4). Up until now, radical cystectomy is the mainstay therapy for muscle-invasive bladder cancer (5). However, the exact molecular mechanisms of bladder tumor formation and progression are not yet completely understood. Thus, genetic and molecular factors may both play an important role in the progression of bladder cancer, which might be an effective target for bladder cancer treatment in the future.

The Rb gene is a recessive gene, located on chromosome 13q14. It is the prototype of a tumor suppressor gene and is well described (6,7). Although the gene was named for its prominent role in the genesis of retinoblastoma, Rb-inactivation seems to be crucial for the development of a variety of other cancers and tumors, including liver cancer, breast cancer and lung cancer (8). Inactivation of the Rb gene, caused by mutations of the coding region or promoter region, as well as the loss of heterozygosity have been reported, which was an important factor, contributing to tumor or cancer progression via p53 and E2F3 modulation (9). However, whether Rb could be a therapeutic target for bladder cancer for future is not clear. Hence, we investigated the role of Rb in bladder cancer.

Autophagy is a process for major intracellular degradation, occuring when the cells undergo stress conditions, including exposure of radiation, nutrient starvation, or cytotoxic compounds, and suffering from cancer, to enhance cell survival or to result in the type II programmed cell death (10). Beclin1 and microtubule-associated protein 1A/1B-light chain 3 (LC3), which are two hallmarks of autophagy, modulating the initialization of mammalian autophagy (11). Beclin1 plays an important role involving in the signaling pathway for autophagy induction and in the onset of the autophagosome formation (12). In addition, apoptosis (programmed cell death), is instead an important physiological process, which occurs in cells during development and normal cellular processes (13). Apoptosis is induced by several cellular signals which alter mitochondrial permeability, leading to a cascade of events such as the release of apoptosis activators from mitochondria (14). Rb has been reported before to be associated with autophagy and apoptosis modulation.

Hence, in the study, Rb knockout mice were used to investigate the role of Rb in urinary bladder cancer progression. Our results indicated that Rb was directly involved in the progress of bladder cancer via suppression of autophagy and apoptosis through p53 and caspase-3 signaling pathways. Rb deficiency is able to accelerate urinary bladder cancer development by

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inhibiting autophagy and apoptosis and promoting cancer cell proliferation.

Materials and methods

Animals. Thirty male, 6-week-old B6 (body weight, 20±20 g) were purchased from Experimental Animal Center of Laboratory Animal Center of Fudan University. The thirty male, 6-week-old B6 Rb knockout mice (Rb^{tm3Tyj}) (body weight, 20±20 g) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All the mice were carefully maintained at room temperature on a 12-h light-dark cycle, with free access to chow and water. This study was approved by the Ethics Committee on Animal Research at the Tangdu Hospital, The Fourth Military Medical University (Shaanxi, China). The mice were randomly divided into 2 groups: i) the Control-WT group; and ii) the Control-$Rb^{-/-}$ (Rb^{tm3Tyj}) group. BIU87 cells ($1x10^7$ cells) were suspended in 100 μl serum-free medium and injected subcutaneously into the left flank of the 6-week old male B6 mice. Tumor size was measured with digital caliper and calculated. Tumor volume were measured every seven days and at the end of ~7 weeks, mice were sacrificed. Tumors were excised, weighed, fixed in 10% neutral formalin, and embedded in paraffin for histological and western blot analysis.

Cells culture. The bladder cancer BIU87 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). They were cultured at the permissive temperature (37˚C) in DMEM medium (GibcoBRL Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and supplemented with 1% penicillin-streptomycin-neomycin provided by GibcoBRL Life Technologies with a humidified incubator in 5% CO_2 atmosphere. Additional introduction of Rb or a control vector into BIU87 cells were administered.

ELISA analysis. The levels of autophagy-related signals, including Beclin1 and MAP1LC3B levels in serum from animals were determined by ELISA, following the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA). Briefly, polyclonal mouse anti-rabbit antibodies were used as capturing antibodies and biotinylated polyclonal mouse anti-rabbit for detection, and the standard curve of these signals was created. Color changes were determined at 450 nm.

Colony formation assays. One hundred bladder cancer BIU87 cells after the vector control or siRb treatment per well in 60-mm plates were cultured in 10% FBS DMEM for 24 h. After another 7 days of incubation, the cell colonies were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min and then stained by Giemsa for 30 min. Each clone with >50 cells was evaluated. Clone forming efficiency for cells was calculated based on colonies/number of inoculated cells x 100%.

Wound width assays. Wound-healing assays were carried out using migration culture dish inserts. Bladder cancer cells of BIU87 after the vector control or siRb treatment were seeded in the chambers of the culture dish insert and transfected. Twenty-four hours after transfection, the insert was removed and fresh culture medium was added to start the migration process. Images were acquired after 0 and 24 h using a Zeiss Axiovert 24 light microscope and an Axiocam MRc camera.

Transwell migration and invasion assay. Bladder cancer cells after treatment were seeded into the upper chamber of a Transwell insert pre-coated with 5 μg/ml fibronectin for migration or a BD™ Matrigel invasion chamber for invasion. Medium with 10% serum was put in the lower chamber as a chemo-attractant, and cells were then incubated for 4 h of migration. Non-migratory cells were removed from the upper chamber by a cotton bud. The cells on the lower insert surface were stained with Diff-Quick. Cells were evaluated as the number of cells observed in five different microscopic fields of two independent inserts. For invasion assay, $5x10^4$ cells were placed on the upper chamber of each insert coated with 150 mg Matrigel. The lower chamber of Transwell was then filled with DMEM medium with 20% FBS. After incubation for invasion assays, the upper surface of the membrane was wiped with a cotton tip and cells attached to the lower surface were stained with crystal violet. The invaded cells were captured and counted in five random fields.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays. Apoptosis assay of samples was determined by TUNEL used an *In Situ* Cell Death Detection kit, Fluorescein (Roche Applied Science, USA) according to the manufacturer's protocol. The number of TUNEL-positive cells was counted under a fluorescence microscope. The percentage of apoptotic cells was calculated. Tissue sections were counter-stained with hematoxylin.

Western blot analysis. The bladder cancer cells and tumor tissue samples were homogenized into 10% (wt/vol) hypotonic buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 μg/ml leupeptin, 1 mM Pefabloc SC, 50 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor, 4 mM benzamidine) to yield a homogenate. Then the final supernatants were obtained by centrifugation at 12,000 rpm for 20 min. Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific, USA) with bovine serum albumin as a standard. The total protein extract will be used for western blot analysis. Equal amounts of total protein of tissues were subjected to 10 or 12% SDS-PAGE followed by immunoblotting using the following primary polyclonal antibodies: rabbit anti-GAPDH (Cell Signaling Technology), rabbit anti-Beclin1 (Cell Signaling Technology), rabbit anti-PARP (Cell Signaling Technology), rabbit anti-E2F3 (Abcam, USA), rabbit anti-Bax (Abcam), rabbit anti-caspase-3 (Abcam), mouse anti-Bcl-2 (Cell Signaling Technology), rabbit anti-P-Rb (Cell Signaling Technology), rabbit anti-LC3-I (Cell Signaling Technology), rabbit anti-LC3-II (Cell Signaling Technology), rabbit anti-Rb (Cell Signaling Technology), mouse anti-Bak (Abcam), rabbit anti-mdm2 (Cell Signaling Technology), mouse anti-Bid (Abcam), mouse anti-Apaf (Abcam), rabbit anti-p53 (Cell Signaling Technology), rabbit anti-PTEN (Cell Signaling Technology), rabbit anti-PI3K (Cell Signaling Technology), rabbit anti-p-AKT (Cell

Signaling Technology), rabbit anti-AKT (Cell Signaling Technology), mouse anti-p21 (Abcam), mouse anti-MAP1 (Abcam) and mouse anti-Cyto-*c* (Abcam). Immunoreactive bands were visualized by ECL Immunoblot Detection system (Pierce Biotechnology, Inc., Rockford, IL, USA) and exposed to Kodak (Eastman Kodak Co., USA) X-ray film. Each protein expression level was defined as grey value (Version 1.4.2b, Mac OS X, ImageJ, National Institutes of Health, USA) and standardized to housekeeping genes (GAPDH) and expressed as a fold of control.

