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The PI3K-AKT-mTOR signaling pathway mediates the cytoskeletal remodeling and epithelial-mesenchymal transition in bladder outlet obstruction

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ABSTRACT

Objective: Partial bladder outlet obstruction(pBOO) is the most common cause of lower urinary tract symptoms (LUTS) and significantly affects the quality of life. Long-term pBOO can cause changes in bladder structure and function, referred to as bladder remodeling. The pathogenesis of pBOO-induced bladder remodeling has yet to be fully understood, so effective treatment options are lacking. Our study aimed to explore how pBOO-induced bladder remodeling brings new strategies for treating pBOO.

Methods: A rat model of pBOO was established by partial ligation of the bladder neck, and the morphological changes and fibrosis changes in the bladder tissues were detected by H&E and Masson trichrome staining. Furthermore, EMT(epithelial-mesenchymal transition) related indicators and related pathway changes were further examined after TGF- β treatment of urothelial cells SV-HUC-1. Finally, the above indicators were tested again after using the PI3K inhibitor. Subsequently, RNA sequencing of bladder tissues to identify differential genes and related pathways enrichment and validated by immunofluorescence and western blotting analysis.

Results: The pBOO animal model was successfully established by partially ligating the bladder neck. H&E staining showed significant changes in the bladder structure, and Masson trichrome staining showed significantly increased collagen fibers. RNA sequencing results significantly enriched in the cytoskeleton, epithelial-mesenchymal transformation, and the PI3K-AKT-mTOR signaling pathway. Immunofluorescence and western blotting revealed EMT and cytoskeletal remodeling in SV-HUC-1 cells after induction of TGF- β and in the pBOO bladder tissues. The western blotting showed significant activation of the PI3K-AKT-mTOR signaling pathway in SV-

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HUC-1 cells after induction of TGF-β and in pBOO bladder tissues. Furthermore, EMT and cytoskeletal damage were partially reversed after PI3K pathway inhibition using PI3K inhibitors.
Conclusions: In the pBOO rat model, the activation of the PI3K-AKT-mTOR signaling pathway can mediate the cytoskeletal remodeling and the EMT to induce fibrosis in the bladder tissues. PI3K inhibitors partially reversed EMT and cytoskeletal damage.

1. Introduction

Partial bladder outlet obstruction (pBOO) is a common problem in urology, leading to significant morbidity in the population. The pBOO is a common chronic disease of the urinary system that causes compression or resistance to the bladder outflow tract from the bladder neck to the urinary orifice and causes the associated lower urinary tract symptoms. The causes leading to pBOO are various, including functional, such as bladder neck obstruction, pelvic floor muscle hyperactivity, and mechanical, such as urethral stenosis, posterior urethral valve, and prostatic hyperplasia [1]. Importantly, pBOO causes voiding dysfunction, detrusor muscle overactivity, vesicoureteral reflux, urinary tract infection, and excessive bladder activity, reducing the patient's quality of life [2]. It is believed that long-term BOO can cause changes in bladder structure and function, known as bladder remodeling [3]. Although much knowledge has been accumulated, the pathogenesis of pBOO-induced bladder remodeling has yet to be fully understood. However, most views argue that the damage caused by pBOO leads from the initial inflammatory phase to bladder smooth muscle hypertrophy, followed by compensatory angiogenesis, eventually leading to bladder fibrosis [4]. Therefore, exploring the mechanisms of bladder fibrosis injury caused by pBOO may help find new therapeutic strategies.

Fibrotic diseases cause significant global health and economic burden and are often fatal. Although fibrosis has traditionally been considered an irreversible process, increasing evidence suggests that organ fibrosis can be reversed in some cases, primarily if the underlying cause of injury can be eliminated [5]. The causes of fibrosis are various, including obstruction factors and drug factors. Among them, damage-triggered epithelial-mesenchymal transition (EMT) dysregulation is crucial to multiorgan fibrosis. EMT is when epithelial cells gradually transform into mesenchymal-like cells and lose their epithelial function and characteristics. Epithelial cells show epithelial connections and apical-basal, while mesenchymal cells show greater motility and invasion, lacking spindle morphology and essential polarity [6]. Increasing evidence suggests that EMT plays a role in physiological and pathological healing. Many studies provide evidence that EMT from myofibroblasts derived from the tubular epithelium contributes to renal fibrosis [7] and that alveolar epithelial cells (AECs) experience EMT and promote pulmonary fibrosis [8]. Lu et al. confirmed that the EMT was also present in the pBOO [9]. EMT includes the down-regulation of epithelial markers and the up-regulation of mesenchymal markers, and many vital biological processes involve EMT. The EMT is characterized by the morphological changes caused by the disappearance of the cell-cell junctions and the actin-cytoskeleton rearrangements [10]. The essence of the EMT is the remodeling of the cytoskeleton. The cytoskeleton is a complex network of protein filaments running through the cytoplasm, supporting the maintenance of cell morphology. Cytoskeleton remodeling facilitates cell shape change and activates movement [11]; It showed that the Ca2 + -binding protein S100A16 can regulate cytoskeletal reorganization and EMT progression to promote renal tubulointerstitial fibrosis [12].

The number of individuals affected by BOO increases yearly [13]. The prevalence of the disease in women ranges from 2.7 % to 29 %. Female pBOO is a common cause of voiding dysfunction in women [14]. Women report fewer typical obstructive symptoms of pBOO than men [15]. As a result, female pBOO is often difficult to treat due to underdiagnosis [16]. Therefore, it is necessary to explore the mechanism of fibrotic injury induced by pBOO in women. We reviewed previous studies, most of which used female rats to construct pBOO animal models. For example, Bridget Wiafe et al. selected female Sprague-Dawley rats for pBOO modeling [17], and Gao et al. also selected female Sprague-Dawley rats to construct the pBOO model [18]. Moreover, female rats had shorter urethras than male mice, with smoother urethras and easier catheter placement. Therefore, female rats were selected for modeling in this study. In addition, studies have placed a catheter laterally to induce obstruction [19], and we agree with this modeling approach. However, ureteral catheterization can better simulate clinical obstruction, although catheterization in the ureter is more difficult. Therefore, this study finally selected the mode of catheter placement in the urethra of female rats for modeling to explain the clinical phenomenon better.

The main treatments for pBOO include alpha-blockers, surgery, or improved drainage through catheterization to reduce the degree of obstruction [20]. However, many bladders still deteriorate to an irreversible, poorly compliant state. This is mainly due to fibrosis progression, and understanding how pBOO induces cystic fibrosis progression may help us search for new treatment strategies. However, the mechanism of pBOO causing bladder fibrosis damage is still poorly defined. Are the EMT and cytoskeletal rearrangements present in the pBOO-induced bladder injury, and which pathways mediate cytoskeletal rearrangements? Therefore, by establishing a female rat pBOO model combined with RNA sequencing, this study aims to identify the key role of EMT and cytoskeleton remodeling in pBOO-induced bladder fibrosis and the key signaling pathways regulating cytoskeleton remodeling and EMT in order to bring new therapeutic strategies for pBOO-induced bladder fibrosis injury.

