

Rb knockdown accelerates bladder cancer progression through E2F3 activation

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Abstract. Bladder cancer is one of the most common cancers diagnosed in the world and leads to significant mortality and morbidity among affected patients. The retinoblastoma (Rb) protein is a main tumor suppressor, controlling cellular responses to potentially oncogenic stimulation. E2F3 was invariably disrupted in different human cancers for its central role in the control of cellular proliferation. Here, we investigated how Rb is integrated to control bladder cancer progression through E2F3 and p53 regulation. The results exhibit that Rb expression is lower in patients with bladder tumor, while E2F3 level is high. Rb knockdown enhanced bladder tumor cell proliferation and migration, aggravated with p53 silence. Interestingly, Rb silence results in E2F3, Myc and mTOR signaling pathway activation, contributing to bladder cancer cell proliferation and apoptosis suppression mainly through caspase-3 inhibition *in vitro* and *in vivo*. Immunohistochemical analysis revealed that Rb is highly expressed in normal bladder cells, but was repressed in tumor tissues of the bladder completely, suggesting a possible role of Rb as a tumor suppressor.

Introduction

Bladder cancer is one of the most common urological malignancy across the world (1). Approximately, 70% of tumors or cancers display indications for non-muscle-invasive bladder cancer (NMIBC) during the initial diagnosis with mutable danger of recurrence and development to invasive disease, hence needing long-term surveillance (2,3). As the gold-standard therapy, intravesical IVES Bacillus-Calmette-Guerin BCG immunotherapy after the lesion transurethral resection has been used for ~40 years (4). However, this therapy was limited among patients during the tumor recurrence and

progression. Therefore, finding an effective target for treating bladder cancer is still necessary.

The retinoblastoma (Rb) protein is a main tumor suppressor, potentially controlling cellular processes or responses to oncogenic stimuli, such as DNA damage, repeated rapid cell division, as well as inappropriate mitogenic signals (5,6). The significance of Rb protein in tumor development was initially showed by the results that an RB allele was unchangeably deleted in retinoblastoma (7). Rb modulates normal cell cycle and responses to stress. The progression of cell cycle is controlled by various cyclin-dependent kinases (CDKs), which could bind to their respective cyclins and promote phosphorylation (8). The activated cyclins and their CDKs subsequently phosphorylate Rb, which is significant for E2Fs activity modulation (9). Rb phosphorylation could disrupt E2F complex formation, resulting in diverse transcription factors dysfunction and some targeting genes to enhance entry into S phase, which is vital for cell proliferation and influences tumor development and progression (10). Additionally, E2F3 and Myc are important transcription factors, which control cellular proliferation. E2F3, as a major transcriptional activator, could be suppressed combined with Rb.

The tumor suppressor p53 regulates several cellular stress responses, such as apoptosis, cell cycle arrest, and genomic stability, through induction of the various transcription of target genes (11,12). Responding to different genotoxic stresses, p53 could be activated via interactivation with various kinases, which include negative cell cycle-related genes and anti- or pro-apoptotic genes of Bcl-2 and Bax (13). Previous reports have suggested that Rb and p53 deletion in cells led to small cell lung cancer (14,15). Also, tumors with Rb and p53 deletion accelerated lung cancer progression through Myc promotion (16).

Despite significant function between E2F and Myc for the control of cell cycle modulation regulated by Rb and p53, the specific molecular mechanisms linking these critical transcriptional programs are poorly understood in bladder cancer progression. We estimated Rb, E2F3 and p53 in cells of the bladder cancer in order to better understand and define how these factors regulate the cell cycle and apoptosis *in vitro* and *in vivo* of bladder cancer.

Materials and methods

Sample collection. A total of 26 bladder cancer samples were obtained from patients undergoing cystectomy at

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Tangdu Hospital, The Fourth Military Medical University (Shaanxi, China), between 2012 and 2014 for further verification. Follow-up data were available for each patient. Tissue samples from primary tumors were collected and prepared for RT-qPCR, immunohistochemical (IHC) staining and western blot analysis. The fresh-frozen tissue samples from 26 bladder patients and matched normal bladder tissues were collected and snap-frozen in liquid nitrogen immediately within 10 min after collection. The tissue sample collection and experimental procedures were conducted with the approval of the Institutional Review Board of Tangdu Hospital.

Cell culture and treatment. The bladder cancer cells of BIU87, as well as human normal bladder epithelial cell of T24 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). They were cultured at the permissive temperature (37°C) in DMEM medium (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) and supplemented with 1% penicillin-streptomycin-neomycin provided by Gibco BRL Life Technologies with a humidified incubator in 5% CO₂ atmosphere. Additional introduction of Rb or a control vector into BIU87 cells were described previously (17).

Colony formation assay. One hundred bladder cancer cells of BIU87 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. Clones with >50 cells were evaluated. Clone forming efficiency for cells was calculated based on colonies/number of inoculated cells x 100%.

Wound-healing assay. 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 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 to play as a chemo-attractant, and cells were then incubated for 4 h of migration. Non-migratory cells were taken away from the upper chamber via scraping with 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 microscope fields of two independent inserts.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays. Apoptosis assay of samples was also determined by TUNEL used an In Situ Cell Death Detection kit, Fluorescein (Roche Applied Science, South San Francisco, CA, USA) according to the manufacturer's

protocol. The number of TUNEL-positive cells was counted under a fluorescence microscope. The percentages of apoptotic cells were calculated from the ratio of apoptotic cells to total cells counted. Tissue sections were counter-stained with hematoxylin. Sections were mounted and observed under light microscopy. The experiment was performed independently three times.

Antibody array detection. In total, 1x10⁶ BIU87 cells expressing a control vector or siRNAs of Rb were seeded into 10-cm culture dishes for 24 h. Then the media were replaced with serum deprived media (0.1% fetal bovine serum), and the bladder cancer cells were cultured for another 48 h. The supernatants of culture were acquired, centrifuged at 1,000 x g, and dialyzed with 1X phosphate-buffered saline with pH 8.0 twice overnight at 4°C. The samples were then labeled with biotin and then incubated with human L1000 Antibody arrays (RayBiotech, Norcross, GA, USA).

