Serum/glucocorticoid-regulated kinase 1-targeted transient receptor potential oxalate subtype 1 regulates bladder smooth muscle cell proliferation due to bladder outlet obstruction in mice via activated T cell nuclear factor transcription factor 2

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Abstract
Bladder outlet obstruction (BOO) is a type of chronic disease that is mainly caused by benign prostatic hyperplasia. Previous studies discovered the involvements of both serum/glucocorticoid-regulated kinase 1 (SGK1) and activated T cell nuclear factor transcription factor 2 (NFAT2) in the proliferation of smooth muscle cells after BOO. However, the relationship between these two molecules is yet to be explored. Thus, this study explored the specific mechanism of the SGK1-NFAT2 signaling pathway in mouse BOO-mediated bladder smooth muscle cell proliferation in vivo and in vitro. In vivo experiments were performed by suturing 1/2 of the external urethra of female BALB/C mice to cause BOO for 2 weeks. In vitro, mouse bladder smooth muscle cells (MBSMCs) were treated with dexamethasone (Dex) or dexamethasone + SB705498 for 12 h and were transfected with SGK1 siRNA for 48 h. The expression and distribution of SGK1, transient receptor potential oxalate subtype 1 (TRPV1), NFAT2, and proliferating cell nuclear antigen (PCNA) were measured by Western blotting, polymerase chain reaction, and immunohistochemistry. The relationship between SGK1 and TRPV1 was analyzed by coimmunoprecipitation. The proliferation of MBSMCs was examined by 5-ethynyl-2′-deoxyuridine and cell counting kit 8 assays. Bladder weight, smooth muscle thickness, and collagen deposition in mice after 2 weeks of BOO were examined. Bladder weight, smooth muscle thickness, the

Abbreviations: BOO, bladder outlet obstruction; BSMC, bladder smooth muscle cell; Dex, dexamethasone; MBSMCs, mouse bladder smooth muscle cells; NFAT2, activated T cell nuclear factor transcription factor 2; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; SGK1, serum/glucocorticoid-regulated kinase 1; TRPV1, transient receptor potential oxalate subtype 1.
collagen deposition ratio, and the expression of SGK1, TRPV1, NFAT2, and PCNA were significantly increased in mice after 2 weeks of BOO. Compared with the control, 10 μM Dex promoted the expression of these four molecules and the proliferation of MBSMCs. After inhibiting TRPV1, only the expression of SGK1 was not affected, and the proliferation of MBSMCs was inhibited. After silencing SGK1, the expression of these four molecules and the proliferation of MBSMCs decreased. Coimmunoprecipitation suggested that SGK1 acted directly on TRPV1. In this study, SGK1 targeted TRPV1 to regulate the proliferation of MBSMCs mediated by BOO in mice through NFAT2 and then affected the process of bladder remodeling after BOO. This finding may provide a strategy for BOO drug target screening.

**KEYWORDS**
bladder outlet obstruction, bladder smooth muscle, cell proliferation, collagen deposition, TRPV1

### 1 INTRODUCTION

Bladder outlet obstruction (BOO) is a common disease that mainly manifests clinically as benign male prostatic hyperplasia.\(^1\) With increasing aging in the population, the incidence of this disease has increased.\(^2\) Long-term BOO can lead to changes in bladder function and structure, such as bladder remodeling. The pathophysiological processes mainly include inflammation, smooth muscle hypertrophy, and proliferation, excessive deposition of extracellular matrix, and decompensated fibrosis.\(^3\) In the early stage of compensation, the proliferation and hypertrophy of bladder smooth muscle cells play an important role. At present, the signal transduction mechanism of BOO-mediated bladder smooth muscle cell proliferation is not fully understood, and there is no effective treatment for bladder remodeling, so further research is needed. Serum/glucocorticoid-regulated kinase 1 (SGK1) is a genomic-regulated kinase that is activated through the phosphoinositide-3-phosphate signaling pathway.\(^4\) According to the literature, SGK1 can regulate several enzymes and transcription factors and participate in a variety of pathophysiological processes, such as transport, hormone release, neural excitability, inflammation, cell proliferation, and apoptosis.\(^5\) There are five members in the nuclear factor of activated T cell (NFAT) family. According to the sensitivity of NFAT to calcineurin, it is divided into calcium regulation (NFAT1-4) and non-calcium regulation subtypes (NFAT5). NFAT regulates the survival of many types of cells. Proliferation and function are very important, including those of mast cells, coronary artery cells, and lymphocytes.\(^6,7\) In addition, many studies have shown that NFAT2 plays an important role in promoting cell proliferation.\(^8,9\) Previous research showed that circulating hydrodynamic pressure promotes the proliferation of human BSMCs (HBSMCs) cultured on scaffolds through the PI3K/SGK1 signaling pathway,\(^10\) and BOO promotes the proliferation of bladder smooth muscle cells through the SGK1-NFAT2 signaling pathway in mice.\(^11\)