Real-time RT-qPCR. Total RNA from bladder cancer cells and tumors were isolated using TRIzol (Invitrogen, USA) following the manufacturer's instructions. The cDNA was synthesized using SuperScript II reverse transcriptase (Thermo Fisher Scientific). Quantitative PCR was performed with SYBR Green Real-Time PCR Master mix (Thermo Fisher Scientific). Finally, the quantitative expression data were collected and analyzed by a 7900 Real-time PCR system (Applied Biosystems, USA). Primers were designed to determine endogenous genes showing as follows and GAPDH using as the endogenous control. Bax forward, 5'-CAG ACG TAG CAA GAC GTT AC-3'; reverse 5'-GTG AGA GTT GCT GGC TTG ATA-3'. Bak forward, 5'-CCA GAC TTC GTA TCT ACG AGG TCG-3'; reverse 5'-GCT CAA TGC ATA GAG TAC TTT TAC-3'. Rb forward, 5'-AAC CCA GGA AGG AAT GGC T-3'; reverse 5'-CTG CGT TCA GGT GAT TGA TG-3'. p53 forward, 5'-CTA CTG CCT GCT TTG CGG CGT-3'; reverse, 5'-GAA GCG GCG TAG GTG CTG AG-3'. Apaf forward, 5'-CGC CAC CGC CAT CTT CTC CA-3'; reverse, 5'-GCA CAA GGC AGC CAG AAG GC-3'. Bid forward, 5'-AGG ATC GCG CTT AGC ATA CTT G-3'; reverse 5'-AAC TGT TCA ATC TCT GTG CTC CGT-3'. GAPDH forward, 5'-CTA AGT CGA ACG CAG ACA GTC AG-3'; reverse, 5'-AAC ATA CCA TCC ACG ACA CGC TC-3'.

Immunofluorescence assays. The tumor tissue in each group was fixed with 10% buffered formalin, imbedded in paraffin and sliced into 4 μm to 5 μm thick sections. Immunofluorescent assay of Cyto-c and caspase-3 were performed according to the manufacturer's instructions. After induction by conditioned culture medium, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS containing 0.5% BSA (PBS-BSA) for 30 min. The cells were subsequently incubated with LC3-II, Beclin1 and MAP1 for 30 min, followed by labeling with Alexa Fluor 488- and 594-conjugated rabbit anti-mouse or goat anti-rabbit IgG antibody. The cells were viewed under a fluorescent microscope.

Immunohistochemistry analysis. Human bladder or bladder cancer tissue samples and animal model bladder cancer tissue specimans were fixed in paraformaldehyde and embedded in paraffn. For hematoxylin and eosin staining (H&E staining), the bladder tumor sections were incubated in a hematoxylin solution for 15 min and then counterstained with eosin for 5 min. After 3 μm thickness sectioning, paraffn-embedded bladder cancer tissues were immunostained with p21, p53, E2F3, caspase-3, Bcl-2 and Ki-67 antibodies. All of the slides were finally observed with x200 magnification by a microscope.

Statistical analysis. Every experiment in our study was conducted at least three times. All data present the mean ± SEM. from three independent experiments. Student's t-test was used for statistical analysis.

Results

Rb-knockout was involved in urinary bladder tumor development. Rb is well known to be involved in many cellular processes, including cell proliferation, apoptosis, invasion, autophagy and migration (15). Previous studies have confirmed that Rb suppression resulted in tumor progression in different cancers, including liver cancer, lung cancer and ovarian cancer (16). Thus, here we attempted to clarify if Rb was involved in urinary bladder cancer progression. Rb knockout mice were used in our study. Fig. 1A shows that, Rb protein levels were highly expressed in the adjacent tumor tissues of the wild-type (WT), while downregulated in the tumor tissue samples in normal mice. In Rb mutant mice (Rb^{tm3Tyj}), Rb was significantly downregulated in the tumor tissue compared to the adjacent tissue samples of Rb^{tm3Tyj} mice. Of note, Rb levels were lower in the tumor tissue samples compared to the wild-type of mice. In contrast, phosphorylated Rb was expressed highly in tumor tissue compared to the adjacent tissues in the wild-type mice. Similarly, the highly phosphorylated Rb was observed in tumor tissue samples of Rb-knockout mice (Fig. 1B). Also, the mRNA levels showed similar trends at lower levels in the tumor tissue of WT mice, while the least in tumor tissue from the Rb knockout mice (Fig. 1C). p53, as an important tumor suppressor, was well investigated in previous studies, inhibiting cancer progression associated with Rb alteration (17). p53 protein and mRNA levels were lower levels in the tumor tissue of wild-type mice compared to the adjacent parts of the mice (Fig. 1D and E). Also, in the Rb knockout mice, p53 protein and mRNA levels were much lower in the tumor tissue. E2F3 was investigated, and it was expressed highly in the tumor tissue of wild-type mice, while being significantly upregulated in Rb-knockout mice, suggesting that Rb deficiency might enhance E2F3 expression, promoting urinary bladder cancer progression (Fig. 1F). Collectively, the data above suggested that Rb might be a key target for urinary bladder cancer progression.

Rb-deficiency promotes urinary bladder tumor growth in nude mice. To confirm whether Rb could directly influence urinary bladder cancer progression, we calculated the tumor size in the wild-type mice and Rb-knockout mice. We found that the tumor size was much higher than that in the WT group, suggetsing that Rb was, at least partly, involved in the urinary bladder cancer development (Fig. 2A). Also, the survival rate indicated that Rb knockout promote the animal death, further suggesting that Rb was of importance in urinary bladder cancer progression (Fig. 2B).