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, USA), rabbit anti-Myc (Cell Signaling Technology), rabbit anti-PARP (Cell Signaling Technology), rabbit anti-E2F3 (Abcam, USA), rabbit anti-caspase-9 (Abcam), rabbit anti-caspase-3 (Abcam), mouse anti-Bcl-2 (Cell Signaling Technology), rabbit anti-P-Rb (Cell Signaling Technology), rabbit anti-Rb (Cell Signaling Technology), mouse anti-cyclin D1 (Abcam), mouse anti-cyclin A (Abcam), mouse anti-CDK4 (Abcam), and mouse anti-CDK2 (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, Carlsbad, CA, USA) according to 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 as follows and GAPDH was used as the endogenous control. eIF4E forward, 5'-CTG ATG TTG CTT GTC GCT TC-3'; reverse, 5'-GTG AGA GTT GCT GGC

TTG AA-3'. eIF4G forward, 5'-CGA GGC TAC GCA TTT AGG AGA A-3'; reverse, 5'-GGG CTA TGA ACA GTC TCT TCT C-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'. E2F3 forward, 5'-CGC CAC CGC CAT CTT CTC CA-3'; reverse, 5'-GCA CAA GGC AGC CAG AAG GC-3'. mTOR forward, 5'-AGG ATC GCG CTT AGC ATA CTT G-3'; reverse, 5'-ATC TCT TCG ACC TCT GTT CTC G-3'. TSC-2 forward, 5'-CGT TCC TCA CGC TGG ACT CCT-3'; reverse, 5'-ACA GCG GAT AAC CTC CTG CTG TT-3'. SESN2 forward, 5'-CCA TTG AAG CCG AGG ATG TGG-3'; reverse, 5'-GTG GCC TCT GTT CAG CTA GGT-3'. GAPDH forward, 5'-CTA AGT CGA ACG CAG ACA GTC AG-3'; reverse, 5'-AAC ATA CCA TCC ACG ACA CGC TC-3'.

Flow cytometry for cell cycle analysis. The bladder cancer cells were fixed with 70% ethanol and washed with PBS with 1% horse serum, and cellular DNA was stained with 100 μ g/ml propidium iodide. Cell cycle and forward scatter profiles were calculated through a Becton-Dickinson FACSCalibur, as determined by ModFit LT 2.0 from Verity Software House Inc.

Animals and treatment. The mouse experiments were conducted in the Animal Laboratory Center. BIU87 cells (1×10^7 cells) treated with the vector control and siRb were suspended in 100 μ l serum-free medium and injected subcutaneously into the left flank of 4- to 6-week old male BALB/c nu/nu nude mice. Tumor size was measured with digital caliper and calculated. Tumor volume were measured every seven days and at the end of ~6 weeks after treatment, mice were sacrificed. Tumors were excised, weighed, fixed in 10% neutral formalin, and embedded in paraffin for histological and western blot analysis.

Immunohistochemistry analysis. Human bladder or bladder cancer tissue samples and animal model bladder cancer tissue specimens were fixed in paraformaldehyde and embedded in paraffin. 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 μ m thickness sectioning, paraffin-embedded bladder cancer tissues were performed for immunostaining with anti-pRb (Cell Signaling Technology), anti-E2F3 (Cell Signaling Technology), anti-P53 (Cell Signaling Technology), anti-Myc (Cell Signaling Technology), anti-Ki67 (Glostrup, Denmark), anti-cleaved caspase-3 (Cell Signaling Technology) and anti-mTOR (Abcam, UK) antibodies. All of the slides were finally observed with x200 magnification by microscopy.

Immunofluorescence assays. 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 E2F3, Myc, mTOR, and p-AKT 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.

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

Results

Patients with bladder cancer express low levels of Rb. Rb has been well known to be of great importance during tumor progression and development, including liver cancer, breast cancer and lung cancer (18). However, whether Rb was involved in bladder cancer was far from clear. In this regard, we first investigated how Rb changed in different tissue samples. In Fig. 1A, we found that Rb expressed highly in normal bladder tissue samples from patients compared to the tumor tissue samples from patients with bladder tumor. Consistently, p53, as significant tumor suppressor, was discovered with lower levels in the tumor tissue segments compared to the normal tissue samples (Fig. 1B) (19). In contrast, mTOR was expressed highly in tumor samples, which has been reported to be linked with cancer progression and growth (Fig. 1C) (20). In agreement with mTOR, E2F3, which was a vital down-streaming signal of Rb and was associated with tumor development, was found to be upregulated in tumor tissue samples in comparison to the normal ones (Fig. 1D) (21). Also, western blot analysis indicated that the phosphorylated Rb, the inactivated form, was increased in tumor tissue samples, which was contrary to the Rb levels in normal and tumor samples (Fig. 1E). Furthermore, immunohistochemical analysis indicated that p-Rb and E2F3 were expressed highly in tumor tissue samples compared to the normal ones with significant difference (Fig. 2A and B). In line with RT-qPCR results, we found that the percentage of p53 positive cells was reduced in tumor samples (Fig. 2C). The data here indicated that Rb was involved in bladder cancer progression, showing suppressed role in bladder cancer regulation.

Alteration of Rb influences apoptosis- and proliferation-related signal expression in vitro. In this regard, RT-qPCR was used to determine Rb, p53, mTOR and E2F3 levels in bladder cancer cells and normal bladder cells. We found that Rb and p53 expressed highly in T24 cells compared to that in the bladder cancer BIU87 cells (Fig. 3A and B). In contrast, mTOR and E2F3 expression was low in normal T24 bladder cells, which was in line with previous results (Fig. 3C and D). To investigate the mechanisms by which Rb expression regulates bladder tumor development, we examined the cell proliferation- and apoptosis-related signals affected by Rb alteration via antibody arrays which analyzed up to 1,000 factors, including chemokines, growth factors, cytokines, as well as other proteins. We cultured BIU87 cells with a control vector or Rb knockdown in serum-deprived media for 48 h before harvesting the cell culture supernatant for the following antibody array analysis. As shown in Fig. 3E, increased levels of mTOR, E2F3, cyclin D1, Myc, Bcl-2 and CDK4 and decreased levels of Bax were observed in the conditioned media derived from Rb-knockdown cells compared to the control vector-expressing BIU87 cells. Quantitative RT-PCR analysis also indicated that the mRNA levels of mTOR, E2F3, cyclin D1, Bcl-2, Myc and CDK4 were also significantly upregulated while Bax was downregulated in Rb-knockdown BIU87 cells compared with that in the control