2. Methods

2.1. Animal model establishment

The experimental animals used in this study were 8-week-old female Sprague-Dawley rats (about 250–300g). We purchased

Sprague-Dawley rats from the Animal Center of Chongqing Medical University. All animals were fed normally. All procedures were approved by the Ethics Committee of the Children's Hospital of Chongqing Medical University (IACUC IACUC Issue No: CHCMU-IACUC20220323005). The surgical procedure was performed according to the previous studies [17,18]. 12 Sprague-Dawley were randomized into two groups: the surgical group (pBOO group): the rats underwent partial ligation of the bladder neck after isoflurane anesthesia, causing some bladder exit obstruction. The bladder was first exposed, and the 18-gauge vessel catheter was inserted into the urethra. The catheter was then tied with 3–0 silk around the urethra, the catheter was removed, and then to close the abdominal wound. The other group was the sham surgery group (Sham group): in which only the pelvic cavity was opened to locate the bladder neck, isolate the surrounding tissue, and then close the abdominal wound. Due to severe obstruction and dysuria after bladder neck ligation in the pBOO group, two rats died. Two rats in the Sham group died due to wound rupture, although we repaired and stitched the wounds, so only eight rats survived. After the obstruction lasted for two weeks, rats were sacrificed, bladder tissue samples were obtained and photographed, some were fixed in 4 % paraformaldehyde, and some were frozen in liquid nitrogen and frozen for-80 °C.

2.2. Hematoxylin and eosin (H&E) staining

Bladder tissues were fixed with 4 % paraformaldehyde, dehydrated, embedded in paraffin, and cut into 4 μm thick sections. Sections were dewaxed, hydrated, and soaked in hematoxylin and eosin, and bladder tissue changes were observed using a light microscope (Nikon).

2.3. Masson trichrome staining

Paraffin sections were routinely dewaxed and then stained by a Masson trichrome staining kit, performed as per the instructions, blue for collagen fibers, red for musculature, and photographed under the microscope.

2.4. RNA sequencing of bladder tissues

Bladder tissues from the pBOO and Sham groups were sent for RNA sequencing, and three samples from each group were sent to LC-BIO Biotechnology Co., Ltd. (Hangzhou, China) for RNA sequencing. The R (R4.4.1) software and the edgeR software package analyzed gene expression data from mRNA. p-value <0.05 and $|\log FC| > 2$ indicated a significant difference. Differential genes were screened, and heat maps and volcano maps were mapped. The KEGG pathway and GO functional enrichment analyses were performed on the differentially expressed genes in the R software using the cluster analyzer software package. Genes with p-value <0.05 regardless of logFC alteration were included in the GSEA with gene set c2. cp.v7.2. symbols.GMT. Pathways with an adjusted p-value <0.05 were considered to have significant differences.

2.5. Cell culture and treatments

The human urothelial SV-HUC-1 cell line was purchased from the American Type Culture Collection (ATCC; Beijing, China) and used in all experiments. Cells were cultured using SV-HUC-1 specific medium (Cat No: CM-0222, Procell), and the cells were routinely cultured in a 37 °C, 5 % CO2 incubator. When cell confluence reached 80 %, fibrotic damage was induced by adding a 10 ng/ml dose of TGF- β for 48h treatment as previously documented [21]. The PI3K inhibitor Wortmannin was used in the rescue test, and 10 μM Wortmannin was added after 6 h of intervention of TGF- β, SV-HUC-1 cells were cultured continue 48h.

2.6. Immunofluorescence

EMT-related indexes in bladder tissues were detected by immunofluorescence staining, paraffin sections were dewaxed, antigen repaired, and then sealed with 5 % bovine serum albumin (BSA) for 1 h. The primary antibody was incubated at 4 °C overnight. Washed with PBS 3 times, incubated with corresponding fluorescent secondary antibody for 1 h, washed with PBS for 3 times, dyed with Hoechst for 30min, and sealed with anti-fluorescent quencher. Finally, photographs were taken using a fluorescence microscope (Olympus Corp, Tokyo, Japan).

The cell climbing slices were prepositioned in a 24-well plate, and SV-HUC-1 cells were placed in a medium to 24-well plate at a rate of 20,000 cells/well. After cell adhesion, TGF-β and PI3K inhibitors were added. The climbing sections were harvested after 48h, and the cells were washed with PBS, fixed with 4 % paraformaldehyde for 30min, washed again with PBS, closed with 0.5 % BSA for 1h, and incubated with primary antibody overnight. The remaining steps are the same as tissue immunofluorescence. The primary antibodies used for immunofluorescence: E-cadherin, N-cadherin, ZO-1, α-SMA, and Vimentin, are the same as the primary antibodies used for western blotting, diluted with 0.5%BSA at a ratio of 1:2000.

2.7. Western blotting

Western blotting was used to detect the EMT-related indicators and the key protein expression of the PI3K-AKT-mTOR signaling pathway. Frozen bladder tissue and treated cells were collected, the protein was lysed using RIPA buffer containing 1 % protease inhibitor, and the BCA assay determined the protein concentration. The exact amount of protein (20 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane

(Microwell, USA). Then blocked with 5 % skim milk for 1 h and incubated with the primary antibody overnight at 4 °C. The primary antibodies were as follows: E-cadherin (1:1000, ZENBIO), ZO-1(1:1000, Affinity Biosciences), α-SMA(1:1000, ZENBIO), N-cadherin (1:1000, Proteintech), Vimentin(1:1000, ZENBIO), PI3K(1:1000, ZENBIO), p-PI3K(1:1000, ZENBIO), AKT(1:1000, ZENBIO), *p*-AKT (1:1000, ZENBIO), mTOR(1:1000, ZENBIO), *p*-mTOR(1:1000, ZENBIO), GAPDH(1:1000, ZENBIO). The next day, they were washed using TBST, secondary antibodies incubated for 1 h at room temperature, three times with TBST, and imaging using chemiluminescence, using ImagLab statistical grey values.

2.8. Cytoskeleton detection

Cytoskeleton was detected using two markers: F-actin and β-tubulin. Paraffin sections were routinely dewaxed and blocked by 0.5 %BSA. F-actin staining was performed with Rhodamine-labeled phalloidine, stained for 1 h, washed with PBS for 3 times, stained with DAPI for 30min, washed again with PBS for 3 times, and sealed with an anti-fluorescence quencher. The β-tubulin staining method is the same as tissue immunofluorescence staining and is photographed under confocal microscopy.

2.9. Statistical analysis

Data analysis was performed using the GraphPad prism(8.0). Data statistics were performed using the Student's T-test. All data was expressed as the mean ± standard deviation (MEAN ± SEM), and a $P < 0.05$ was statistically significant.

3. Results

3.1. The pBOO causes structural changes and increased fibrosis in the bladder tissues

We established an animal model of pBOO by partial ligation of the bladder neck of SD rats, and the results showed that the bladder volume was significantly larger in the pBOO group compared with the Sham group (Fig. 1A); HE staining showed a normal bladder tissue structure in the Sham group, with a normal bladder mucosa with a urothelium, and a normal smooth muscle layer morphology; in the pBOO group, bladder epithelial thinning and loss of standard tissue structure (Fig. 1B); Masson trichrome staining confirmed increased collagen fibers in pBOO group (Fig. 1C). The above results confirm that pBOO does cause bladder fibrosis injury.

