NR4A3 suppresses bladder cancer progression by modulating autophagy *via* the PI3K/AKT/mTOR pathway

Li Fan,* Feng Xu,* Shouyong Liu, Ding Wu, Suchun Wan, Xin Pan, Yulin Zhou, Le Qu, Wenquan Zhou

Department of Urology, Jinling Hospital, Affiliated Hospital of Medical School, Nanjing University, Nanjing, Jiangsu, China

**These authors have contributed equally to this work.*

ABSTRACT

Bladder cancer (BC) is a prevalent and aggressive malignancy with high recurrence. Autophagy plays a dual role in cancer, acting as a tumor suppressor early on and promoting survival in later stages. NR4A3, a nuclear receptor with tumor-suppressive effects in other cancers, has not been explored in BC. NR4A3 expression was analyzed using TCGA data and validated in clinical BC samples *via* immunohistochemistry and RT-qPCR. NR4A3-overexpressing BC cell lines (5637, T24) were created using lentiviral vectors. Cell viability, proliferation, migration, and invasion were assessed through CCK-8, EdU, and Transwell assays. Autophagy was measured by microtubule-associated protein 1A/1B-light chain 3 (LC3), autophagy-related protein 5 (ATG5), Beclin-1 and p62 expression *via* immunofluorescence and Western blotting. The phosphoinositide 3-kinase (PI3K) / protein kinase B (AKT) / mammalian target of rapamycin (mTOR) pathway was examined by assessing phosphorylation levels. It was found that NR4A3 was significantly downregulated in BC tissues. Overexpression of NR4A3 inhibited BC cell proliferation, migration, and invasion, while promoting apoptosis. NR4A3 overexpression increased autophagy markers and suppressed PI3K/AKT/mTOR signaling. Autophagy inhibition reversed these effects. In conclusion, NR4A3 suppresses BC progression by promoting autophagy *via* the PI3K/AKT/mTOR pathway. Targeting NR4A3-mediated autophagy may provide a novel therapeutic strategy for BC.

Key words: bladder cancer; NR4A3; autophagy; PI3K/Akt/mTOR pathway; tumor suppressor.

Correspondence: Wenquan Zhou, Department of Urology, Jinling Hospital, Medical School of Nanjing University, No. 305 East Zhongshan Road, Nanjing 210002, Jiangsu, China. E-mail: Zwquan866jnh@163.com

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Availability of data and materials: the datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Introduction

Bladder cancer (BC) ranks among the most prevalent urogenital malignancies, with over 550,000 new cases annually and a mortality rate exceeding 40%.[1,2] Its high recurrence and asymptomatic early stages complicate timely diagnosis and treatment, underscoring the need to elucidate molecular drivers of progression and identify novel therapeutic targets.[3]

Autophagy -a lysosome-mediated recycling process- plays dual roles in cancer: suppressing tumorigenesis by clearing damaged organelles in early stages, yet promoting survival in advanced disease.[4-6] In BC, elevated autophagy facilitates invasion and chemoresistance. Studies demonstrate that silencing autophagy-related genes (*e.g.*, ATG7) induces apoptosis, highlighting its therapeutic relevance.[7-9] NR4A3, a transcriptional regulator in the nuclear receptor subfamily,[10] exhibits tumor-suppressive properties in lymphoma and gastrointestinal cancers.[11,12] Recent studies also suggest that NR4A3 may regulate autophagy-related processes.[13,14] Given the established link between the PI3K/AKT/mTOR pathway and autophagy,[15] and evidence that NR4A1 (a homolog of NR4A3) has been shown to modulate autophagy *via* the PI3K/AKT/mTOR pathway,[16] we hypothesized that NR4A3 inhibits BC progression *via* PI3K/AKT/mTOR-mediated autophagy.

In this study, we aimed to investigate the role of NR4A3 in BC progression, with a particular focus on its impact on autophagy and the underlying molecular mechanisms. By examining the expression levels of NR4A3 in both clinical BC samples and public databases, we evaluated the potential of NR4A3 as a tumor suppressor in BC. We also explored how the overexpression of NR4A3 affects BC cell behavior, including proliferation, migration, and invasion. Additionally, we examined the effects of NR4A3 overexpression on autophagy and the PI3K/AKT/mTOR signaling pathway, validating these results through rescue experiments. The findings from this study provide valuable insights into the involvement of NR4A3 in BC and suggest a new therapeutic strategy for the prevention and treatment of BC, by targeting autophagy regulation *via* the PI3K/AKT/mTOR pathway.

Materials and Methods

Bioinformatics analysis

RNA sequencing (RNA-seq) data and clinical data for BC tumors (n=404) and normal tissues (n=28) were obtained from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov). The GEPIA online tool (http://gepia.cancer-pku.cn) was utilized to assess the expression levels of NR4A3 in both BC and normal tissues, generating box plots for visualization.