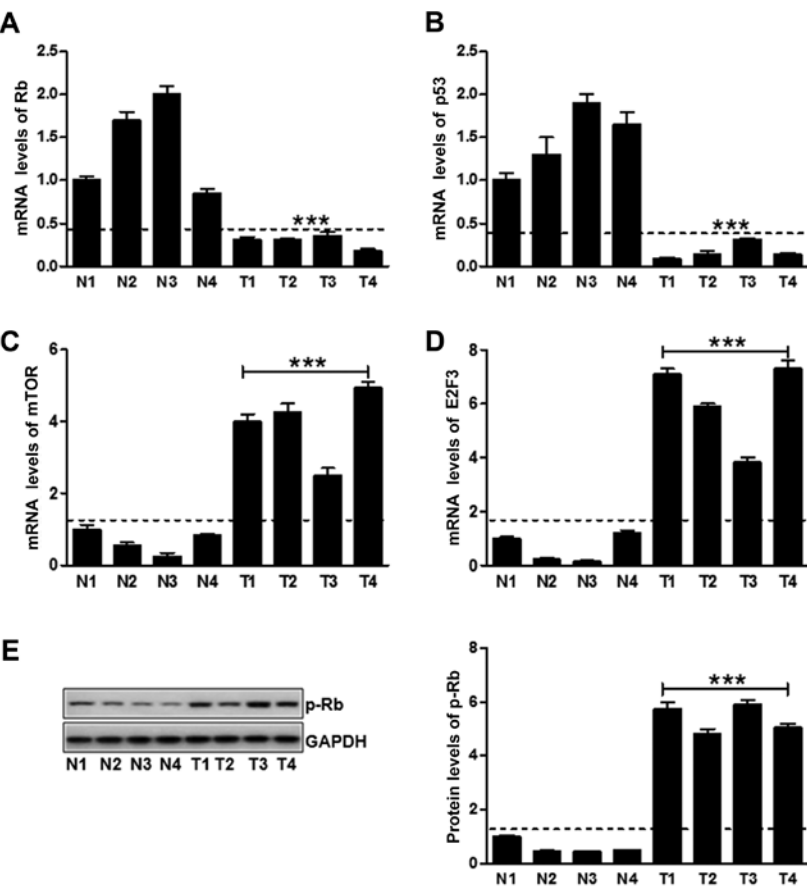


Figure 1. Patients with bladder cancer express low levels of Rb. (A) RT-qPCR was used to explore Rb mRNA levels in the normal tissue samples and bladder tumor tissue samples, respectively. (B) RT-qPCR was used to explore p53 mRNA levels in the normal tissue samples and bladder tumor tissue samples, respectively. (C) RT-qPCR was used to explore mTOR mRNA levels in the normal tissue samples and bladder tumor tissue samples, respectively. (D) RT-qPCR was used to explore E2F3 mRNA levels in the normal tissue samples and bladder tumor tissue samples, respectively. (E) Western blot analysis was performed to investigate p-Rb protein levels in the normal tissue samples and bladder tumor tissue samples, respectively. Data are expressed as the mean \pm SEM. ***p<0.001 versus the normal control.

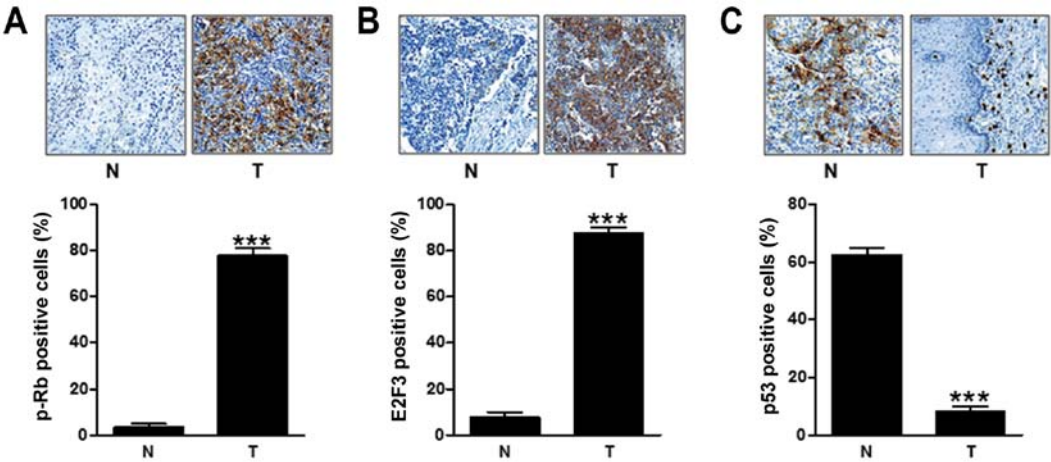


Figure 2. High expression of p-Rb is related to bladder cancer development. Immunohistochemical analysis was used to determine (A) p-Rb, (B) E2F3 and (C) p53 levels in the normal tissue samples and bladder tumor tissue samples, respectively. Data are expressed as the mean \pm SEM. ***p<0.001 versus the normal control.

vector-expressing cells (Fig. 3F). Therefore, the findings above suggested that Rb inhibition in bladder cancer cells promoted expression of mTOR, E2F3, cyclin D1, Bcl-2, Myc and CDK4 companied by Bax downregulation at both the transcriptional and protein levels.

Knockdown of Rb accelerates E2F3 and influences the regulatory proteins of proliferation and apoptosis. Previous study indicated that the phosphorylation of Rb influenced cell proliferation and cell apoptosis through modulating related signaling pathway (22). Therefore, we demonstrated that Rb

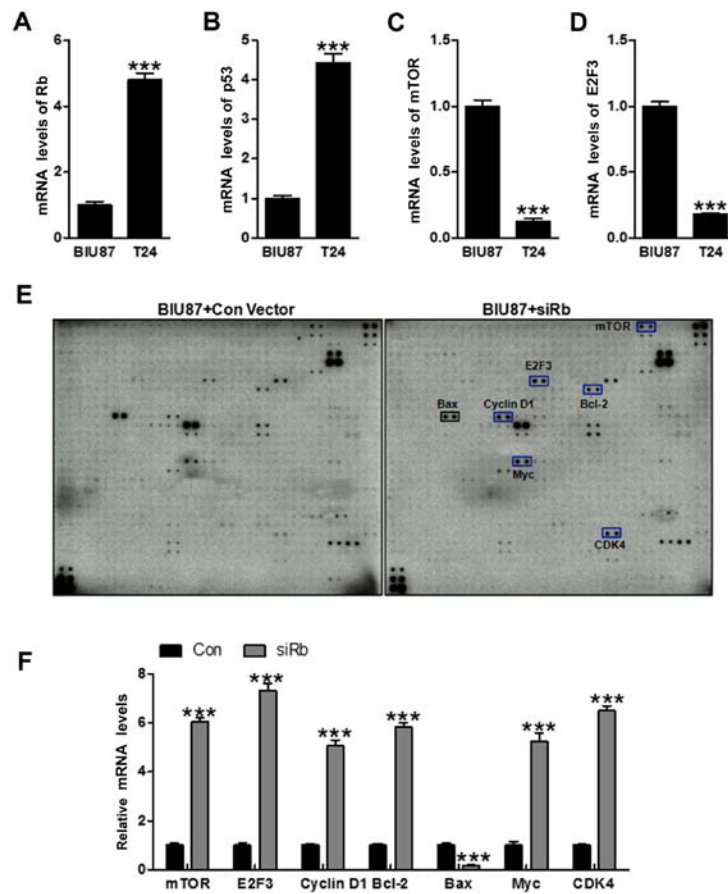


Figure 3. Alteration of Rb influences apoptosis- and proliferation-related signal expression. mRNA levels of (A) Rb, (B) p53, (C) mTOR and (D) E2F3 expressed differently in normal and tumor bladder cells of BIU87 and T24, respectively. (E) Antibody array analyses of cell culture supernatants of BIU87 expressing a control vector or silence of Rb. BIU87 cells were cultured in serum-deprived media with 0.1% FBS for 48 h. Then the cell culture supernatants were collected and labeled with biotin before incubation with array membranes for detection. Upregulated or downregulated proteins are displayed in blue and black colour, respectively. Also, representative images from three independent experiments are exhibited. (F) Quantitative RT-qPCR analysis of mTOR, E2F3, cyclin D1, Bcl-2, Bax, Myc and CDK4 mRNAs in BIU87 cells expressing a control vector and siRb. BIU87 cells were cultured for 48 h before harvesting for total RNA analysis. Data are expressed as the mean \pm SEM. ***p<0.001 versus the vector control.