3.2. RNA sequencing and bioinformatics analysis of bladder tissues

We performed RNA sequencing of bladder tissues in the pBOO group and sham group, and sequencing results showed 1390 up-regulated genes and 608 down-regulated genes (Fig. 2A), and the volcano plot showed the same results (Fig. 2B); the differential gene heat map showed good clustering effect and significant differences between groups (Fig. 2C). GO analysis of differential genes showed extensive enrichment to EMT and cytoskeleton-related processes (Fig. 2D); The KEGG results showed that the PI3K-AKT signaling pathway and the cytoskeleton regulation-related pathway were widely enriched (Fig. 2E). We further performed a GSEA enrichment analysis of the relevant pathways, and the results showed that the PI3K-AKT-mTOR was significantly activated (Fig. 2F). Based on the sequencing results, it was speculated that pBOO induced the activation of the PI3K-AKT-mTOR signaling pathway, which

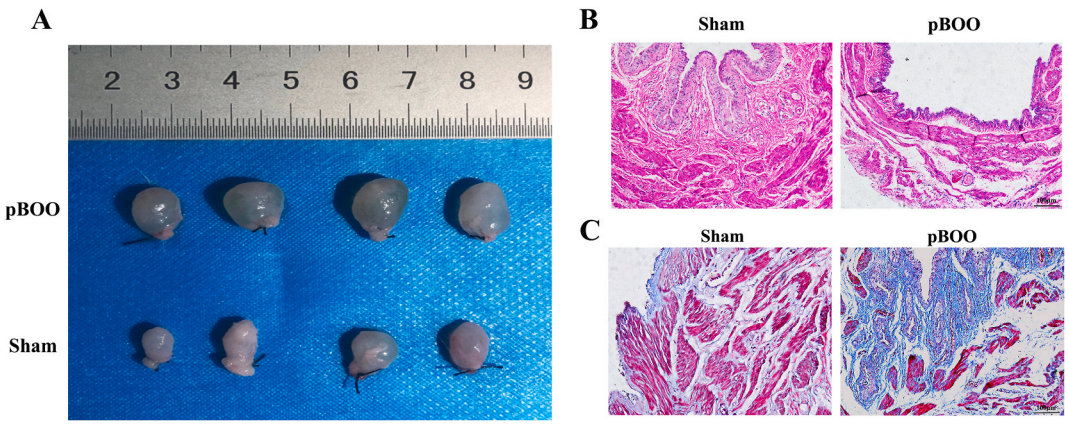

Fig. 1. Gross picture of the bladder, H&E staining, and Masson trichrome staining. A: Gross picture of the bladder shows that the bladder volume in the pBOO group was greater than that in the sham group; B: H&E staining showed a normal bladder tissue structure in the Sham group, with a normal bladder mucosa with a urothelium, and a normal smooth muscle layer morphology; in the pBOO group, bladder epithelial thinning and loss of standard tissue structure; C: Masson trichrome staining confirmed increased collagen fibers in pBOO group (blue for collagen fibers, red for musculature). (Scale bar:100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

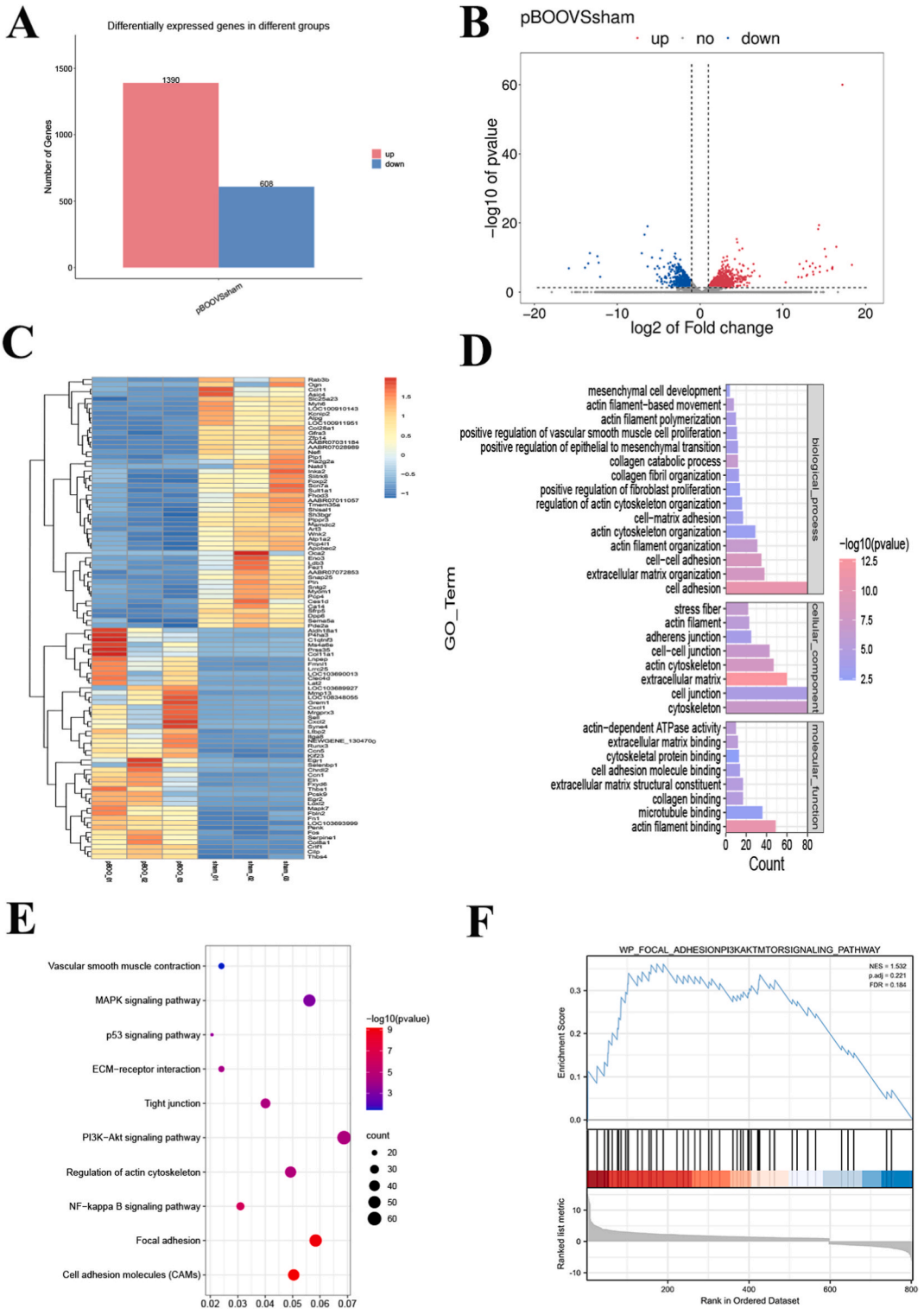

Fig. 2. The transcriptome sequencing and bioinformatic analysis of bladder tissue in the Sham and pBOO groups. A: bar chart of differential genes; B: volcano chart of differential genes; C: cluster heat map of differential genes; D: GO enrichment analysis of differential genes; E: KEGG enrichment analysis of differential genes; F: GSEA enrichment analysis of differential genes.

regulates EMT and cytoskeletal remodeling, leading to fibrotic damage in the bladder.