Rb-deficiency-induced urinary tumor growth is related to autophagy and apoptosis. Autophagy and apoptosis are two main molecular mechanism, which regulate growth and progression of many tumors (18). Immunohistochemical analysis suggested that E2F3 was highly expressed in Rb

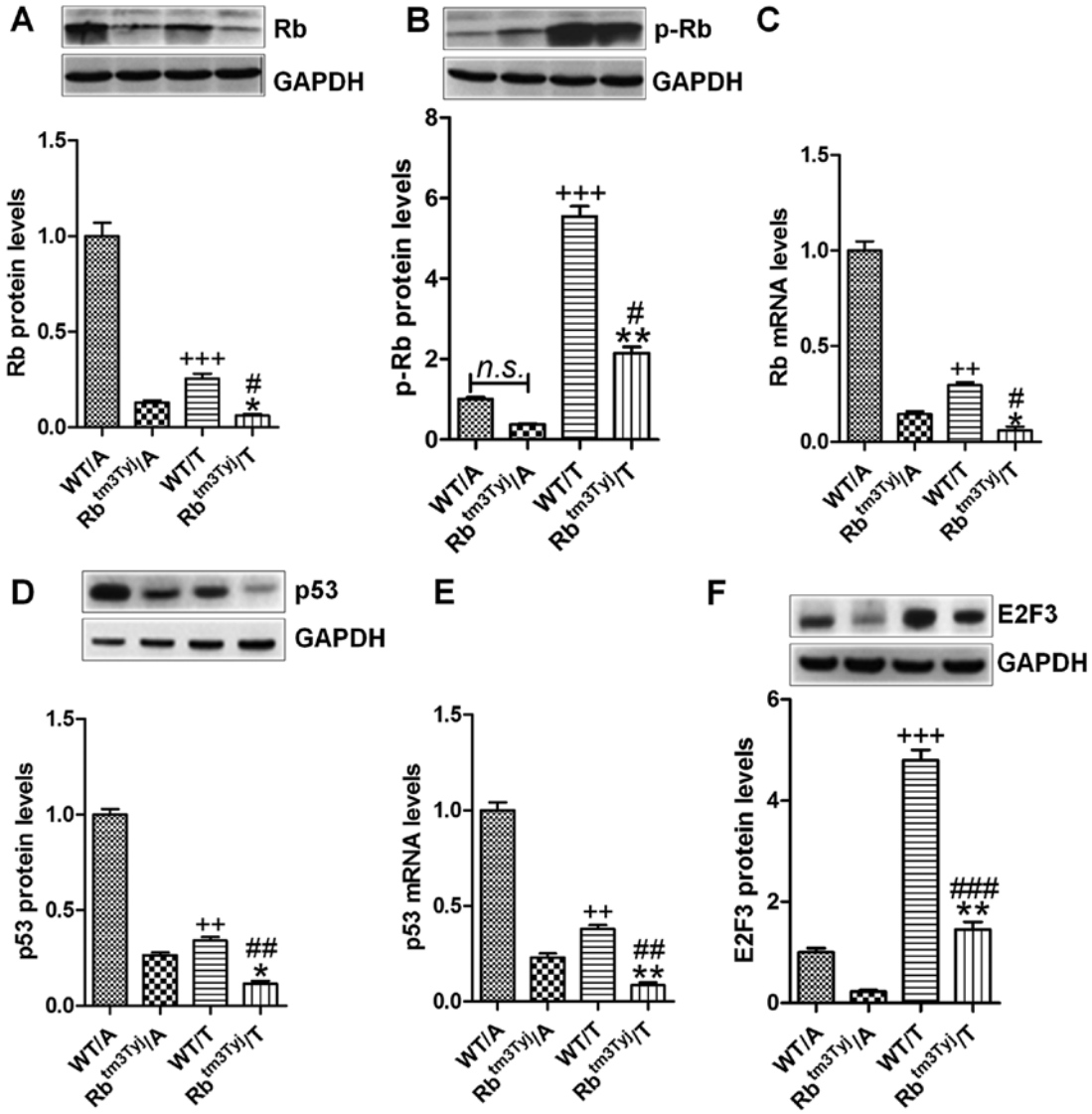

Figure 1. Rb-knockout was involved in urinary bladder tumor development. (A and B) Western blot analysis was performed to determine Rb and p-Rb levels in tumor and adjacent cancer tissue in normal mice and the Rb-knockout mice (Rb^{tm3Tyj}). (C) RT-qPCR was used to determine Rb mRNA levels in different groups of normal mice and Rb-knockout mice. p53 protein levels (D) and mRNA levels (E) were calculated in different groups of normal mice and Rb-knockout mice. (F) E2F3 protein levels were evaluated in different groups from the normal mice and Rb-knockout mice. Data are expressed as the mean ± SEM. $^{++}p<0.01$ and $^{+++}p<0.001$ versus the WT/A group; $^{*}p<0.05$ and $^{**}p<0.01$ versus the Rb^{tm3Tyj} group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ versus the WT/T group.

knockout mice in comparison to the wild-type, while p53 was downregulated in mice in the absence of Rb compared to the wild-type ones (Fig. 3A). Additionally, caspase-3 was significantly reduced for Rb deficiency, inhibiting apoptotic response in mice with urinary bladder cancer. In contrast, Bcl-2 was highly expressed in tumor mice without Rb expression (Fig. 3B). Bcl-2, as an important anti-apoptotic factor, is always overexpressed in tumor tissue samples (19). Finally, TUNEL and Ki-67 were evaluated in both two tumor tissue samples from the wild-type mice and Rb-deficient mice. As shown in Fig. 3C, TUNEL levels were downregulated in Rb-deficient mice, indicating that apoptosis was suppressed for Rb knockout. However, Ki-67, a factor in tumor progression, was upregulated in tumor tissue samples without Rb expression. The results indicated that Rb deficiency was the main reason contributing to autophagy and apoptosis suppression and leading to urinary bladder cancer development.

Apoptosis suppression was involved in Re-deficiency mice with urinary bladder cancer. As shown above, we supposed that apoptosis was the main contributor, regulating bladder cancer development for Rb absence. Thus, we attempted to investigate which molecular mechnism was included. Mitochondaria dysfunction-induced apoptosis is the main protocol, resulting in cell death in many cellular progresses (20). Here, the protein and mRNA levels of Bax, Bak, Bid and Apaf were found to be reduced in mice in the absence of Rb, inhibiting apoptotic response in urinary bladder cancer (Fig. 4A and B). Furthermore, immunofluorescent analysis indicated that Cyto-c and caspase-3 were both downregulated in Rb knockout mice, which are two factors promoting apoptosis in cells and leading to cell death in tumor treatment (Fig. 4C). The data above indicated that Rb knockout resulted in urinary bladder cancer progression was attributed to apoptosis suppression through caspase-3 signaling pathway disruption.