As a member of the transient receptor potential (TRP) family, the transient receptor potential vanilloid (TRPV) channel is a tetrameric protein channel that is widely distributed in the human body and can allow \(\text{Ca}^{2+}, \text{Mg}^{2+}, \text{Na}^{+}\) and variety of cations, such as \(\text{K}^{+}\), to pass through, and plays an important role in cell function and signal pathway transmission.\(^12,13\) There are six members in the TRPV subfamily. The capsaicin receptor TRPV1 was the first discovered and cloned member of the TRPV family, and it is also the most widely studied channel protein.\(^12\) Early research showed that TRPV1 is mainly expressed in nociceptive sensory neurons and is mainly involved in the transmission of nociceptive signals and pain modulation in the body.\(^14\) However, in recent years, increasing evidence has shown that TRPV1 is involved in not only pain transmission, but also various pathophysiological processes, such as thermoregulation, vascular and ventricular remodeling, inflammation, cell proliferation, airway smooth muscle contraction, and mechanical sensory conduction.\(^15-20\) TRPV1 is distributed in the skin, respiratory tract, gastrointestinal tract, blood vessels, heart, bladder, and urethra.\(^21\) There are reports showing that TRPV1 induces apoptosis in colorectal cancer cells by activating the calcineurin-NFAT2-p53 signaling pathway.\(^22\) It can be hypothesized that TRPV1 plays a role in the SGK1-NFAT2 signaling pathway to mediate the proliferation of bladder smooth muscle cells.
In the early stage of BOO compensation, as the pressure in the bladder gradually increases, bladder smooth muscle thickens to overcome urination resistance. During this process, bladder smooth muscle cell proliferation is an important factor that leads to thickening of the bladder wall. Therefore, in this study, we successfully constructed a mouse BOO model through the 1/2 suturing method of the urethral external opening; that is, while suturing the 1/2 external urethral opening, the 1/2 external urethral opening was exposed to avoid direct damage to the bladder. In our previous study, only SGK1 and NFAT2 were involved in the proliferation of bladder smooth muscle cells in the context of murine BOO, but the relationship between the two factors is still unclear. Therefore, we examined the specific mechanism by which SGK1 and NFAT2 mediate bladder smooth muscle proliferation after BOO in mice in vivo and in vitro, which may provide some ideas for the development of BOO therapeutic drug targets.

2 | MATERIALS AND METHODS

2.1 | Animals

Twenty-four female BALB/c mice (aged 6–8 weeks; body weight 20–30 g) were purchased from the Dashuo Laboratory Animal Technology Co. The mice were housed at 24°C with a 12-h light/dark cycle, 35%–40% humidity, and free access to food and water.

The mice were randomly divided into three groups (8 mice in each group): the control group, the sham group and the BOO 2W group. Animals in the BOO 2W group received isoflurane inhalation anesthesia before the operation, and BOO was induced as previously described. In our previous study, only SGK1 and NFAT2 were involved in the proliferation of bladder smooth muscle cells in the context of murine BOO, but the relationship between the two factors is still unclear. Therefore, we examined the specific mechanism by which SGK1 and NFAT2 mediate bladder smooth muscle proliferation after BOO in mice in vivo and in vitro, which may provide some ideas for the development of BOO therapeutic drug targets.

2.2 | Cell culture

MBSMCs were cultured in DMEM (cat. no. 12430054; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (cat. no. 16000044; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO2 and 95% O2. The experiment was performed with cells from passages 3 to 7.

2.3 | Overexpression of SGK1 in vitro and the TRPV1 inhibitor

Dexamethasone (Dex), which induces the overexpression of SGK1 in vitro, was purchased from Solarbri (cat. no. ID0170; Solarbio), and the specific TRPV1 inhibitor SB705498 was purchased from Selleck Chemicals (cat. no. S2773; Selleck Chemicals). To overexpress SGK1 in MBSMCs, according to the literature, the concentrations of Dex were 0, 1, 5, 10, 15 and 20 μmol/L, and culture medium containing 10% FBS was used. After 6 and 12 h, total cellular protein or supernatant was collected. After the Dex concentration and incubation time were determined, the MBSMCs were divided into the control (CTR) group, Dex-10 μM group, and Dex-10 μM + SB705498 group. The Dex-10 μM + SB705498 group was pretreated with SB705498 (0.1 μmol/L) for 6 h, and then the three groups were incubated for 12 h before proceeding to follow-up experiments.