3.3. In vivo and in vitro experiments demonstrated the presence of the EMT phenomenon in pBOO

Based on the results of the GO analysis, we further verified whether EMT existed in bladder fibrosis injury caused by pBOO. The immunofluorescence and the western blotting showed that the bladder tissues' epithelial-related indexes ZO-1 and E-cadherin were significantly downregulated in the pBOO group compared to the sham group. At the same time, Vimentin, α-SMA, and N-cadherin were significantly up-regulated (Fig. 3A and B), indicating the phenomenon of EMT exists in the bladder injury caused by pBOO. In addition, cell experiments observed significant downregulation of epithelial-related indicators ZO-1 and E-cadherin and upregulation of Vimentin, α-SMA, and N-cadherin in SV-HUC-1 cells after TGF- β treatment (Fig. 4A and B). The above results confirmed the presence of the EMT phenomenon in pBOO.

3.4. Both in vivo and in vitro experiments confirmed the presence of cytoskeletal rearrangements in pBOO bladder tissue

GO analysis was widely enriched in the cytoskeleton, and cytoskeleton rearrangement was also the key to EMT. Therefore, we further tested the cytoskeleton in the bladder tissues of pBOO modeling. The results showed that in the bladder epithelial tissues of the

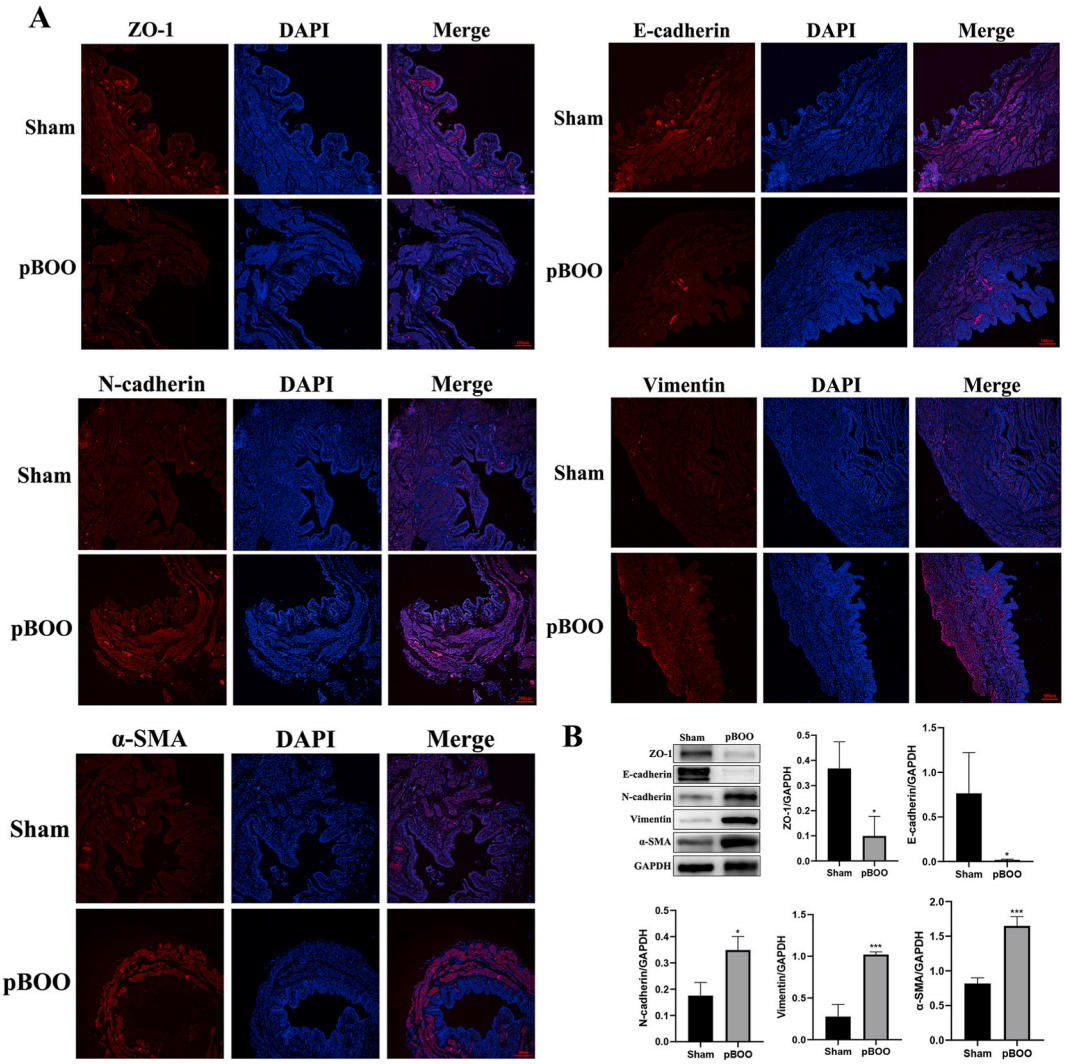

Fig. 3. Expression of EMT-related indicators in the bladder tissue. A: The immunofluorescence showed that the bladder tissues' epithelial-related indexes ZO-1 and E-cadherin were significantly downregulated in the pBOO group compared to the sham group. At the same time, Vimentin, α-SMA, and N-cadherin were significantly up-regulated. (Scale bar:100 μm). B: Western blotting showed that ZO-1 and E-cadherin were significantly downregulated in the pBOO group compared to the sham group. At the same time, Vimentin, α-SMA, and N-cadherin were significantly up-regulated.*$p < 0.05$.***$p < 0.001$.