Collection of clinical samples

Tumor samples and adjacent normal tissues were collected from three BC patients who underwent surgical treatment at the PLA Eastern Theater Command General Hospital. All participants provided written informed consent. None of the patients had received preoperative chemotherapy or radiotherapy. The study was approved by the Clinical Ethics Committee of PLA Eastern Theater Command General Hospital (Approval No. 2023DZKY-037-01).

Immunohistochemical staining

Fixed tumor and adjacent normal tissues were paraffin-embedded and sectioned. After dewaxing in xylene and rehydration through a graded ethanol series, antigen retrieval was performed by heating the sections in 10 mM citrate buffer (pH 6.0) at 95°C for 20 min using a microwave oven. Endogenous peroxidase activity was quenched by incubating the sections in 3% hydrogen peroxide in methanol for 10 min at room temperature. Non-specific binding was blocked with 5% normal goat serum for 30 min. The sections were incubated overnight at 4°C with a primary antibody (ab188752, 1:200 dilution; Abcam, Cambridge, UK). As a negative control, parallel sections were processed under identical conditions, except the primary antibody was replaced with isotype-matched IgG at the same concentration. The following day, sections were washed with PBS, incubated with HRP-labeled secondary antibody (ab205718, 1:5000 dilution; Abcam), and developed with DAB reagent (Yeasen Biotechnology, Shanghai, China). Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) was used for counterstaining. The sections were then mounted and examined under a microscope (Olympus BX53; Tokyo, Japan) with 40× objective lenses.

Cell culture

Normal urothelial cells (SV-HUC-1) and BC cell lines (5637, T24, HT-1376, RT-112) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SV-HUC-1 cells were cultured in Ham's F-12K medium (Invitrogen, Carlsbad, CA, USA), while BC cell lines were maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT, USA) and 1% penicillin/streptomycin (Yuanye, Shanghai, China).

Lentivirus transduction

Lentiviral vectors encoding NR4A3 overexpression (OE-NR4A3) or the control vector (OE-vector) were constructed and packaged by Cyagen Biosciences (Suzhou, China). 5637 and T24 cells were seeded and infected with lentivirus in serum-free RPMI-1640 medium. After 48 h, the medium was replaced with complete RPMI-1640. At 72 h post-infection, stable cell lines were selected with puromycin (1 mg/L; Yeasen Biotechnology). The transduction efficiency was confirmed by measuring NR4A3 mRNA levels using RT-qPCR, which served as a direct indicator of successful gene overexpression.

RT-qPCR

Total RNA was extracted from cells using TRIzol reagent (Absin, Shanghai, China) and reverse-transcribed into cDNA. RT-qPCR was performed with five replicates per group using Takara's (Tokyo, Japan) kits. The cycling conditions were as follows: initial denaturation at 95°C for 3 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The primer sequences are provided in Table 1. The expression levels of target genes were normalized to GAPDH and analyzed using the ΔΔCt method.

CCK-8 assay

Cell viability was assessed using the CCK-8 kit (Absin). Transfected 5637 and T24 cells were seeded in 96-well plates. On the second day, 10 µL of CCK-8 reagent was added to each well, and the cells were incubated for 2.5 h. Absorbance at 450 nm was measured using a microplate reader (Thermo-Fisher Scientific, Carlsbad, CA, USA).

EdU assay

Cell proliferation was assessed using the EdU assay kit (RiboBio Co., Ltd., Guangzhou, China). 5637 and T24 cells were seeded in 24-well plates and allowed to adhere. EdU solution was added for 2 h, and the cells were then fixed and permeabilized with 4% paraformaldehyde and Triton X-100 (Macklin Biochemical

Co., Ltd., Shanghai, China), respectively. Cells were incubated with Apollo®567 staining solution for 25 min in the dark. After nuclear staining with DAPI, fluorescence microscopy (Olympus IX83) was used to capture images at 400× magnification. At least five randomly selected high-power fields (HPFs) per sample were analyzed. The EdU positive rate was calculated as the percentage of EdU-positive cells relative to the total number of DAPI-stained nuclei.

TUNEL assay

Apoptosis was detected using the TUNEL assay (Beyotime Biotech, Inc., Haimen, China). Cells were fixed, permeabilized, and incubated with the TUNEL reaction mixture at 37°C for 1 h. After washing with PBS, an anti-fade mounting medium with DAPI was applied. TUNEL-positive cells were observed under a fluorescence microscope (Olympus IX83) under 400× magnification. At least five randomly selected HPFs per section were analyzed. The apoptosis rate was calculated as the percentage of TUNEL-positive cells among the total DAPI-stained nuclei.

Transwell assay

For invasion assays, Matrigel (Corning Inc., Corning, NY, USA) was diluted with cold RPMI-1640 at a 1:8 ratio and applied to the upper chamber of the Transwell apparatus. Transfected cells (1×10^6 cells/mL) were seeded in the upper chamber, and complete medium was added to the lower chamber. After 48 h, the non-invading cells were removed, and the remaining cells were fixed, stained with 0.5% crystal violet (Macklin Biochemical Co., Ltd), and examined under a microscope. For migration assays, the procedure was identical, except Matrigel was omitted from the chamber.