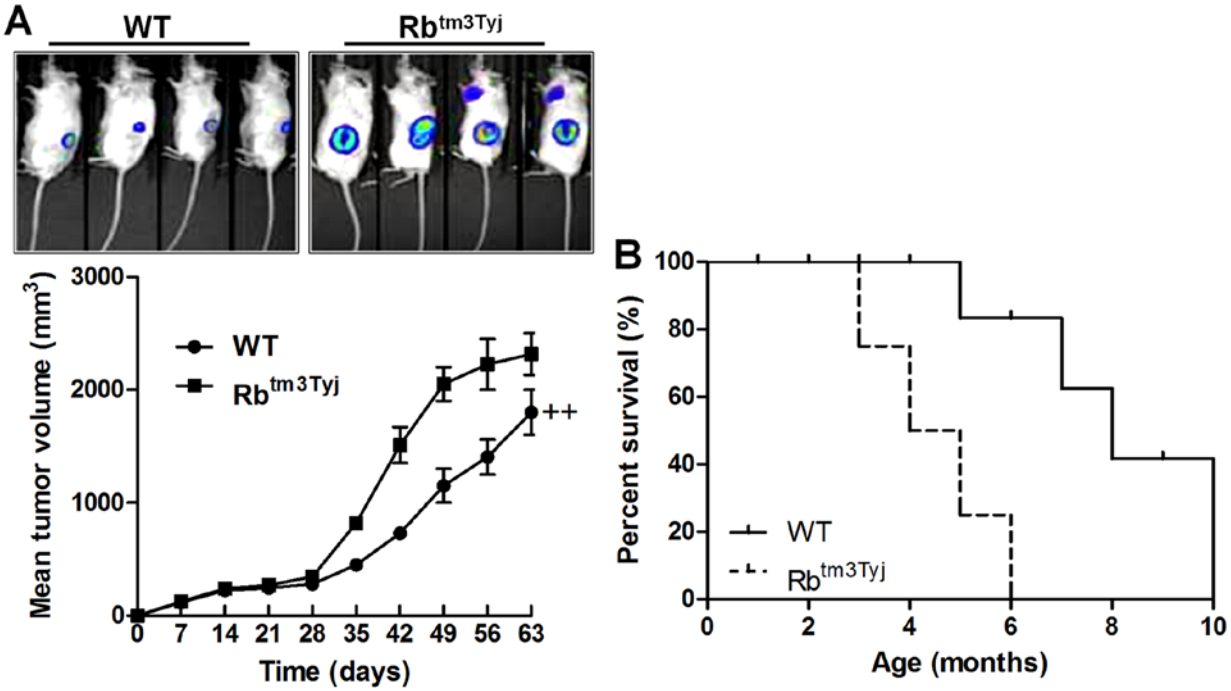

Figure 2. Rb-deficiency promotes urinary bladder tumor growth in nude mice. (A) The tumor size and volume were evaluated in normal mice and Rb-knockout mice. (B) The survival rate was calculated during the whole phase. Data are expressed as the mean ± SEM. $^{++}p<0.01$ versus the WT group.

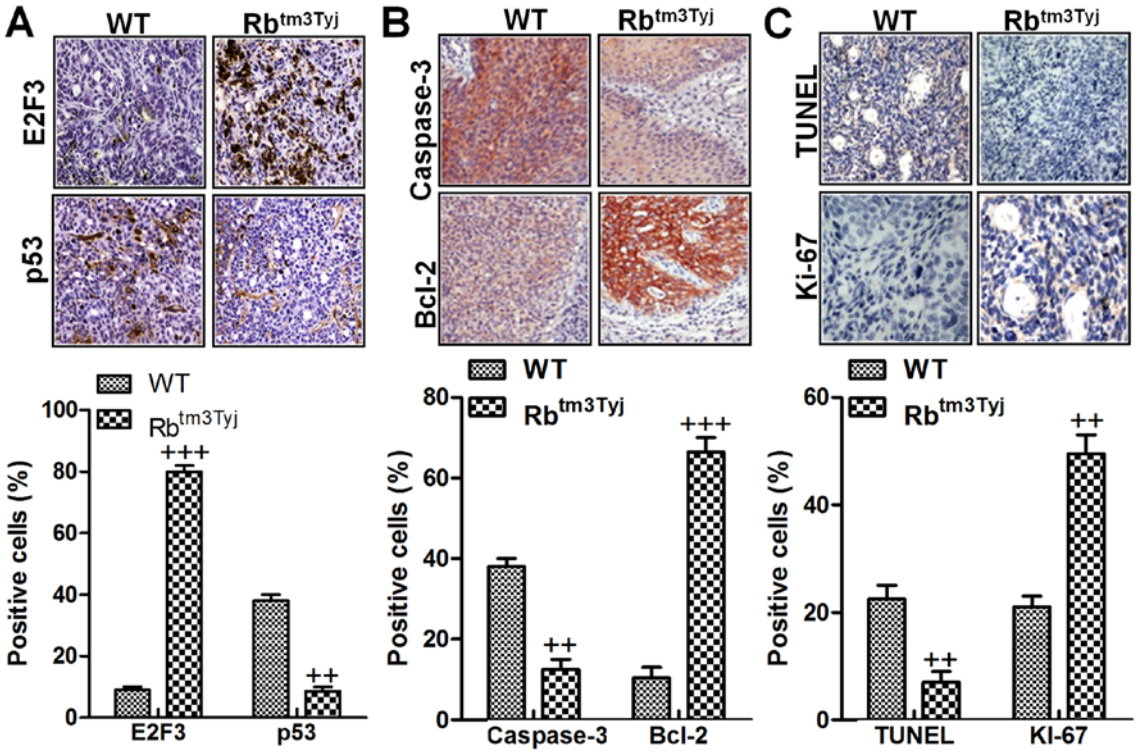

Figure 3. Rb-deficiency-induced urinary tumor growth was related to autophagy and apoptosis. Immunohistochemical analysis was used to analyze (A) E2F3 and p53, (B) caspase-3 and Bcl-2 in different groups of mice. (C) TUNEL and Ki-67 were evaluated in mice. Data are expressed as the mean ± SEM. $^{++}p<0.01$ and $^{+++}p<0.001$ versus the WT group.

Autophagy suppression is associated with urinary bladder cancer progression in Rb-knockout mice. We found that autophagy was the main mechnism regulating bladder cancer progression. Thus, in order to establish how Rb modulated urinary bladder cancer progression, the signals associated with autophagy development were investigated throuh ELISA kits, which suggested that Beclin1 and MAP1LC3B were significantly downregulated in the serum of mice without