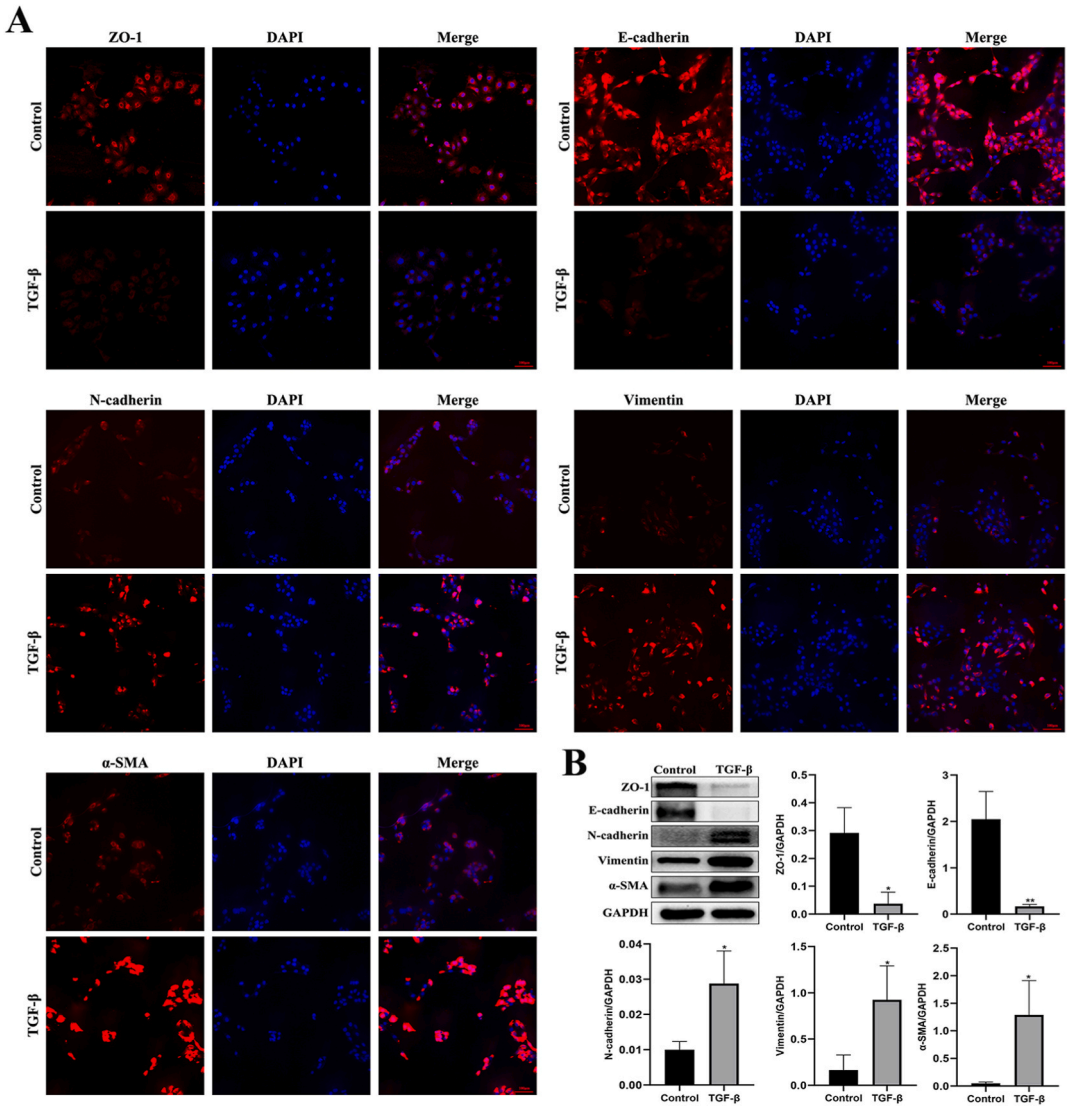

Fig. 4. Expression of EMT-related indicators in SV-HUC-1 cells. A: Immunofluorescence showed that ZO-1 and E-cadherin were downregulated in TGF-β treated SV-HUC-1 cells, while N-cadherin, Vimentin, and α-SMA were up-regulated compared with control cells. (Scale bar:100 μm). B: Western blotting showed ZO-1 and e-cadherin were downregulated in TGF-β treated SV-HUC-1 cells, while n-cadherin, Vimentin, and α -SMA were up-regulated compared with control cells. *$p < 0.05$.**$p < 0.01$.

sham group, cells were intact with good continuity, and the actin skeleton (F-actin) and microtubules (β -Tubulin) structures were intact. However, the bladder epithelial cells were disordered in the pBOO group, cell continuity was poor, and the F-actin and β -Tubulin structures were significantly destroyed (Fig. 5A), indicating significant cytoskeleton remodeling in the bladder tissue of the pBOO group. In addition, in vitro experiments observed significant changes in F-actin and β -Tubulin structure in SV-HUC-1 cells after TGF-β treatment and poor continuity between cells (Fig. 6A).

3.5. Both in vivo and in vitro experiments confirmed the presence of PI3K-AKT-mTOR signaling pathway activation in pBOO bladder tissue

Based on the results of KEGG and GSEA, we further used western blotting to detect the expression of key proteins in the PI3K-AKT-mTOR pathway in the bladder tissues of the pBOO and sham groups. The western blotting results showed that the key proteins were significantly up-regulated in this pathway (Fig. 5B), indicating that this pathway was significantly activated, consistent with the GSEA results. In addition, cell experiments also observed that the expression of key proteins of the PI3K-AKT-mTOR-pathway was also significantly up-regulated after TGF- β induction (Fig. 6B), which is consistent with the in vivo results.

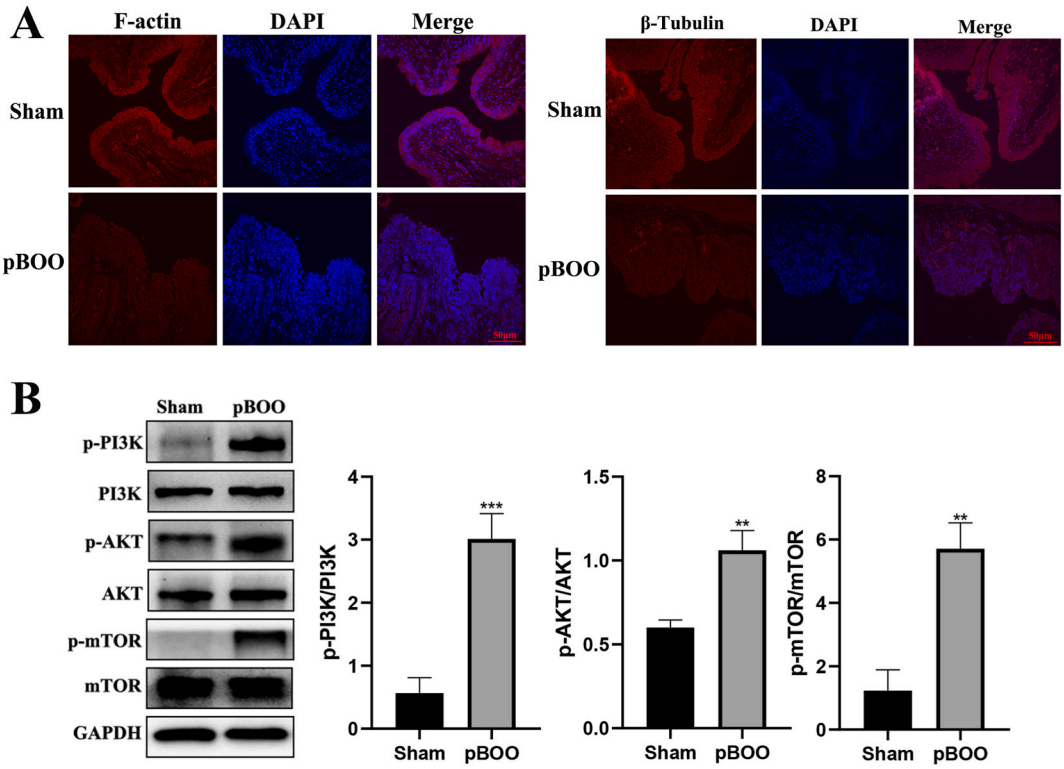

Fig. 5. Cytoskeletal changes and PI3K-AKT-mTOR pathway changes in bladder tissues. A: Fluorescence image of F-actin: compared with the Sham group, the bladder epithelial cells were disordered in the pBOO group, cell continuity was poor, and the F-actin structures were significantly destroyed; Fluorescence image of β -Tubulin： compared with the Sham group, the β -Tubulin structures were significantly destroyed. (Scale bar:50 μm). B. Western blotting showed that the key protein expression of the PI3K-AKT-mTOR-related pathway was elevated in the bladder tissue of the pBOO group compared to the sham group.**$p < 0.01$,***$p < 0.001$.

3.6. The PI3K-AKT-mTOR signaling pathway regulates the EMT and the cytoskeleton

To confirm whether PI3K-AKT-mTOR signaling regulates EMT and cytoskeleton, we added PI3K inhibitor into the TGF- β treatment group. We observed a reduction of EMT after the addition of the PI3K inhibitor as compared with the TGF- β treatment group (Fig. 7A and B). In addition, we observed downregulation of PI3K-AKT-mTOR signaling and partial reduction of cytoskeletal damage after adding PI3K inhibitors compared with the TGF- β treatment group (Fig. 8A and B), indicating that EMT and cytoskeleton are indeed regulated by PI3K-AKT-mTOR signaling.