Immunofluorescence staining

Cells were seeded on slides in 48-well plates and fixed with 4% paraformaldehyde. After permeabilization and blocking with 5% BSA (Sangon Biotech, Shanghai, China), the cells were incubated overnight with anti-LC3 antibody (1:1000, ab232940; Abcam). After incubation with a secondary antibody (1:1000, ab150077; Abcam) for 30 min in the dark, slides were mounted with DAPI-containing medium (Beyotime Biotech, Inc). Fluorescence images were acquired using an Olympus IX83 fluorescence microscope equipped with 40× objectives under consistent exposure settings. Cells showing clear and specific fluorescent signals localized to cytoplasm were considered positive. At least five randomly selected HPFs were imaged per sample. Quantification was performed by measuring fluorescence intensity using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blotting

Cell lysates were prepared, and protein concentrations were determined. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (MilliporeSigma, Burlington, MA, USA). After blocking with 5% BSA, the membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature. Protein bands were detected using enhanced chemiluminescence reagents (MedChemexpress, Monmouth Junction, NJ, USA) and imaged with a chemiluminescence system (Chemisscope 6300; Clinx Science, Shanghai, China). Band intensity was quantified using ImageJ software, and GAPDH was used as the loading control. Detailed information on all antibodies used (including host species, dilution, catalog numbers, and manufacturers) is provided in Table 2.

Statistical analysis

All data represent the results from at least three independent experiments and are expressed as the mean ± SD. Statistical analyses were performed using SPSS version 23.0. A p-value <0.05 was considered statistically significant.

Results

NR4A3 is downregulated in bladder cancer and correlates with poor prognosis

To explore the potential role of NR4A3 in BC, we first analyzed its expression in BC tissues using data from The Cancer

Table 1. Primer sequences for RT-qPCR.

Gene	Primer sequences (5'-3')
NR4A3-F	AAAGTTCCGCAAGTCAGAGTAC
NR4A3-R	ACCAGCACCTGGATTAAAAGT
GAPDH-F	GGAGCGAGATCCCTCCAAAAT
GAPDH-R	GGCTGTTGTCATACTTCTCATGG

Table 2. Information of antibodies used in Western blot.

Antibody name	Host	Dilution	Catalog No.	Manufacturer
Anti-LC3	Rabbit	1:2500	14600-1-AP	Proteintech
Anti-ATG5	Rabbit	1:2500	10181-2-AP	Proteintech
Anti-Beclin1	Rabbit	1:5000	11306-1-AP	Proteintech
Anti-p62/SQSTM1	Rabbit	1:20000	18420-1-AP	Proteintech
Anti-PI3K	Rabbit	1:500	20584-1-AP	Proteintech
Anti-AKT	Rabbit	1:5000	10176-2-AP	Proteintech
Anti-mTOR	Rabbit	1:5000	28273-1-AP	Proteintech
Anti-PI3K p85 beta (phospho Y464)	Rabbit	1:1000	ab138364	Abcam
Anti-Phospho-AKT (Ser473)	Rabbit	1:2500	28731-1-AP	Proteintech
Anti-Phospho-mTOR (Ser2481)	Rabbit	1:500	28879-1-AP	Proteintech
Anti-β-actin	Rabbit	1:10000	20536-1-AP	Proteintech
HRP-goat anti-rabbit	Goat	1:10000	RGAR001	Proteintech

Genome Atlas (TCGA) database. The results revealed a significant downregulation of NR4A3 in BC tissues compared to normal tissues (Figure 1A). Further validation was performed using clinical BC samples. RT-qPCR and immunohistochemical staining showed that NR4A3 expression was markedly reduced in BC tissues compared to adjacent normal tissues (Figure 1 B,C). Moreover, the NR4A3 mRNA levels in four BC cell lines, including 5637, T24, HT-1376, and RT-112, were examined and compared with those in SV-HUC-1 cells. The findings revealed a significant reduction in NR4A3 expression across all four BC cell lines (Figure 1D). These findings suggest that NR4A3 is downregulated in BC and may be associated with the progression of the disease.

Overexpression of NR4A3 inhibits the malignant behavior of BC cells

To investigate the functional role of NR4A3 in BC, we established NR4A3 overexpression (OE-NR4A3) stable cell lines in 5637 and T24 BC cells. The efficiency of NR4A3 overexpression was confirmed by quantitative RT-qPCR (Figure 2A). Cell viability assays (CCK-8) demonstrated that overexpression of NR4A3 significantly inhibited the proliferation of both 5637 and T24 cells (Figure 2B). The EdU proliferation assay further confirmed that NR4A3 overexpression reduced the proportion of proliferating cells (Figure 2C). Additionally, TUNEL assay verified that overexpression of NR4A3 elevated the apoptosis level of BC cells (Figure 2D). Next, we examined the effect of NR4A3 on the migratory and invasive capacities of BC cells using Transwell assay. Overexpression of NR4A3 dramatically reduced the migratory and invasive ability of both 5637 and T24 cells (Figure 2E). These results suggest that NR4A3 suppresses the aggressive behaviors of BC cells.