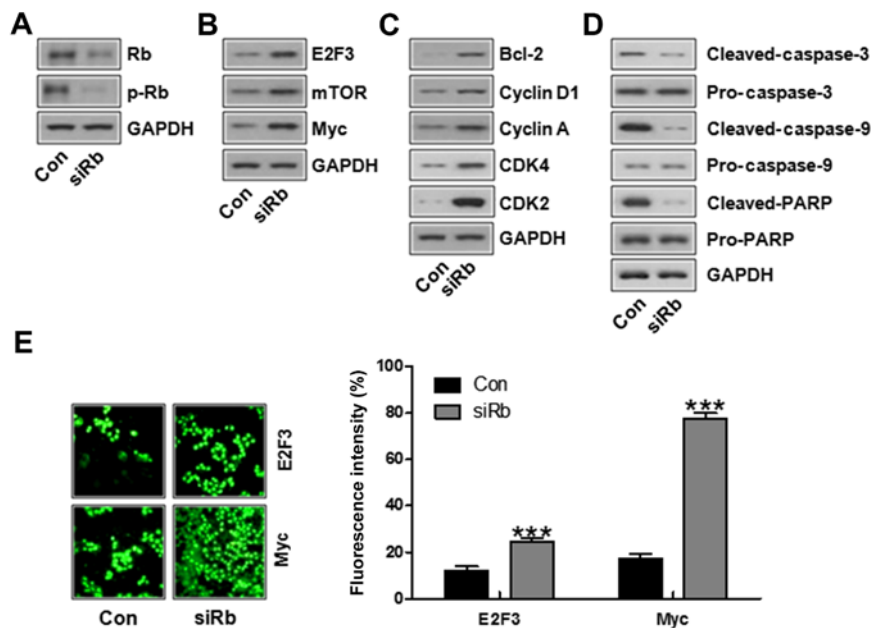


Figure 4. Knockdown of Rb accelerates E2F3 and influences the regulatory proteins of proliferation and apoptosis. (A) Western blot analysis of Rb and p-Rb in BIU87 cells treated with a control vector and siRb. (B) Western blot analysis of E2F3, mTOR and Myc in BIU87 cells treated with a control vector and siRb. (C) Western blot analysis of proliferation-related proteins in BIU87 cells treated with a control vector and siRb. (D) Western blot analysis of apoptosis-related proteins in BIU87 cells treated with a control vector and siRb. (E) Immunofluorescent analysis of E2F3 and Myc in BIU87 cells treated with a control vector and siRb. Data are expressed as the mean \pm SEM. ***p<0.001 versus the vector control.

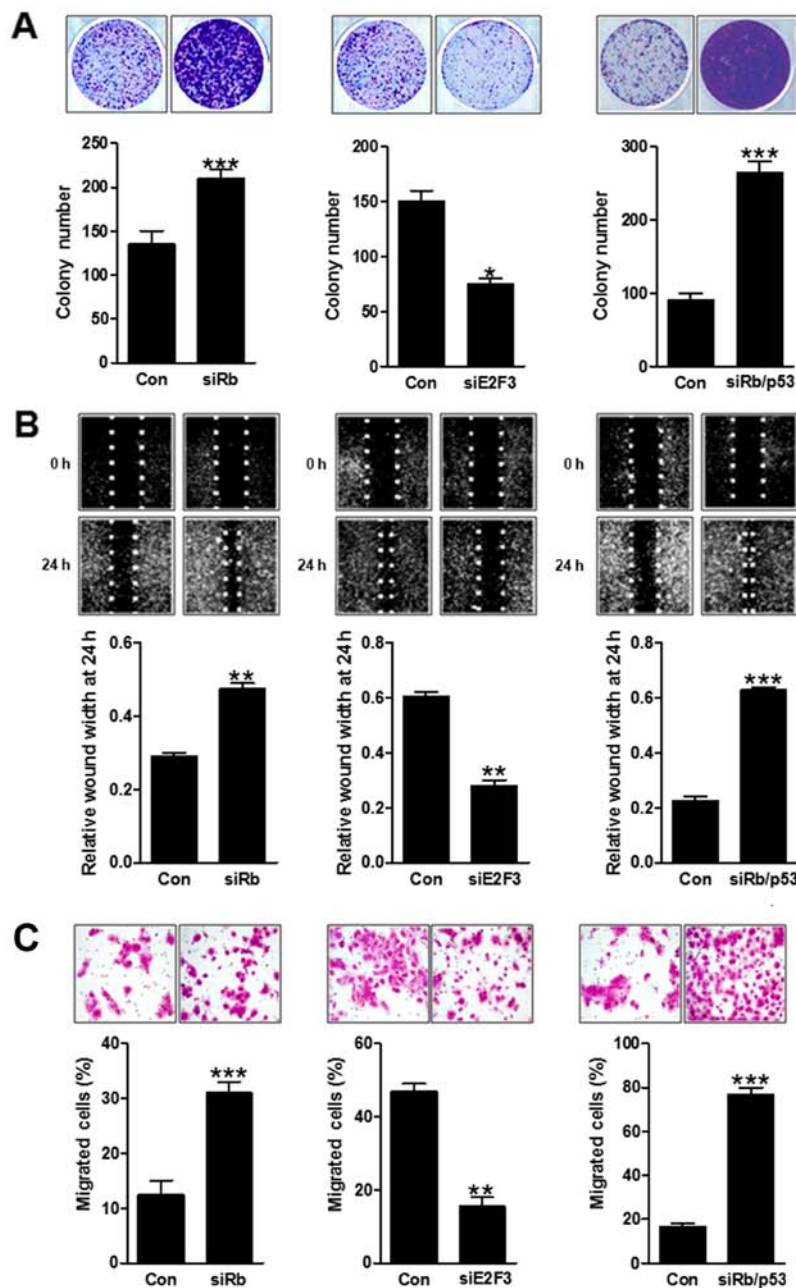


Figure 5. Rb silence promotes bladder cancer cell proliferation and migration. (A) Effect of Rb, E2F3 and p53 expression of BIU87 cells treated with a control vector and siRb or siE2FE and siRb combined with p53 on clonogenic growth of BIU87 cells. (B) The changes in BIU87 cell morphology and spreading after treatment of a control vector and siRb or siE2FE and siRb combined with p53 for 24 h were studied, and BIU87 cells were photographed via a microscope equipped with digital camera. (C) The migration of BIU87 after treatment of a control vector and siRb or siE2FE and siRb combined with p53, and BIU87 cells were photographed via a microscope equipped with digital camera. Data are expressed as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the vector control.