4. Discussion

Common problems with the bladder or posterior urethra valve that have significant morbidity and potential mortality in urology. BOO is the most common cause of lower urinary tract symptoms (LUTS) and significantly affects the patient's quality of life [22]. Recent studies have shown that mechanical obstruction caused by BOO causes structural and functional changes in the bladder detrusor, including the extracellular matrix (ECM) deposition. However, the outcome is bladder fibrosis [4]. Previous studies suggest many signaling pathways are involved in remodeling the obstruction-exposed urinary bladder. To figure out the specific mechanism of pBOO causing bladder fibrosis, we established an animal model of pBOO. We found that the altered bladder tissue structure and a significant increase in collagen fibers suggested the possibility of bladder fibrosis. Previous studies showed that BOO induced collagen fiber thickening and deposition of these fibers in the detrusor layer [23,24], which is consistent with our results.

Fibrosis is an active biosynthetic process characterized by an abnormal accumulation of the extracellular matrix in a chronic injury caused by ischemia, infection, physical injury, or immune attack [5]. Fibrosis resembles the dysregulation of a permanently "open" repair process, and fibrogenesis continues after harmful stimuli and inflammation subside, causing stromal enlargement [25]. Despite the different etiologies and clinical manifestations, most chronic fibrotic diseases have a continuous stimulus that stimulates the deposition of connective tissue to progressively reshape and disrupt the typical tissue structures [26]. However, the mechanism of pBOO causing bladder fibrosis is still poorly understood. To determine how pBOO leads to bladder fibrosis damage, we performed RNA sequencing of bladder tissues and found extensive enrichment to EMT, cytoskeleton, and PI3K-AKT-mTOR signaling. Previous literature showed that PI3K-AKT-mTOR signaling is essential in EMT and cytoskeleton regulation. Therefore, we speculated that pBOO regulates cytoskeletal rearrangement to promote EMT by activating PI3K-AKT-mTOR signaling.

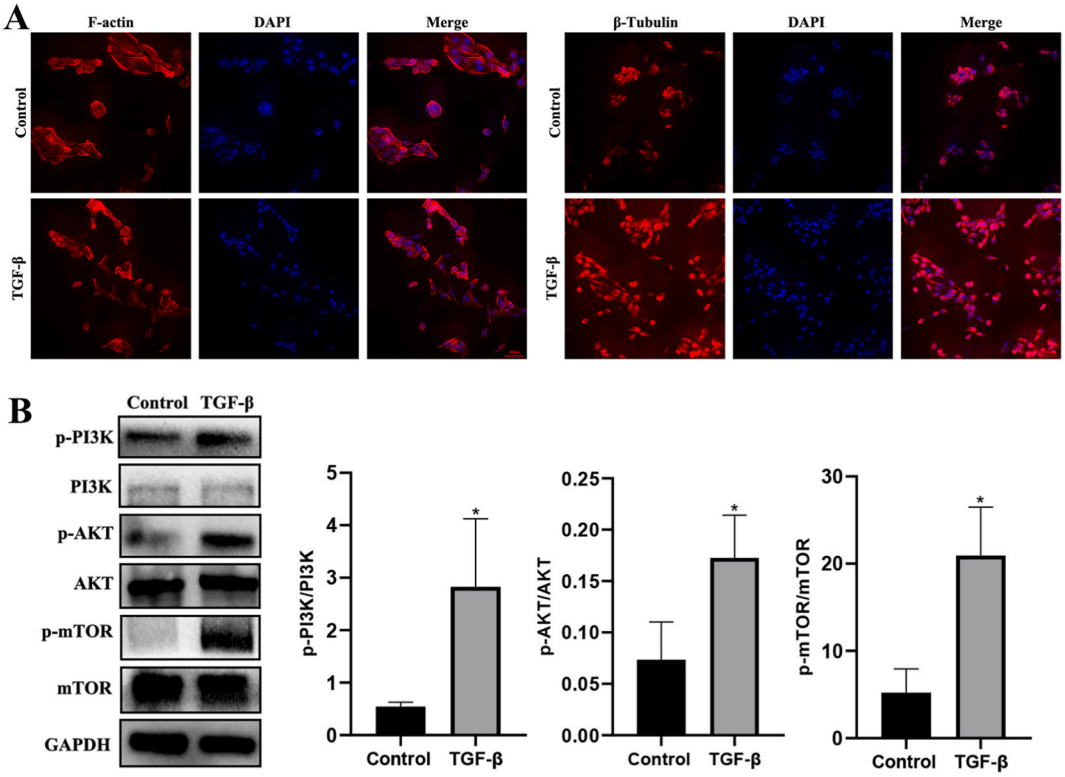

Fig. 6. Cytoskeletal changes and PI3K-AKT-mTOR pathway changes in SV-HUC-1 cells. A: Significant changes in F-actin and β -Tubulin structure in SV-HUC-1 cells after TGF-β treatment and poor continuity between cells. (Scale bar:50 μm). B. Western blotting showed that key proteins of the PI3K-AKT-mTOR-related pathway were elevated in TGF-β treatment SV-HUC-1 cells compared to the Control group.*$p < 0.05$.

Hay first identified EMT in the early 1980s as an essential mechanism of embryogenesis and organ development, aiming to create cells that can move and produce the matrix [27]. EMT is usually divided into three subtypes by biological background: type I, which occurs during embryogenesis; type II, which occurs during tissue repair; and type III, which is involved in the metastatic spread of cancer [28]. EMT plays a crucial role in many biological processes, such as type III EMT, promoting cancer migration and invasion [29]. However, type II EMT is a crucial driver of fibrosis in many organs. Federica Limana et al. showed that EMT plays a role in fibrosis after cardiac injury, producing mesenchymal cells characteristic of stem cells and myofibroblasts [30]. Since then, it has been increased the recognition that EMT is a component of tissue fibrosis [31]. Studies have confirmed EMT's role in pBOO-induced bladder fibrosis [32]. Dunton et al. also confirmed EMT in both the acute and chronic BOO models, and the EMT phenomenon was also observed in urothelial cells isolated from the acute and chronic BOO rat models [33]. The above studies support our conclusion that EMT plays a key role in pBOO. The first step in EMT is the loss of epithelial markers, including E-cadherin and ZO-1 down-regulation. The presence of E-cadherin was first proposed in the late 1970s and was soon found to be a key mediator of epithelial-cell adhesion. E-cadherin is one of the main targets of EMT-induced transcription factors, highlighting its role in inhibiting cell migration [34]. Iguchi et al. found that E-cadherin was downregulated in PBOO mice compared with sham-operated mice [35]. Healthy ectodermal cells lacking E-cadherin cannot correctly express the tight junction protein ZO-1, a protein associated with cell adhesion and junction [36]. Celthen develops toward the mesenchymal phenotype by acquiring mesenchymal markers and abilities. Mesenchymal markers include N-cadherin, Vimentin, and α-SMA. During EMT, N-cadherin gradually replaces the expression of E-cadherin and enhances the cell migration ability [37]. Vimentin is an intermediate filament that reduces the transport of E-cadherin to the cell surface [38]. α-SMA is also one of the typical mesenchymal markers [28]. Alessia Omenetti et al. found that co-culture of mouse cholangiocytes with myofibroblast HSCs in vitro led to EMT in cholangiocytes, exhibiting increased cell migration, reduced epithelial markers, and induced mesenchymal markers [39]. Our results also showed that pBOO could lead to the downregulation of epithelial markers in bladder tissue and the up-regulation of mesenchymal markers, suggesting the involvement of EMT in pBOO-induced bladder fibrosis.