NR4A3 regulates autophagy in BC cells

Given that autophagy plays a crucial role in the progression of BC, we next assessed whether NR4A3 modulates autophagy. Immunofluorescence staining for LC3, a marker of autophagosomes, revealed that overexpression of NR4A3 led to a significant increase in LC3-positive puncta in both 5637 and T24 cells (Figure 3A). Western blot analysis further confirmed that NR4A3 overexpression increased the levels of autophagy markers, including LC3, ATG5 and Beclin-1, while decreasing the expression of p62, an autophagy substrate (Figure 3B).

NR4A3 regulates autophagy via the PI3K/AKT/mTOR signaling pathway

We next investigated the molecular mechanisms through which NR4A3 regulates autophagy. Western blot analysis showed that NR4A3 overexpression decreased the phosphorylation levels of PI3K, AKT and mTOR in both 5637 and T24 cells (Figure 4A). These findings suggest that NR4A3 might modulate autophagy through the PI3K/AKT/mTOR pathway.

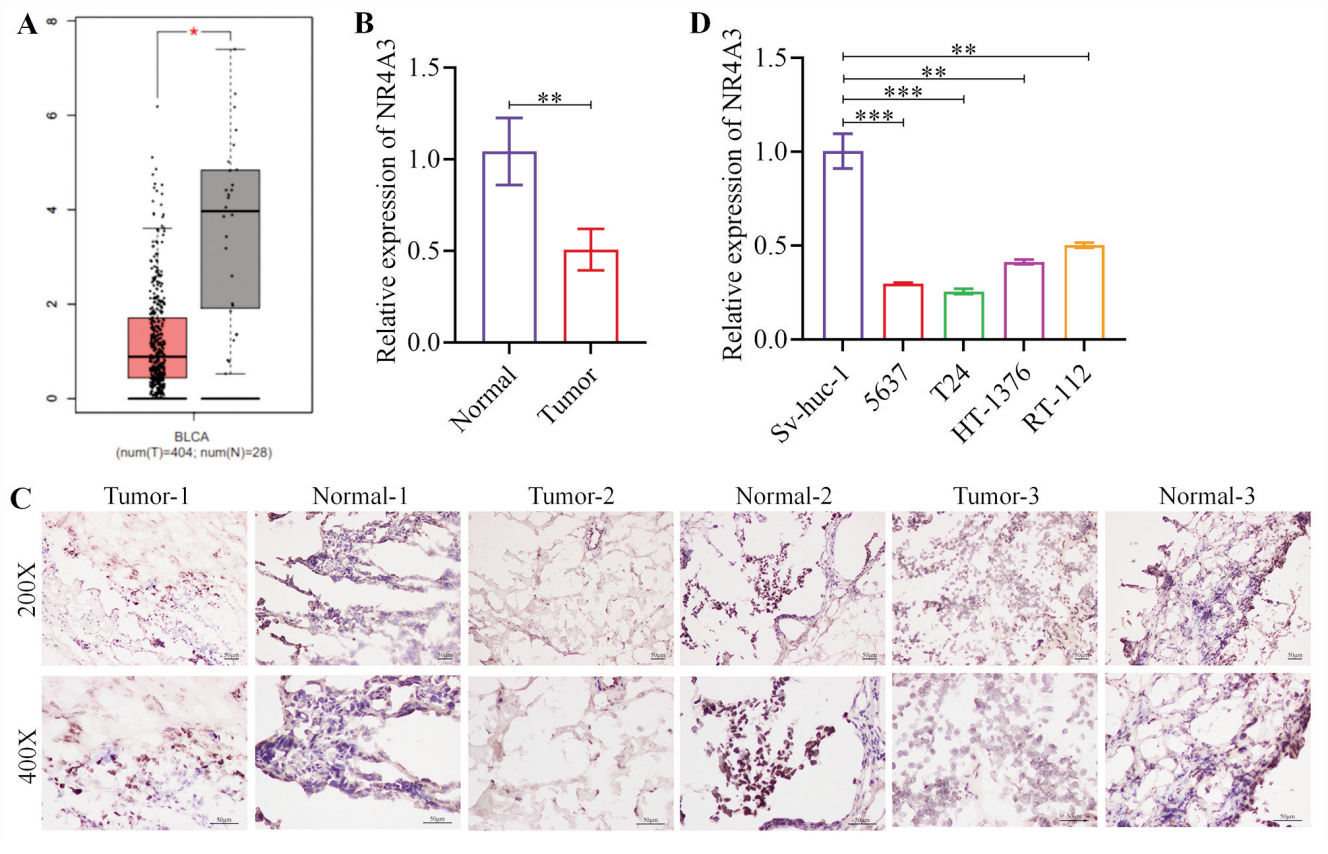

Figure 1. NR4A3 was low-expressed in bladder cancer. **A**) Analysis of the RNA-seq data of BC tumor and adjacent normal tissues obtained from the TCGA database; BLCA, bladder cancer.) **B**) RT-qPCR analysis of the relative mRNA expression of NR4A3 in clinical tumor and adjacent normal tissues. **C**) Immunohistochemical staining of NR4A3 in clinical samples. **D**) The relative mRNA expression of NR4A3 in BC cell lines, including 5637, T24, HT-1376 and RT-112. *p<0.05; **p<0.01; ***p<0.001.