phosphorylation induced E2F3 activity through carcinogenic gene induction and apoptosis suppression in different cancer cell lines (23). In order to further determine how Rb performs its role in bladder cancer progression, we knocked down Rb gene in BIU87 bladder cancer cells. In Fig. 4A, we found that Rb was successfully silenced with lower expression accompanied with relatively higher level of phosphorylated Rb. Next, E2F3, mTOR and Myc were upregulated significantly in siRb group (Fig. 4B). E2F3, mTOR and Myc are well known to play important roles in contributing to tumor or cancer development (24). Next, the cell proliferation- and apoptosis-related signals were investigated. As shown in Fig. 4C, Bcl-2, a crucial

anti-apoptosis factor, was found to be upregulated, inhibiting apoptosis in bladder cancer. Also, cyclin D1, cyclin A, CDK4 and CDK2 were also upregulated in bladder cancer cells with Rb silenced, suggesting that Rb deficiency was, at least partly, involved in bladder cancer progression through regulating apoptosis and proliferation of cells. Furthermore, cleaved caspase-3, cleaved caspase-9 and cleaved PARP were reduced significantly in BIU87 cells with Rb knockdown, indicating that Rb was associated with apoptosis, influencing bladder cancer development (Fig. 4D). Additionally, immunofluorescence analysis suggested that E2F3 and Myc were expressed highly in siRb cells compared to the vector control (Fig. 4E).

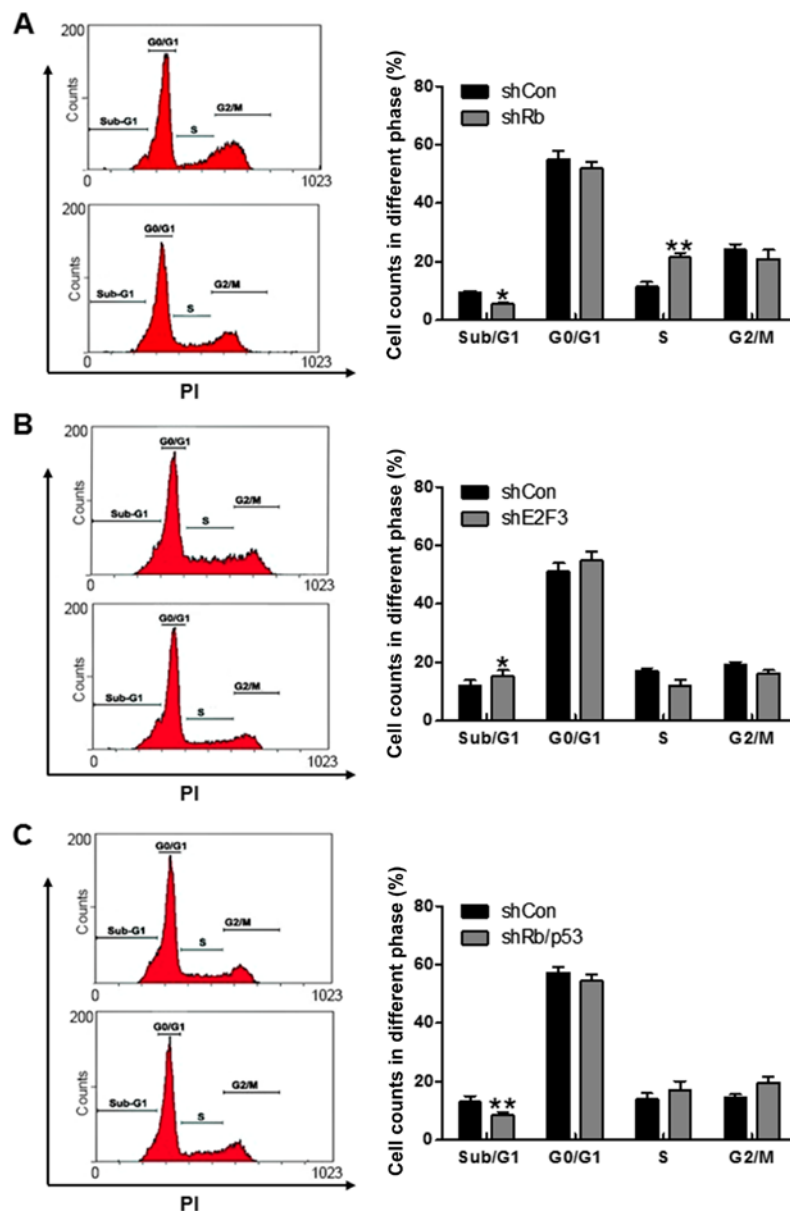


Figure 6. Rb knockdown inhibits apoptosis in BIU87 cancer cells. (A) Cell cycle phase distribution was analysed by flow cytometry in BIU87 cells treated with a control vector and siRb. (B) Cell cycle phase distribution was analysed by flow cytometry in BIU87 cells treated with a control vector and siE2F3. (C) Cell cycle phase distribution was analysed by flow cytometry in BIU87 cells treated with a control vector and siRb combined with sip53. Data are expressed as the mean \pm SEM. * $p < 0.05$, and ** $p < 0.01$ versus the vector control.

Taken together, the data in this part illustrated that Rb was indeed involved in bladder cancer progression through regulating bladder cancer cell proliferation and apoptosis.

Rb silence promotes bladder cancer cell proliferation and migration. In this regard, we investigated the role of Rb in bladder cancer cell proliferation and migration. As shown in Fig. 5A, the colony formation assay showed that Rb silence promoted the colony number, while inhibiting E2F3 expression, the colony number was reduced, indicating that E2F3 had a potential role in enhancing bladder cancer cell proliferation. In addition, inhibiting Rb and p53 simultaneously, the colony formation number was higher. Further, the relative wound width at 24 h indicated that Rb silence could increase the relative wound width. Also, E2F3 knockdown significantly decreased the relative wound width at 24 h. Similarly, Rb and

p53 silence could further promote the wound width at 24 h (Fig. 5B). Finally, we investigated the number of migrated cells modulated by Rb, E2F3 and p53. As shown in Fig. 5C, the number of migrated bladder cancer cells was significantly increased in Rb-knockdown cells. In E2F3-silence cells, the number of bladder cancer cells was decreased remarkably. Significantly, the percentage of migrated cells was increased in Rb and p53 knockdown cells concurrently. Together, the data above indicated that Rb has a potential role in suppressing bladder cancer cell proliferation and migration, which was related to E2F3 and p53 modulation.