Cytoskeletal remodeling is a crucial driver of EMT, during which it is reshaped to form pseudopodia and allows cells to move in the surrounding environment [40]. Moreover, the morphology and movement of the mesenchymal cells during the EMT process also occur through cytoskeleton reconstruction [41]. The cytoskeleton comprises microfilaments, microtubules, and intermediate filaments; actin, as the main component of the microfilaments, can interact with many accessory proteins to generate the actin cytoskeleton [42]. Microtubules are one of the non-covalent polymers that constitute the cytoskeleton, consisting of heterodimers of spherical tubulin and tubulin molecules. They are functionalized by various evolutionarily conserved post-translational modifications [43]. Lack of E-cadherin and ZO-1 causes defects in the organization of the actin cytoskeleton [44]. Evidence shows that the disruption of actin and

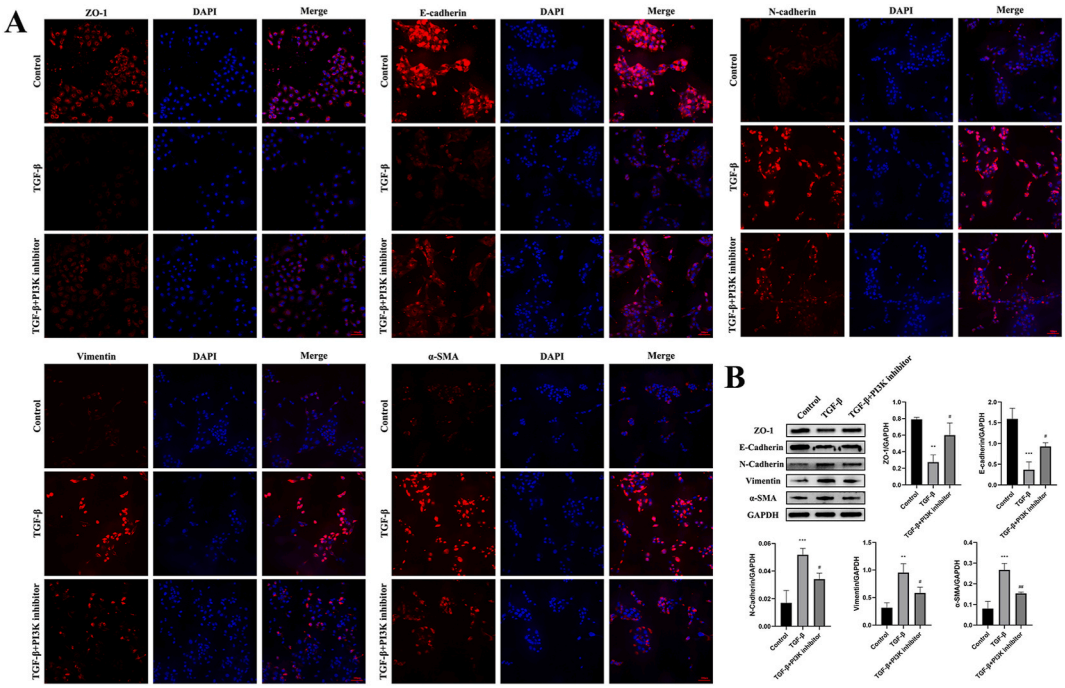

Fig. 7. Changes in the EMT-associated fingers in SV-HUC-1 cells after treatment with the PI3K inhibitor. A: Immunofluorescence showed that ZO-1 and E-cadherin were increased in SV-HUC-1 cells after treatment with the PI3K inhibitor. At the same time, N-cadherin, Vimentin, and α-SMA were decreased compared with the TGF-β treatment group. (Scale bar:100 μm). B: Western blotting showed that ZO-1 and E-cadherin were increased in SV-HUC-1 cells after treatment with the PI3K inhibitor, while N-cadherin, Vimentin, and α-SMA were decreased compared with TGF-β treatment group. *compared with the Control group. #compared with the TGF-β group.#$p < 0.05$,**,##$p < 0.01$,***$p < 0.001$.

microtubules in the cytoskeleton is associated with renal fibrosis [45]. Guan et al. found that microtubule networks are essential for hypertension-related renal fibrosis [46]. Our results showed that in the pBOO group, the bladder tissue has a disordered cell structure and poor continuity between cells and cells, which indicates that pBOO causes marked rearrangement of the epithelial cytoskeleton in the bladder tissue, leading to bladder tissue remodeling, which induces fibrosis.

Multiple signaling pathways regulate the EMT process. Dabin Choi et al. showed that the mTOR signaling pathway could regulate the EMT-like changes in renal podocytes due to TGF-1 [47]. The PI3K -AKT-mTOR signaling pathway plays an essential role in normal physiological and pathogenic processes by regulating gene expression in cell survival, differentiation, growth, motility, and apoptosis [48]. Previous literature showed that the PI3K-AKT-mTOR signaling pathway plays an essential role in the EMT and cytoskeleton regulation and that the activation of TOR complexes 1 (mTORC1) and 2 (mTORC2) regulates the cytoskeleton rearrangement and the use of PI3K inhibitors can prevent EMT [49]. In EMT, mTORC1 contributes to increased cell size, protein synthesis, motility, and invasion, and mTORC2 is required for the EMT process. The study of Alexandra Milena Cuartas-Lopez et al. showed that the activation of the PI3K -AKT is accompanied by a reorganization of the actin cytoskeleton [50]. Zhiyu Fan et al. showed that the PI3K-AKT pathway is vital in regulating cytoskeletal rearrangement and phenotypic turnover in pulmonary artery smooth muscle cells (PASMC) [18,51]. Our results showed that pBOO is accompanied by decreased epithelial markers, increased mesenchymal markers, and a cytoskeleton disruption and that the PI3K-AKT-mTOR signaling pathway is activated in this process. We also observed the activation of the PI3K signaling pathway after TGF- β induction in vitro experiments. Furthermore, a partial reversal of EMT and cytoskeletal damage was observed with PI3K inhibitors treatment, confirming that PI3K signaling plays a critical role in regulating EMT and cytoskeleton.

Current treatment modalities for pBOO still need to be revised. Neither clean intermittent catheterization nor oral anticholinergic drugs can target physiological pathways to inhibit the pathological progression of bladder fibrosis. Therefore, bimodality therapies that attenuate the underlying pathological process of pBOO while promoting bladder tissue regeneration may be a novel therapeutic strategy. Jiang et al. used tanshinone IIA sodium sulfonate to inhibit the TGF- β/Smad pathway to improve bladder fibrosis in pBOO rats [52]. In the study of Chen et al., the efficacy of metformin on pBOO-induced bladder fibrosis was also achieved by inhibiting bladder remodeling [53]—all the above studies reduced bladder fibrosis by attenuating the underlying pathological process of pBOO. Our study mainly clarified the regulatory mechanisms of EMT and cytoskeletal remodeling during bladder fibrosis induced by pBOO. It confirmed that this regulatory pathway PI3K inhibitor could improve EMT and cytoskeletal remodeling in vitro. We also alleviated fibrosis by attenuating the underlying pathological process of pBOO, but antifibrotic treatment is a complex and long-term clinical problem. Current research on tissue regeneration is the current trend. Regarding promoting bladder tissue regeneration in pBOO, previous studies have demonstrated the antifibrotic effect of MSC treatment on the pBOO model in vitro and in vivo [54,55]. The study