Autophagy inhibition reverses the effects of NR4A3 overexpression on BC cell behavior

To confirm that autophagy is essential for NR4A3-mediated suppression of BC progression, we performed rescue experiments. BC cells were co-treated with NR4A3 overexpression and MHY1485, an autophagy inhibitor and a mTOR activator.[17] The results showed that inhibition of autophagy reversed the suppressive effects of NR4A3 on cell proliferation, migration, and invasion (Figure 5 A,B,D). The TUNEL assay demonstrated that NR4A3 overexpression increased apoptosis, an effect that was diminished by autophagy inhibition (Figure 5C). Additionally, MHY1485 treatment decreased the levels of LC3, ATG5, and Beclin1, while upregulated p62 level (Figure 5 E,F). Moreover, the results revealed increased phosphorylation levels of PI3K, AKT, and mTOR in the OE-NR4A3 + MHY1485 group, while the total protein levels of PI3K, AKT and mTOR remained constant (Figure 5G). Collectively, these results highlight the critical role of autophagy in mediating the tumor-suppressive effects of NR4A3 in BC cells.

Discussion

BC remains one of the most challenging urogenital cancers, characterized by high recurrence rates, metastasis, and significant mortality.[1] Our study aimed to elucidate the role of NR4A3 in BC progression, focusing on its relationship with autophagy and the PI3K/AKT/mTOR pathway. Our findings demonstrate that NR4A3 is significantly downregulated in BC tissues and cell lines compared to normal urothelial cells, highlighting its potential as a tumor suppressor. Overexpression of NR4A3 in BC cells notably suppressed malignant behaviors,[18] including cell proliferation, migration, and invasion.[19] These results are consistent with previous studies that identify NR4A3 as a tumor suppressor in various cancers, such as lymphoma[12] and hepatocellular carcinoma.[20] Specifically, our study showed that NR4A3 overexpression significantly inhibited BC cell proliferation, promoted apoptosis, and reduced invasive and migratory capacities.[19] These findings suggest that NR4A3 plays a crucial role in curbing BC progression by targeting key cellular processes involved in cancer cell survival and metastasis.