Rb knockdown inhibits apoptosis in BIU87 cancer cells. In order to further explore how Rb suppressed BIU87 bladder cancer cell progression, flow cytometry analysis was performed. In Fig. 6A, the counts of bladder cancer cells in

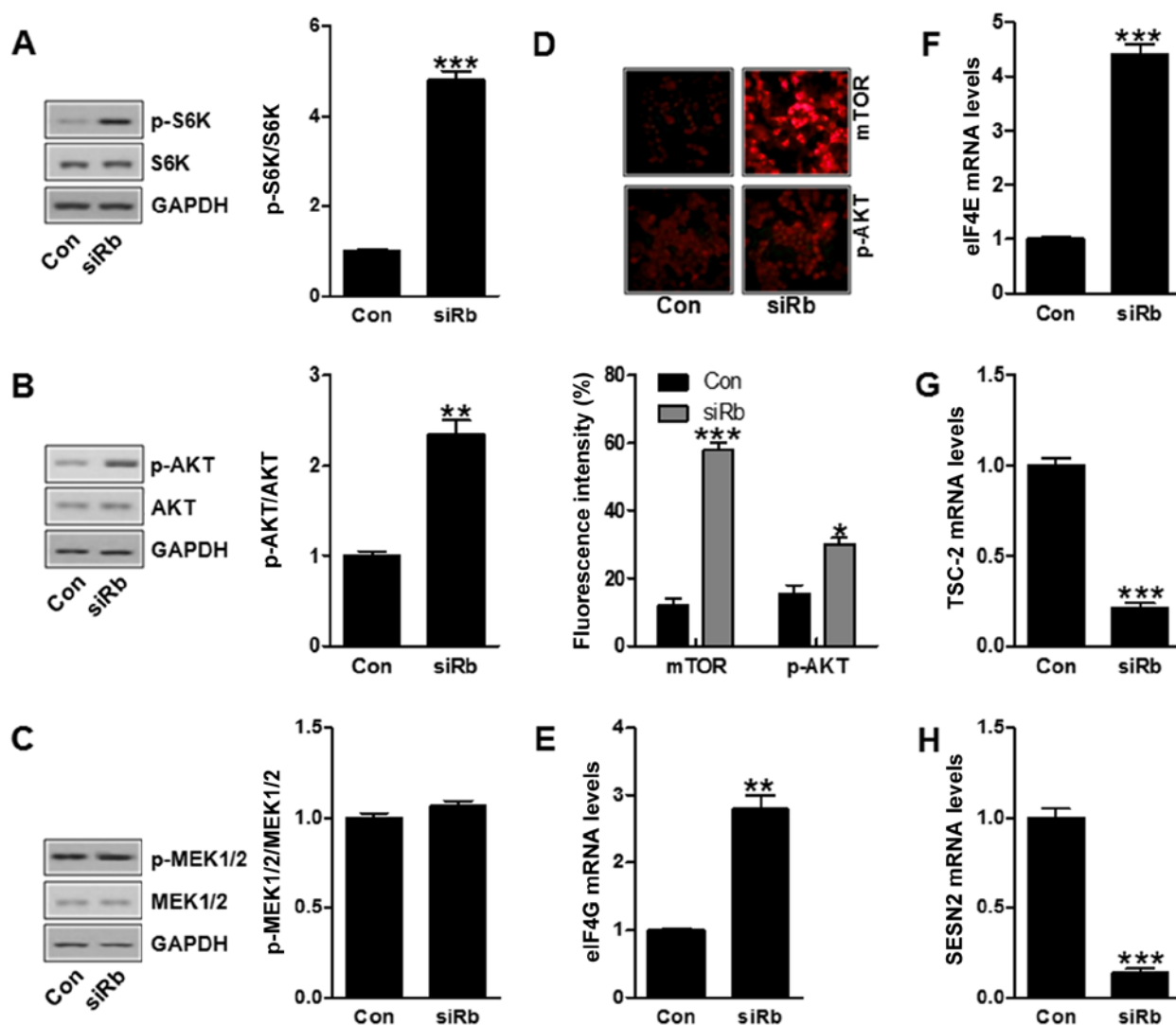


Figure 7. Rb-modulated bladder cancer progression is related to mTOR signaling pathway. (A) p-S6K was significantly increased in siRb as compared with the control with similar total S6K levels. (B) p-AKT was expressed highly in siRb as compared with the control with similar total AKT levels. (C) p-MEK was significantly downregulated in siRb in comparison with the control ones with similar total MEK expressed levels. (D) Immunofluorescent analysis of mTOR and p-AKT in BIU87 cells treated with a control vector and siRb. Quantitative RT-qPCR displayed significantly upregulated expression of eIF4G (E) and eIF4E (F) and downregulated TSC-2 (G) and SESN2 (H) levels compared to the control. Data are expressed as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the vector control.

sub/G1 phase were lower in the vector control ones in comparison to the Rb-silenced group, further indicating apoptosis was inhibited for Rb suppression. In addition, E2F3 silence significantly increased the number of BIU87 cells in Sub/G1 phase, inhibiting apoptosis in cells (Fig. 6B). Significantly, silencing Rb and p53 simultaneously reduced the number of bladder cancer cells in Sub/G1 phase (Fig. 6C). The results above indicated that Rb expressed levels were associated with apoptosis development, which could be a potential target for bladder cancer treatment.

Rb-modulated bladder cancer progression is related to mTOR signaling pathway. S6K, p-AKT and MEK1/2 has been reported to be involved in tumor progression, which are important in mTOR pathway, regulating the cellular process (25). Thus, here we attempted to investigate how Rb modulated bladder cancer progression via mTOR signaling pathway. Fig. 7A shows that S6K phosphorylated level was

higher in Rb-silenced cells, while total S6K was not observed with significant difference. Additionally, phosphorylated AKT levels were also expressed highly in bladder cancer cells after Rb knockdown compared to the vector control (Fig. 7B). In contrast, activated MEK1/2 was found to be reduced after Rb silence, which was linked with tumor inhibition (Fig. 7C). Moreover, immunofluorescence analysis was used to determine how mTOR and p-AKT changed in siRb bladder cells. As shown in Fig. 7D, mTOR and p-AKT fluorescent intensity was significantly upregulated in BIU87 cells with Rb silence. Additionally, Eif4g and Eif4e were important factors helping to activate mTOR signaling pathway, which was increased by Rb knockdown (Fig. 7E and F). In contrast, TSC-2 and SESN2, which inhibit mTOR signaling pathway activity, were found to be downregulated with significant difference (Fig. 7G and H). Together, the above results indicated that Rb-regulated bladder cancer progression was closely related to mTOR activation.