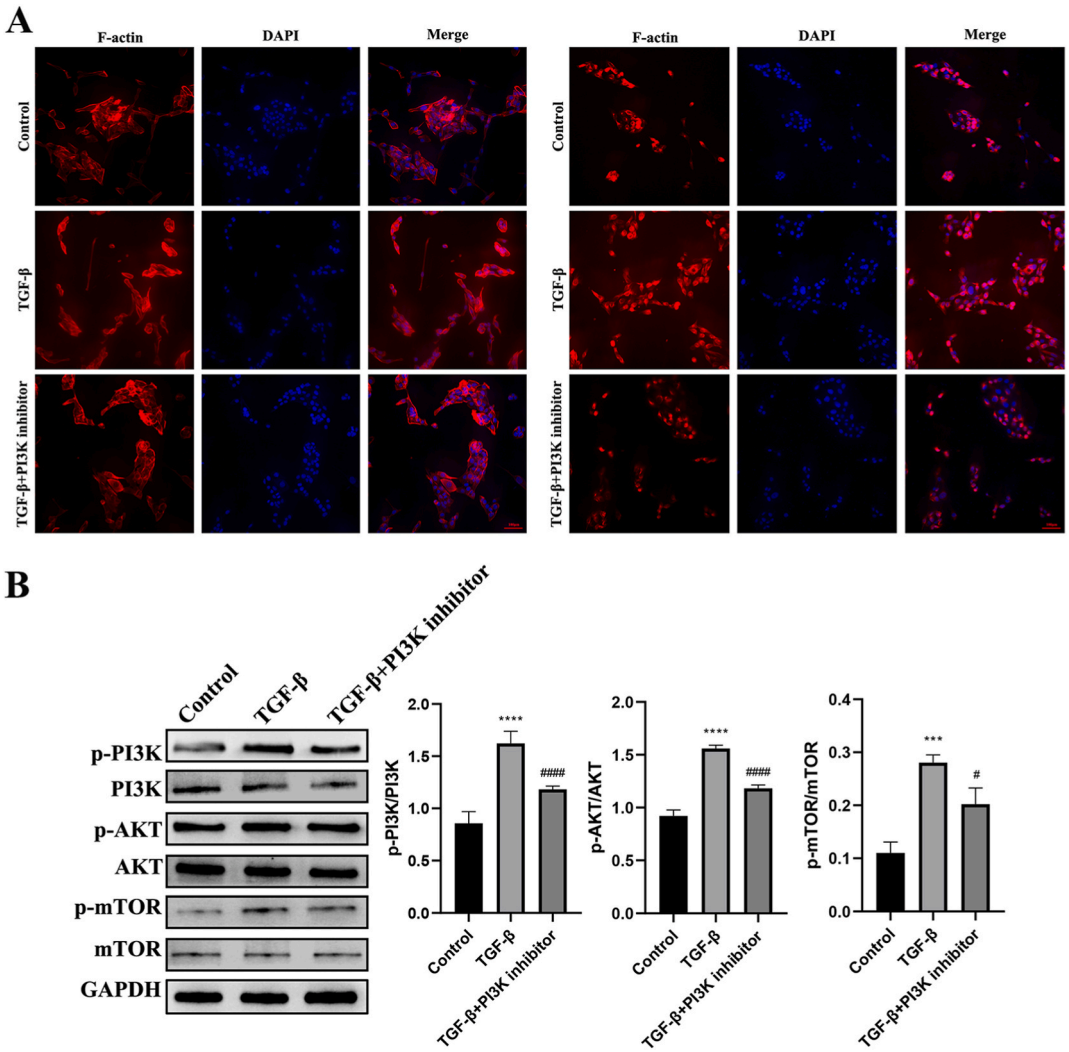

Fig. 8. Cytoskeletal changes and PI3K-AKT-mTOR pathway changes in SV-HUC-1 cells after PI3K inhibitor treatment. A: F-actin and β-tubulin structures were significantly changed in PI3K inhibitor treatment SV-HUC-1 cells compared to the TGF-β treated group. (Scale bar:50 μm). B: Western blotting showed that the key proteins of the PI3K-AKT-mTOR-related pathway in SV-HUC-1 cells after PI3K inhibitor treatment were decreased compared to the TGF-β group. *compared with the Control group. #compared with the TGF-β group.#$p < 0.05$,***$p < 0.001$,****, ####$p < 0.0001$.

of Rutuja Kadam et al. also demonstrated that MSC can improve EMT during pBOO-induced bladder fibrosis [32]. Bridget Wiafe et al. also found that intraperitoneal injection of MSC improved the symptoms of pBOO [17], and these studies provided a feasible basis for bimodality therapy. At the same time, the MSC-derived extracellular vesicles have gradually replaced the MSCs themselves. In future studies, we might combine the field of regenerative medicine to adopt mesenchymal stem cell-derived extracellular vesicles to promote bladder tissue regeneration while combining PI3K inhibitors to attenuate the potential pathological process of pBOO. Dual-mode therapy is expected to provide better therapeutic results for pBOO.

In addition, our study has several important limitations. Although we tried to replicate the situation of clinical pBOO best, we only selected female rats for modeling, and it would be more clinically meaningful to model both male and female rats and investigate sex differences. In addition, TGF-β treated urothelial cells were used as the cell model in this study, but the cell model corresponding to pBOO injury could not be completely simulated. Primary bladder epithelial cells should be extracted from pBOO bladder tissue for in vitro testing. However, expanding primary cells is very difficult, and our experimental conditions cannot meet the experimental requirements. In future studies, efforts will be made to improve on these limitations.

5. Conclusions

In conclusion, this study explored the phenotype and the specific mechanism of pBOO-induced bladder injury by establishing a

pBOO animal model combined with RNAsequencing. Our study found that pBOO causes bladder fibrosis damage, EMT and cytoskeleton remodeling play a key role in pBOO-induced bladder fibrosis injury, and the PI3K-AKT-mTOR signaling pathway probably regulates cytoskeleton remodeling and EMT. However, previous studies have also reported the involvement of EMT in bladder injury caused by pBOO. However, to our knowledge, our study is the first to reveal the presence of cytoskeletal remodeling and the activation of the PI3K-AKT-mTOR signaling pathway in the pBOO-induced bladder fibrosis damage. Moreover, PI3K inhibitors could reduce bladder fibrosis damage by reversing EMT and cytoskeletal damage and thus induced by pBOO.

Ethics approval and consent to participate

All experimental animal procedures used in the study were approved by the Ethics Committee of the Children's Hospital of Chongqing Medical University (IACUC Issue NO: CHCMU-IACUC20220323005).

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Consent for publication

None.

CRediT authorship contribution statement

Zhaoxia Zhang: Conceptualization, Data curation, Methodology, Writing – original draft. **Chenghao Zhanghuang:** Conceptualization, Writing – review & editing. **Tao Mi:** Data curation. **Liming Jin:** Methodology. **Jiayan Liu:** Investigation. **Maoxian Li:** Writing – review & editing. **Xin Wu:** Writing – review & editing. **Jinkui Wang:** Software. **Mujie Li:** Formal analysis. **Zhang Wang:** Writing – review & editing. **Peng Guo:** Writing – review & editing. **Dawei He:** Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21281.

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