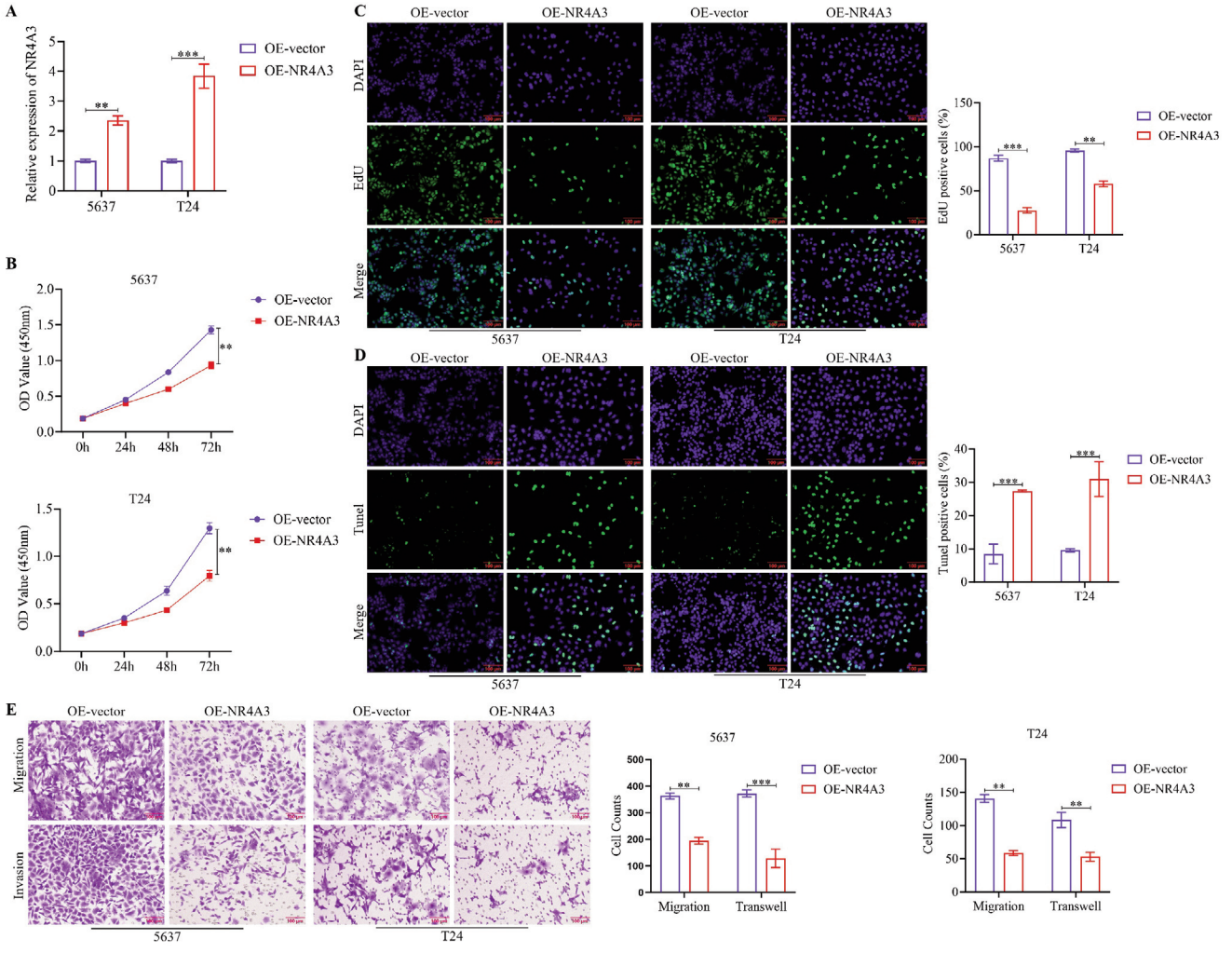

Figure 2. Overexpression of NR4A3 inhibits the malignant behavior of bladder cancer cells. **A**) The efficiency of OE-vector and OE-NR4A3 was validated by RT-qPCR. **B**) The cell viability of 5637 and T24 cells overexpressing NR4A3 was detected by CCK-8 assay. **C**) EdU assay was used to evaluate cell proliferation. **D**) Cell apoptosis was visualized by TUNEL assay. E) The migration and invasion capacity of 5637 and T24 cells was detected by Transwell assay. $^{**}p<0.01$; $^{***}p<0.001$.

One of the novel aspects of this study is the role of NR4A3 in modulating autophagy in BC.[21-23] Autophagy is a complex cellular process with dual roles in cancer, acting as both a tumor-suppressive and tumor-promoting mechanism depending on the stage of cancer.[7] In this study, we observed that overexpression of NR4A3 significantly upregulated autophagy-related proteins such as LC3, ATG5, and Beclin1, while downregulating p62/SQSTM1 in BC cells. This suggests that NR4A3 may facilitate the autophagic process, which is consistent with its proposed role in maintaining cellular homeostasis.[24] Moreover, our study revealed that NR4A3 overexpression suppressed the PI3K/AKT/mTOR signaling pathway, a critical regulator of autophagy.[15] This pathway is often dysregulated in cancer, leading to uncontrolled cell growth and inhibition of autophagy. We found that inhibiting the PI3K/AKT/mTOR pathway by NR4A3 promoted autophagy in BC cells,[15] further confirming that NR4A3 may exert its tumor-suppressive effects through modulation of this pathway. Although the exact mechanism remains unclear, NR4A3 may inhibit the PI3K/AKT/mTOR pathway by upregulating upstream suppressors such as PTEN or TSC1/2, or through indirect transcriptional regulation, as reported for other NR4A family members.[18,25] Further studies are needed to confirm these possibilities. Interestingly, activation of mTOR using MHY1485 reversed the effects of NR4A3 overexpression,[26] leading to enhanced cell viability,[15] increased proliferation, and restored migration and invasion abilities. These results underscore the significance of the PI3K/AKT/mTOR pathway in mediating the antitumor effects of NR4A3 and its potential for therapeutic targeting in BC.[27] Although targeting the PI3K/AKT/mTOR pathway holds therapeutic promise, systemic inhibition of this axis may lead to adverse effects due to its role in normal physiology. Strategies such as nanoparticle-based drug delivery, isoform-selective inhibitors, or combination therapies may help mitigate toxicity. Future studies should explore these approaches to improve clinical applicability. Besides targeting PI3K/AKT/mTOR, direct activation of NR4A3 may represent a complementary strategy. However, given the dual role of autophagy in cancer -suppressing early tumorigenesis but potentially supporting survival in advanced stages- careful modulation is needed to avoid undesired effects. Taken together, our findings provide compelling evidence that NR4A3 functions as a tumor suppressor in BC by promoting autophagy through the inhibition of the PI3K/AKT/mTOR pathway.[28] This novel insight into the molecular mechanisms of NR4A3 opens new therapeutic avenues for BC treatment. Targeting the PI3K/AKT/mTOR signaling pathway, in combination with strategies to enhance NR4A3 expression, could provide a promising approach for overcoming BC progression and metastasis. However, the potential clinical application of these findings requires further validation, including *in vivo* studies and exploration of potential side effects related to the modulation of the PI3K/AKT/mTOR pathway, which is essential for normal cellular functions. Future animal studies using xenograft models are planned to assess NR4A3-related effects on tumor growth, autophagy activity, and systemic toxicity.