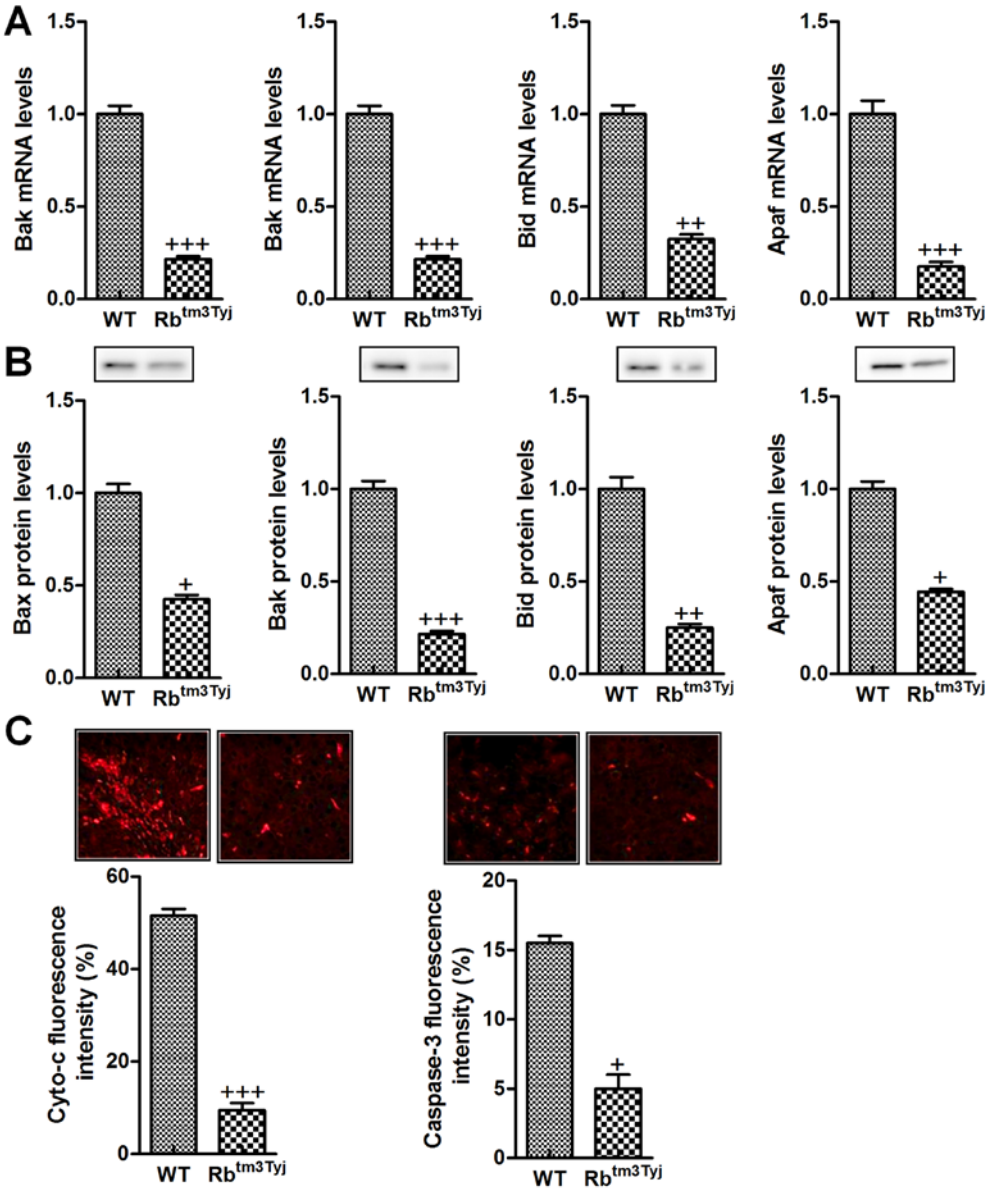

Figure 4. Apoptosis suppression is involved in Re-deficiency mice with urinary bladder cancer. (A) RT-qPCR was conducted to analyze Bax, Bak, Bid and Apaf mRNA levels in mice. (B) Western blot analysis was used to determine Bax, Bak, Bid and Apaf protein levels in mice. (C) Cyto-c and caspase-3 levels were analyzed via immunofluorescent analysis. Data are expressed as the mean ± SEM. $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ versus the WT group.

Rb expression (Fig. 5A and B). As shown in Fig. 5C, western blot analysis further indicated that Beclin1 and MAP1 were reduced in Rb knockout. In addition, LC3-I and LC3-II were both decreased in Rb absence, which have been reported as significant autophagy induction signals (Fig. 5C). Also, our data here were in line with the results above that autophagy was impeded in mice in Rb knockout. Moreover, LC3-II immunofluorescent intensity was also observed with downregulation in Rb-deficient mice (Fig. 5D). The data above confirmed that autophagy-related signals were involved in Rb-regulated urinary bladder cancer.

Rb knockout-induced urinary bladder tumor progression is dependent on p53 inhibition. p53 has been considered as a crucial signal regulating cell proliferation through its downstream signals, including p21, PTEN and PI3K (21). Fig. 6A shows that p21 was significantly downregulated in Rb knockout mice. Similarly, the protein levels of p21 were also found to be downregulated compared to the mice in wild-type group (Fig. 6B). At the same time, PTEN, the downstreaming signal of p53, was expressed at low level in Rb deficiency (Fig. 6C). In contrast, Rb knockout resulted in PI3K activation, which is important for tumor growth through AKT phosphorylation (Fig. 6D and E). mdm2 is an important factor regulated by PI3K/AKT signaling pathway and modulate cell proliferation (22). However, in our study, we found that mdm2 was not significantly altered for Rb deficiency, suggesting that Rb-regulated urinary bladder cancer was not dependent on mdm2 (Fig. 6F). Taken together, the results above indicated that p53 and its related signaling pathway was closely related to Rb-regulated urinary bladder cancer progression.

Rb knockdown leads to urinary bladder cancer cell proliferation in vitro. To further confirm that Rb was a key in urinary

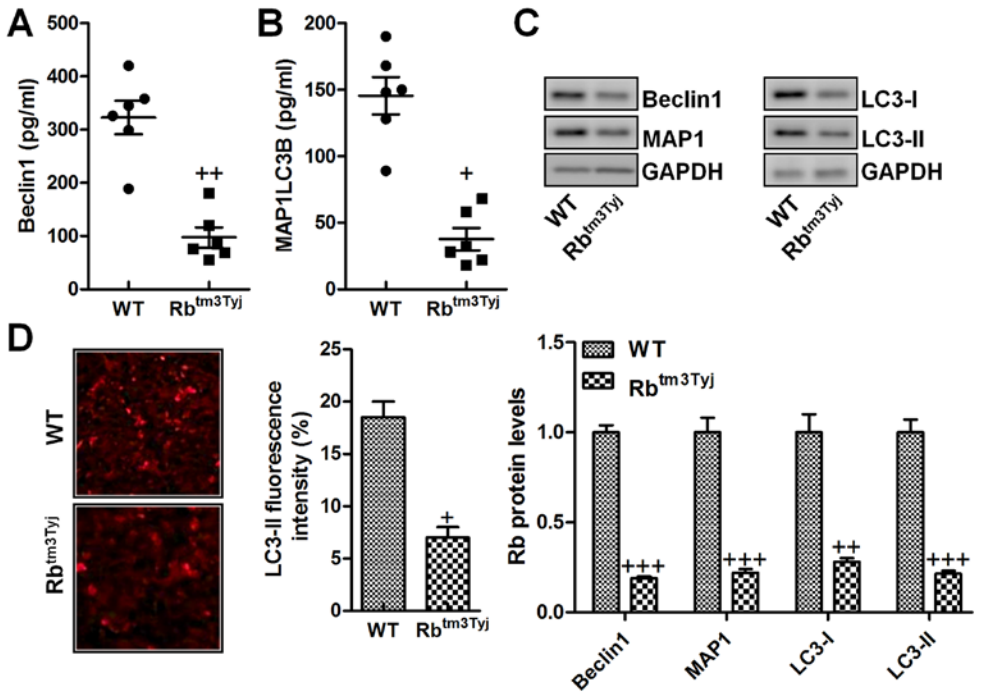

Figure 5. Autophagy suppression is associated with urinary bladder cancer progression in Rb-knockout mice. (A) Beclin1 and (B) MAP1LC3B were evaluated in the serum of mice from different groups via ELISA. (C) Western blot analysis was used to determine Beclin1, MAP1, LC3-I and LC3-II protein levels in mice. (D) Immunofluorescent analysis was used to analyze LC3-II levles in tumor tissue samples. Data are expressed as the mean ± SEM. $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ versus the WT group.