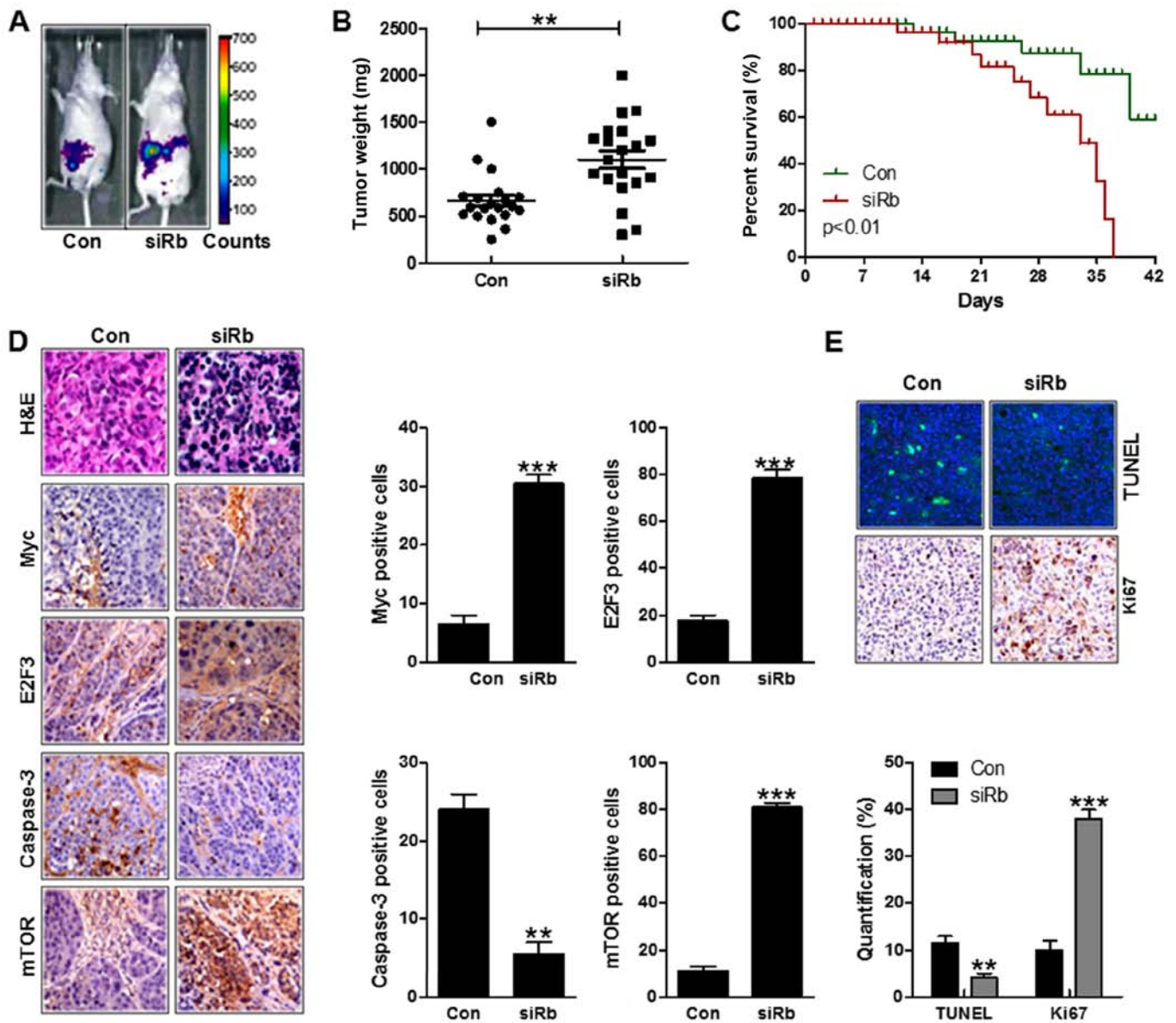


Figure 8. Rb knockdown enhances bladder tumor growth *in vivo*. (A) Representative images show an increase in luminescence in the lower expression of Rb group compared with the control group. (B) Rb silence upregulated tumor weight in mouse models. (C) Rb knockdown decreased the survival rate of mice with bladder cancer. (D) Immunohistochemical analysis of H&E, Myc, E2F3, caspase-3 and mTOR in bladder tumor samples of mice. (E) TUNEL and Ki67 analysis were determined in bladder tumor samples in animal models. Data are expressed as the mean \pm SEM. ** $p < 0.01$, *** $p < 0.001$ versus the vector control.

Rb knockdown enhances bladder tumor growth *in vivo*. We implanted BIU87+Luc cells expressing inducible vector control siRNA or siRb by intraperitoneal injection into the nude mice. After 42 days, we found that inducible suppression of Rb durably enhanced tumor growth (Fig. 8A). In addition, the tumor weight was also higher in the nude mice injected with Rb-silenced cells compared to the control ones (Fig. 8B). As shown in Fig. 8C, and the results showed that Rb silence significantly reduced survival rate in mice. Next, H&E staining suggested the morphology of tumor tissue sample in the control group and the siRb group. In addition, immunohistochemical analysis indicated that Myc, E2F3 and mTOR were expressed highly in Rb-silenced tumor tissue samples with significant difference compared to the control, while caspase-3 was found to be downregulated after Rb knockdown, which was in line with the above

results (Fig. 8D). Further, TUNEL and Ki67 expressed levels were also explored. As shown in Fig. 8E, we found that the TUNEL positive tissue samples were lower after Rb silence. In contrast, Ki67 expressed highly in Rb-silenced tissue samples. Finally, the cell proliferation- and apoptosis-related signaling pathway was further investigated *in vivo*. As shown in Fig. 9A, phosphorylated Rb was expressed highly, a main contributor leading to E2F3 expression. Additionally, Bcl-2, cyclin D1, cyclin A, CDK4 and CDK2 were all upregulated in siRb bladder tumor tissue samples (Fig. 9B). Also, the levels of cleaved caspase-3, and cleaved PARP were significantly reduced in Rb-silenced tissue samples, suppressing apoptosis in bladder tumor samples (Fig. 9C). The data above, which was in line with the results *in vitro*, further suggested that Rb-regulated bladder cancer was related to cell proliferation and apoptosis signaling pathway.