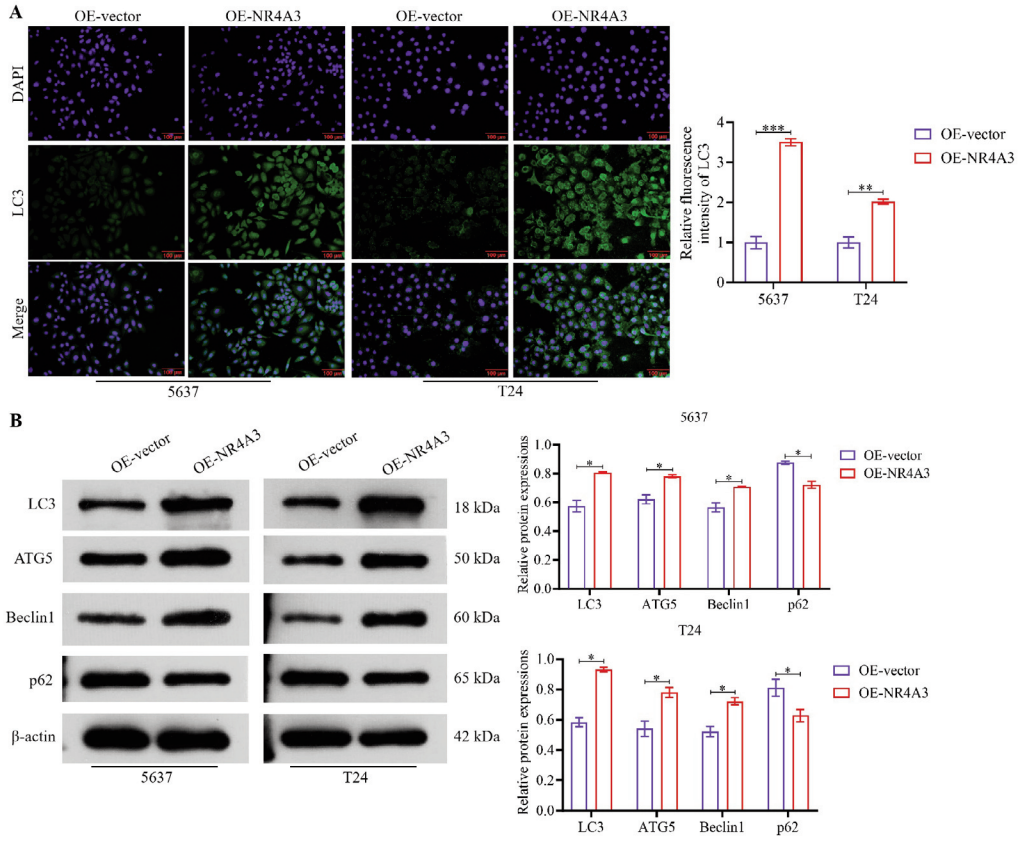

Figure 3. Overexpression of NR4A3 promotes autophagy in bladder cancer cells. **A**) The expression of LC3 in 5637 and T24 cells was determined by immunofluorescent staining. **B**) The protein expression of LC3, ATG5, Beclin1, and p62 was detected through Western blotting assay. *p<0.05; **p<0.01; ***p<0.001.