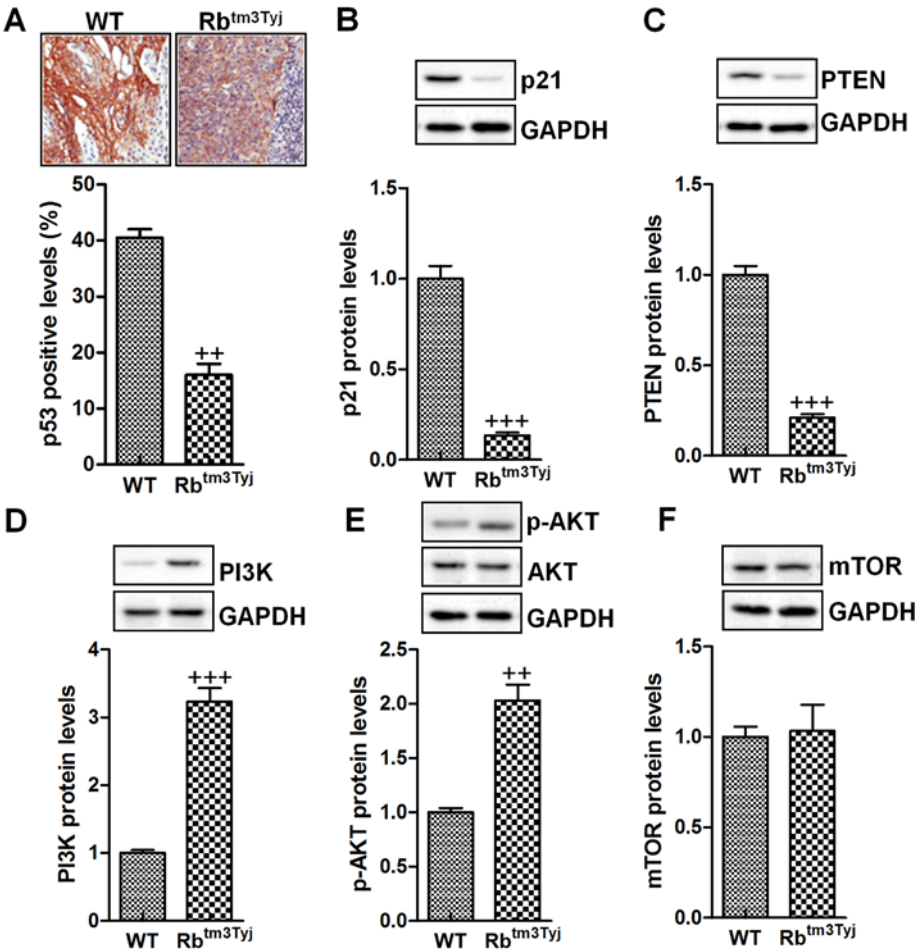

Figure 6. Rb knockout-induced urinary bladder tumor progression is dependent on p53 inhibition. (A) Immunohistochemical analysis was used to analyze p21 levels in urinary tumor tissue samplaes of mice. Western blot analysis was used to determine (B) p21, (C) PTEN, (D) PI3K, (E) p-AKT and (F) mdm2 protein levels in mice. Data are expressed as the mean ± SEM. $^{+}p<0.05$, $^{++}p<0.01$ and $^{+++}p<0.001$ versus the WT group.

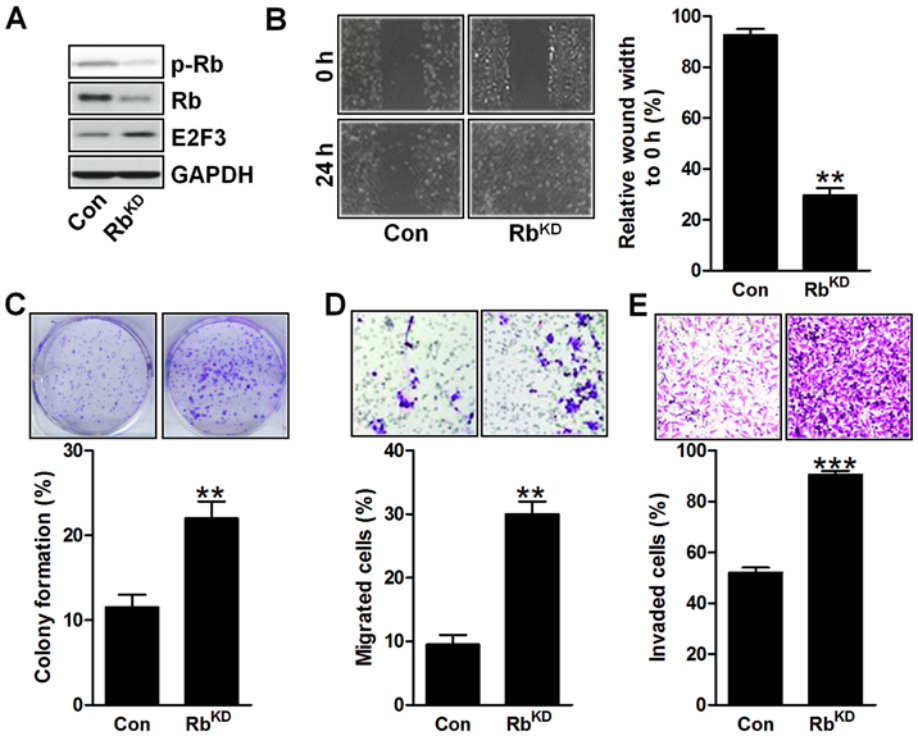

Figure 7. Rb knockdown leads to urinary bladder cancer cell proliferation *in vitro*. (A) Western blot analysis was used to determine Rb, p-Rb and E2F3 levels in cells after Rb knockdown. (B) The changes in BIU87 cell morphology and spreading after treatment of a control vector and knockdown of Rb for 24 h were studied, and BIU87 cells were captured via a microscope equipped with digital camera. (C) The colony formation of BIU87 after treatment of a control vector and Rb knockdown was calculated. (D) The migration of BIU87 after treatment with a control vector and Rb knockdown, and BIU87 cells were captured via a microscope equipped with digital camera. (E) The invasive cells of BIU87 after treatment with a control vector and Rb knockdown, and BIU87 cells were captured via a microscope equipped with digital camera. Data are expressed as the mean ± SEM. **p<0.01 and ***p<0.001 versus the Con group.