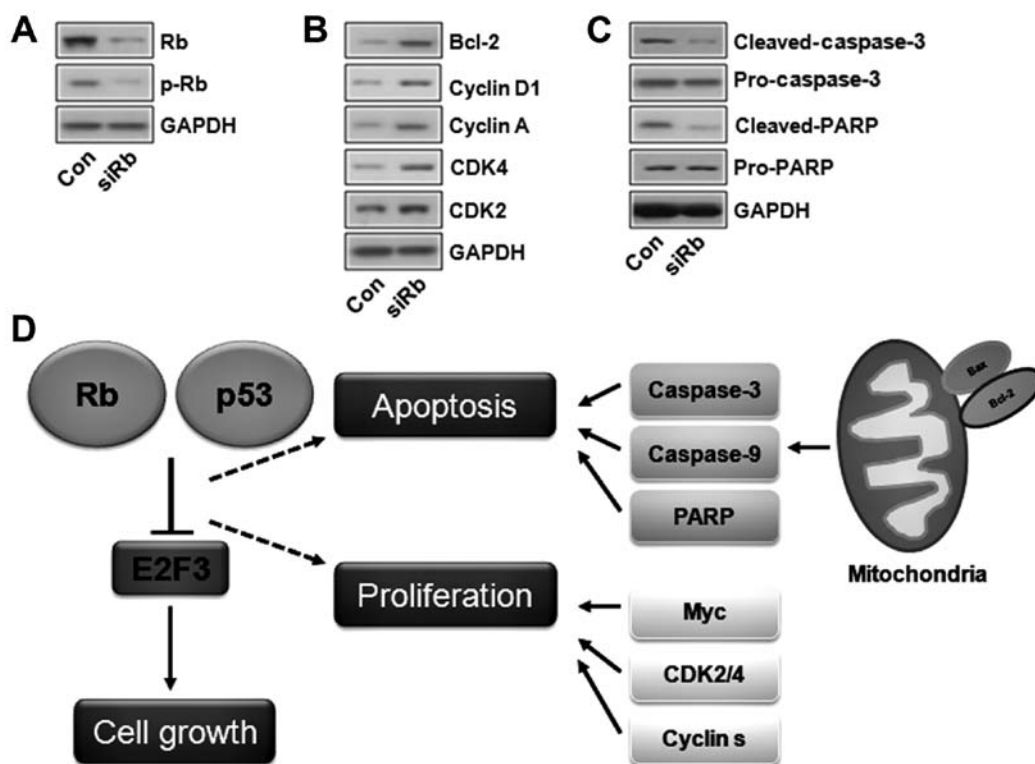


Figure 9. Knockdown of Rb accelerates E2F3 and influenced the regulatory proteins of proliferation and apoptosis *in vivo*. (A) Western blot analysis of Rb and p-Rb in bladder tumor tissues. (B) Western blot analysis of proliferation-related signals in bladder tumor tissues. (C) Western blot analysis of apoptosis-related signals in bladder tumor tissues. (D) The working model of Rb-regulated bladder tumor progression and development. Data are expressed as the mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the vector control.

Discussion

Bladder cancer is one of the most frequent types of cancer among males and a main cause leading to death in the world (26). Initially, although most patients are diagnosed with non-muscle-invasive bladder cancer, a large number of such tumors experience recurrence after therapy and exit muscle invasion eventually (27,28). Recently, researchers have focused on using therapeutic strategies against cancer cells to inhibit cell proliferation and induce apoptosis, representing a programmed self-killing molecular mechanism, which includes extrinsic and intrinsic signaling pathways (29). However, the targeted genes or proteins that impede bladder cancer progression, is still limited. Thus, further research of the molecular mechanisms by which bladder cancer cells migrate and progress are necessary.

Cell cycle dysregulation is known as a hallmark for tumor cells (30). The G1/S checkpoint comprises cyclin D1 and E, CDK2, 4, and 6, and Rb. Rb plays the role in constraining the G1/S transition in many mammalian cells. cyclin D-CDK4/6 as well as cyclin E-CDK2 complexes result in the Rb phosphorylation and inactivation cooperatively (31,32). Phosphorylated Rb regulates E2F3 activation, which is required for the progression into late phase of G1 and S (33). This sequential regulation has additional specificity in modulating alternative cell fates, including differentiation and proliferation, and plays an important role in tumor development and progression (34). The Rb phosphorylation, resulting in the sequestration of E2F3, and then influencing the expression of Myc, which is

known as a significant factor, contributing to tumor progression (35). In line with previous reports, our study indicated that Rb expression was low in bladder tumor tissue samples and bladder cancer cells *in vivo* and *in vitro*, respectively. On the contrary, the phosphorylated Rb was highly expressed in bladder cancer samples and cells, inducing cells cycle arrest in bladder cancer cells, which was dependent on cyclin D1, cyclin A and CDK2/4, as well as E2F3 signaling pathway accompanied by Myc overexpression, contributing to cell proliferation. In addition, E2F3 knockdown ameliorated bladder cancer proliferation and migration, further suggesting that Rb-inhibited bladder cancer progression was at least partly dependent on E2F3 suppression. Tumor suppressor p53 is well known as a critical protein, in apoptosis (36). In our study, we found that combination of Rb and p53 knockdown could aggravate bladder cancer cell proliferation and apoptosis, suggesting that Rb might interact together with p53 to modulate bladder cancer progression.

Bcl-2 family proteins function in various roles in the modulation of cell apoptosis and primarily influence the mitochondrial signaling pathway (37). Bcl-2 stabilizes the cell mitochondrial membrane and inhibits the cytochrome *c* release and other pro-apoptotic factor expression, while Bax enhances apoptosis. Furthermore, the ratio of Bcl-2/Bax is usually considered as a criterion for programmed cell death (38). In addition, p53 has a close relationship with apoptosis (39). Bcl-2 promoter has a p53-negative responding element, which may be the reason for p53-regulated Bcl-2 transrepression (40). In addition, Rb has been reported to influence caspase-3

activation, promoting apoptosis in tumor development (41). In our study, we found that Rb silence increased Bcl-2 expression, while decreased cleaved caspase-3 and cleaved PARP levels *in vitro* and *in vivo*, inhibiting apoptosis in bladder cancer cells. Furthermore, E2F3 knockdown promoted apoptosis, which was in agreement with previous reports (42). Also, Rb silence combined with p53 could further downregulate apoptosis in bladder cancer cells through flow cytometry analysis. Collectively, our data above indicated that Rb could combine with p53 affect apoptosis in bladder cancer development.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is ubiquitously expressed in mammalian cells. mTOR is well known to be associated with tumor development in many cancers (43,44). Also, AKT/mTOR signaling pathway was involved in a variety of tumor types (45). Similarly, our study suggested that mTOR was upregulated in Rb silence. Notably, mTOR activation increased eIF4E and eIF4G via RT-qPCR in siRb group, while TSC-2 and SESN2 were downregulated, which were two negative factors for mTOR activation (46). The S6 kinase (S6K) proteins are some of the main downstream effectors of the mTOR and act as key modulators for protein synthesis and cell growth. S6K activation is overexpressed in a variety of human tumors (47). Similarly, in our study, phosphorylated S6K was observed in Rb-silenced bladder cancer cells. However, no significant difference of MEK1/2 and phosphorylated MEK1/2 was determined, suggesting that Rb-regulated bladder cancer progression was not dependent on MEK1/2 alteration.

Taken together, our data reveal that Rb functions as a potent tumor suppressor in bladder cancer. Patients with bladder tumor face a clinical prognosis with lower Rb, contributing to cell proliferation and apoptosis inhibiting through E2F3, p53 and mTOR modulation (Fig. 9D). It is essential that we carry out research to understand the pathways through which Rb and other tumor-suppressing genes control the process of tumor initiation and metastatic progression.

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