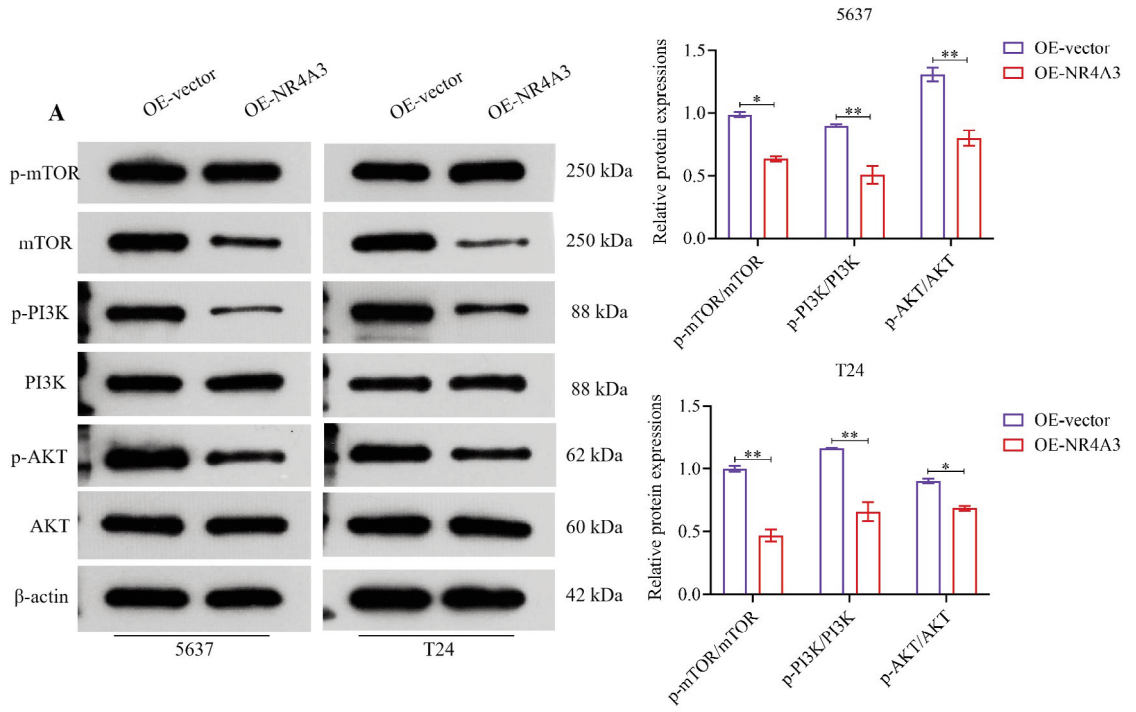

Figure 4. Overexpression of NR4A3 inhibits the PI3K/AKT/mTOR pathway. **A**) The activation of PI3K/AKT/mTOR pathway was evaluated. ns, non-significant;*p<0.05; **p<0.01.

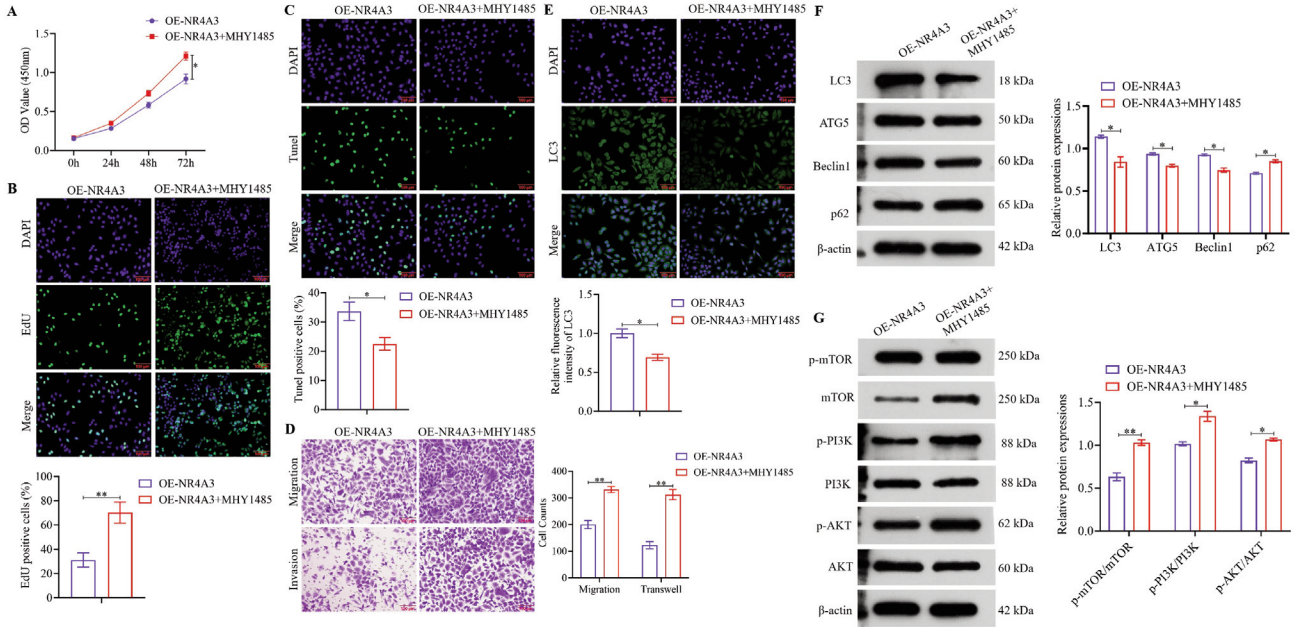

Figure 5. Activation of mTOR reverses the effect of NR4A3 overexpression. The mTOR activator MHY1485 was used to treat BC cells. The changes of cell viability (**A**), proliferation (**B**), apoptosis (**C**), migration and invasion (**D**) were determined. **E**) The expression of LC3 in OE-NR4A3 and OE-NR4A3 + MHY1485 groups. **F**) Protein expression levels of LC3, ATG5, Beclin1, and p62 in OE-NR4A3 and OE-NR4A3 + MHY1485 groups. **G**) The effect of MHY1485 on the activation of PI3K/AKT/mTOR pathway. ns, non-significant;*p<0.05; **p<0.01.

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