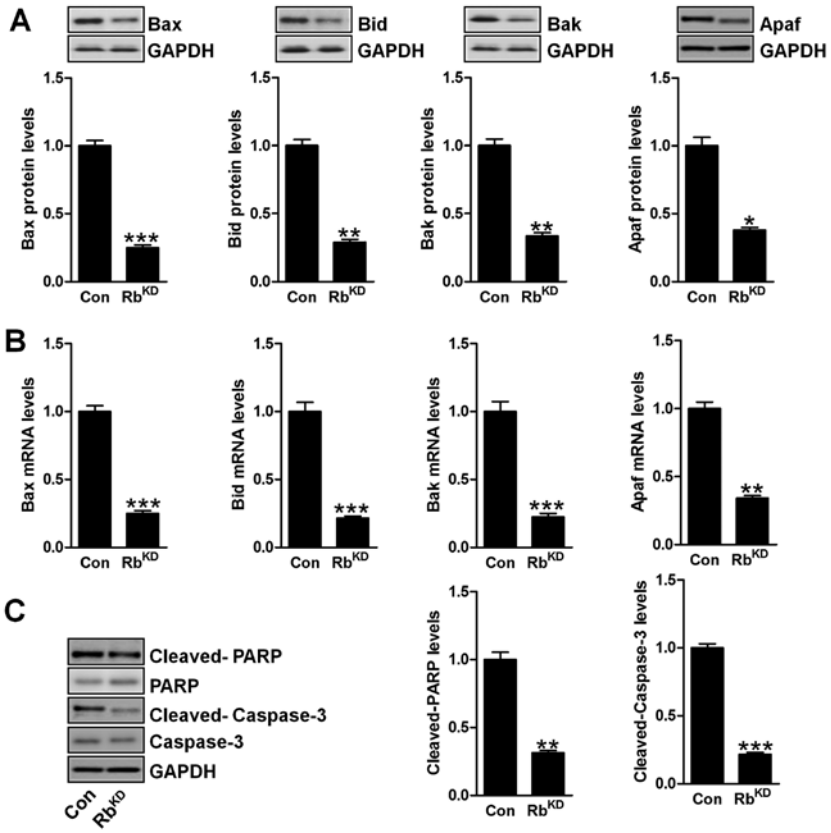

Figure 8. Downregulation of Rb induces apoptosis suppression *in vitro*. (A) Western blot analysis was used to determine Bax, Bak, Bid and Apaf protein levels in BIU-87 cells after silencing Rb. (B) RT-qPCR was conducted to analyze Bax, Bak, Bid and Apaf mRNA levels in BIU-87 cells after Rb knockdown. (C) Cleaved PARP and caspase-3 levels were analyzed via western blot analysis. Data are expressed as the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 versus the Con group.

bladder tumor progression, the *in vitro* study was conducted. In Fig. 7A, the Rb gene was silenced in BIU-87 cells, which indicated that Rb was successfully knocked down, causing its downregulation of phosphorylated Rb and E2F3 expression in bladder cancer cells. In addition, the results of wound width to 0 h indicated that the BIU-87 cell proliferation was enhanced for Rb silence with decreased wound width (Fig. 7B). As shown in Fig. 7C, the colony formation was highly upregulated in BIU-87 cells for Rb knockdown. Furthermore, much more migrated and invaded cells of BIU-87 were observed for Rb silence, which suggested that Rb suppression could result in urinary bladder cancer cell proliferation (Fig. 7D and E). The data above illustarted that Rb downregulation indeed caused urinary bladder cancer development *in vitro*, which was in line with the *in vivo* results as mentioned.

Downregulation of Rb induces apoptosis and autophagy suppression in vitro. Since the data *in vivo* implied that apoptosis inhibition was the main mechnism for bladder cancer progression, here we further studied the molecular mechnism by which the urinary bladder cancer was modulated for Rb. As shown in Fig. 8A and B, we found that the protein and mRNA levels of pro-apoptotic factors, including Bax, Bak, Bid and Apaf, were significantly downregulated in BIU-87 cells due to Rb silence. Additionally, the cleaved PARP and caspase-3 were also discovered with lower levels than the protein levels (Fig. 8C), which was in agreement with the data *in vivo* above.

Furthermore, autophagy-related signals of Beclin1, MAP1 and LC3-II were also observed with downregulated levels via immunofluorescent analysis in comparison to the Con group (Fig. 9A-C). Western blot analysis also showed that Beclin1, MAP1, LC3-I and LC3-II were obviously downregulated for Rb silence, further indicating that autophagy was suppressed in BIU-87 cells with Rb knockdown (Fig. 9D). Collectively, the data *in vitro* confirmed that Rb-regulated urinary bladder cancer progression was dependent on autophagy alteration.

Rb suppression-induced BIU-87 progression rely on p53 signaling pathway in vitro. Finally, the p53 signaling pathway was investigated *in vitro* with reduced p53 fluorescent intensity in BIU-87 cells after Rb knockdown (Fig. 10A). Similarly, p53, p21 and PTEN were significantly downregulated in bladder cancer cells with Rb silence (Fig. 10B). However, PI3K/AKT signaling pathway was activated in Rb-knockdown group, intensifying bladder cancer progression (Fig. 10C). Of note, the mdm2 signal was not changed in either group, indicating that mdm2 might not be involved in Rb-regulated urinary bladder cancer progression. The data above *in vitro* further indicated that p53 was of importance in urinary bladder cancer progression regulated by Rb.

Discussion

Bladder cancer is one of the most common urological malignancies and displays a significant reason for morbidity and mortality across the world (23). The disease includes two principal forms of cancer, the superficial and the invasive, with the majority of bladder carcinomas as the former type at the time of diagnosis (24). The superficial cancers show papillary and multifocal tumor growth and progression, which usually recurs following transurethral surgery and progresses to become an invasive disease occasionally. In contrast, invasive cancer is often known as nodular, which could metastasize to distant organs during the early phase of the disease and possesses a poor prognosis (25). The treatment used for these cancers or tumors usually includes transurethral resection of the bladder tumors or a combination of immunotherapy, chemotherapy and radical cystectomy (26). However, a large number of patients suffer from the disease recurrence and progression. Thus, a greater understanding of the molecular mechnism involved in urinary bladder cancer progression is necessary to find improved and more effective therapeutic treatments.

Retinoblastoma protein (Rb) is a classical tumor suppressor for its role in cell cycle checkpoint of G1/S, but recent data have suggested that Rb participats in many other cellular functions, such as apoptosis regulation (27). Dysfunction of apoptosis-related genes are known as a critical mechanism for cancer development (28), which is recognized as the most important type of cell death. The B-cell lymphoma 2 protein (Bcl-2) family display genetic alterations in various cancers, helping to escape apoptosis through removing pro-apoptotic genes and promoting anti-apoptotic genes (29). Bad/Bcl-2 heterodimerization isolates Bcl-2 and results in Bax permeation of both the outer and inner mitochondrial membranes, leading to the release of cytochrome c, and the downstream activation of the caspase cascade culminating in caspase-3 cleavage (30). In our study, we found that Rb knockout resulted in significant Bcl-2 upregulation, while Bid, Bax and Bak were obviously downregulated, leading to Apaf decrease. Apaf is known as an important activator responding to the up-streaming apoptotic factor activation, activating the downstream signals, such as Cyto-c, contributing to apoptotic response (31). Caspase is the executor of apoptosis (32). As the downstream signal of Bcl-2 pathway, we found that caspase-3, consistently, was inactivated in Rb deficiency or silenced, accompanied with downregulation of cleaved PARP, which function as important pro-apoptotic genes *in vitro* and *in vivo* studies. These results indicated that suppressing Rb activation might be the main mechanism by which the urinary bladder cancer was enhanced through apoptosis inhibition.

In addition, the tumor suppressor p53, could also induce cell cycle arrest and apoptosis, resulting in conserved genome stability integrity responding to cellular stress and DNA damage (33,34). The expression of p21 has been investigated in the development of chemotherapeutic drugs, which could disrupt tumorigenesis via suppressing cell cycle in cancer cells, contributing to the suppression of cell proliferation. These results indicated that Rb deficiency downregulated p53 and p21 levels, as well as PTEN, which is known as a significant downstream signal of p53, helping to suppress cell proliferation (35). Accordingly, with the reduced p53 levels, we found that PTEN was also decreased, further indicating the role of Rb in regulating urinary bladder cancer development, knockout of which was the main reason, leading to bladder cancer progression. E2F3 was invariably disrupted in different human cancers for its central role in the control of cellular proliferation. Phosphorylated Rb regulates E2F3

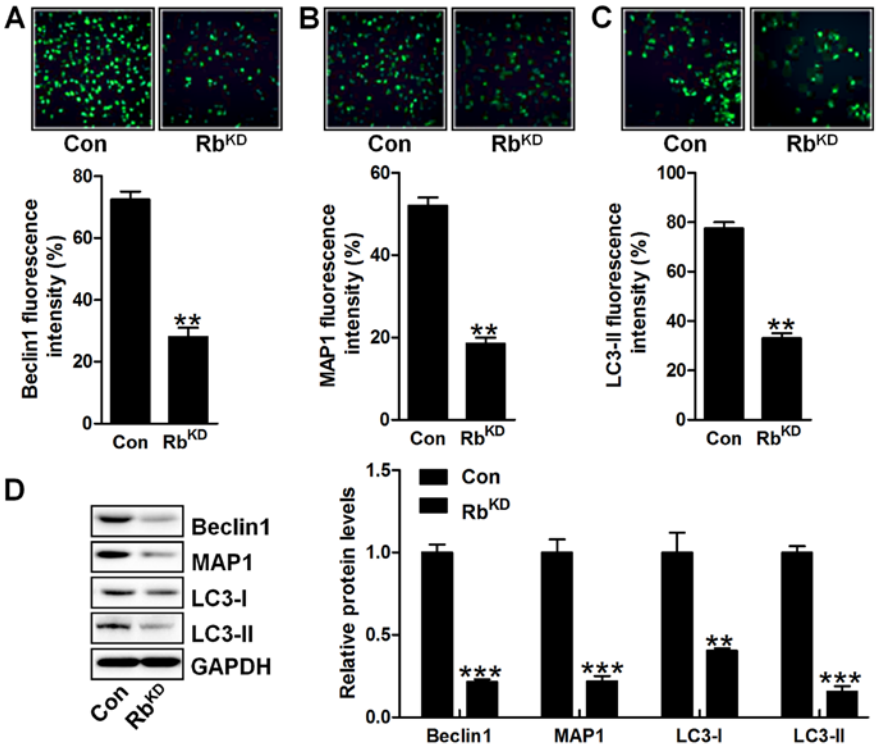

Figure 9. Rb silence results in autophagy suppression *in vitro*. Immunofluorescence analysis was used to analyze (A) Beclin1, (B) MAP1, and (C) LC3-II levels in BIU-87 cells after Rb knckdown. (D) Western blot analysis was performed to determine Beclin1, MAP1, LC3-I and LC3-II levels in cells after Rb knockdown. Data are expressed as the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 versus the Con group.

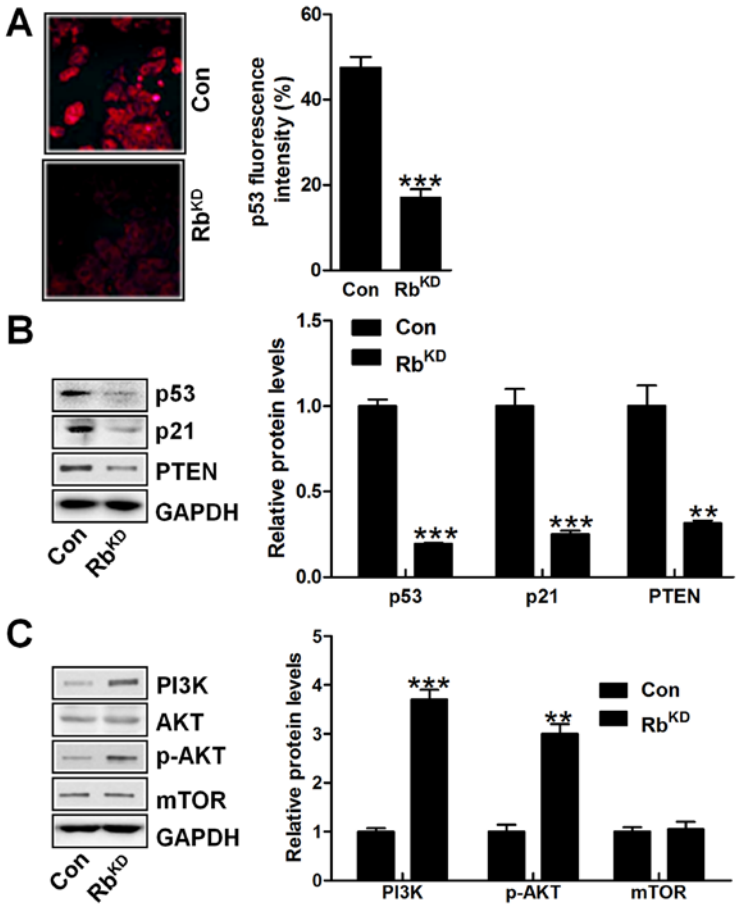

Figure 10. Rb suppression-induced BIU-87 progression rely on p53 signaling pathway *in vitro*. (A) Immunofluorescence analysis was used to analyze p53 levels in BIU-87 cells after Rb silence. (B) Western blot analysis was performed to determine p53, p21 and PTEN levels in cells after Rb knockdown. (C) PI3K, p-AKT and mdm2 protein levels in BIU-87 cells were determined through western blot analysis. Data are expressed as the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 versus the Con group.

activation, which is required for the progression into late phase of G1 and S (36). This sequential regulation exhibits additional specificity in modulating alternative cell fates, including differentiation and proliferation, and plays an important role in tumor development and progression (37). In line with the results of enhanced colony formation, migrated and invaded cells for Rb suppression, we found that E2F3 was also upregulated.

Furthermore, as a negative regulator of p53, mdm2 interacts with p53 protein to inhibit the transcriptional activation of p53, leading to cell proliferation in a tumor (38). In contrast to p53 alteration, mdm2 was upregulated for Rb inhibition, further confirming our results of Rb suppression to promote urinary bladder cancer development. The PI3K-AKT pathway has been reported to be activated in many malignant tumors due to abnormalities in various genes (39). Studies have found that AKT pathway plays an important role in lung cancer, intestine cancer and pancreatic carcinoma (40). Here, we found that PI3K/AKT signaling pathway was stimulated for Rb suppression *in vitro* and *in vivo*, which was in agreement with p53 and PTEN downregulation, enhancing urinary bladder cancer progression.

In conclusion, we demonstrated that Rb was frequently downregulated in urinary bladder cancer tissues and cell lines. Rb suppression played a crucial role in the malignant progression of urinary bladder cancer cells through inactivation of p53 and caspase-3, inhibiting autophagy and apoptosis. Therefore, targeting Rb has the potential to be a valuable therapeutic strategy for urinary bladder cancer.

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