2.4 | Histological analysis and immunohistochemistry

The bladder was fixed with 4% paraformaldehyde, embedded in paraffin and sliced transversely at a thickness of 5 μm. Hematoxylin and eosin (H&E) and Masson’s trichrome staining were used to determine the histopathology. Masson’s three-color staining method was used at room temperature for 15 min for histopathological analysis. The sample was observed and imaged using an orthogonal fluorescence microscope (Nikon Corporation) at 160× magnification. The levels of muscle tissue (red) and collagen (blue) were quantified with an image analysis system (ImageJ 1.80). Bladder weight, bladder smooth muscle thickness, and the collagen/muscle ratio were used as indicators for evaluating bladder remodeling.

For immunohistochemical staining, bladder tissue sections were stained with antibodies against the following proteins: SGK1 (cat. no. ab43606, 1:100, Abcam), TRPV1 (cat. no. ab6166, 1:100, Abcam), and NFAT2 (cat. no. ab25916, 1:50, Abcam).

2.5 | Western blotting

RIPA lysis buffer (cat. no. CW2333S; CoWin Biosciences) containing protease inhibitors and phosphatase inhibitors was used to isolate proteins from mouse bladder tissue.
and cultured mouse bladder smooth muscle cells. The protein concentration was determined with a quinolinic acid protein detection kit (cat. no. BL521A; Biosharp). The homogenate was centrifuged at 15,000 × g for 10 min at 4°C to obtain the supernatant. Equal amounts of protein (40 μg per lane) were separated by SDS–PAGE and transferred to a PVDF membrane. Nonspecific binding was blocked with 5% skimmed milk powder at room temperature for 1 h. Subsequently, the membrane was incubated overnight at 4°C with primary antibodies against the following proteins: SGK1 (cat. no. ab43606; 1:1000; Abcam), TRPV1 (cat. no. ab6166; 1:700; Abcam), NFAT2 (cat. no. ab175134; 1:1000; Abcam), proliferating cell nuclear antigen (PCNA; cat. no. D3H8PXP; 1:1000; CST) and GAPDH (cat. no. SA30-01; 1:1000; Huabio). Then, the membrane was washed 3 times in TBST containing 0.1% Tween-20 for 10 min each. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. HA1001; 1:2000; Huabio) and slowly shaken at room temperature for 1 h. Then, the membranes were washed three times with TBST containing 0.1% Tween-20 for 10 min 10 min each. The protein bands were passed through a ChemiDoc XRS + system (Bio–Rad Laboratories, Inc.) using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.) for visualization. Image Lab software 5.2.1 (Bio–Rad Laboratories, Inc.) was used to quantify protein expression.

### 2.6 | RNA extraction and real-time qPCR

According to the manufacturer’s instructions, TRIzol reagent (cat. no. 15596026, Thermo Fisher Scientific) was used to extract total RNA. RNA concentration and purity were determined by measuring the optical density at 260 and 280 nm. The PrimeScript™ RT kit (Takara) and a StepOnePlus™ real-time PCR system (Applied Biosystems) were used for the reverse transcriptase reaction. For quantitative PCR, SYBR Green RT–PCR (Takara Biotechnology Co., Ltd.) was performed on the cDNA fragments using a StepOne Plus system. The PCR cycle conditions were 95°C for 30 s, 40 cycles of 95°C denaturation for 5 s, 55°C annealing for 30 s, and 72°C extension for 30 s. The 2−ΔΔCT method was used to obtain a quantitative measurement and normalize the results to the expression of β-actin. The following primers for each target gene were used: SGK1: forward 5'-GGTTCTTCTGGCTAGCACAAGG-3', reverse 5'-TTCCGCTCTGACATAATATGCTTCTCC-3'; TRPV1: forward 5'-GGAGGTGATCGCCTACAGTAGCAGTG-3', reverse 5'-GCCGATAGTAAGCAGCCGTTGTGAA-3'; NFAT2: forward 5'-GGCAGGAGATCACAGTGTGAAA-3', reverse 5'-GAGAGGTGATCGCCTACAGTAGCAGTG-3'; PCNA: forward 5'-GAAGTTTTCTGCAAGTGGAGAG-3', reverse 5'-CAGGCTCATTCATCTCTATGGT-3'; and β-Actin: forward 5'-GTGCTATGTTGCTTAGACTTCG-3', reverse 5'-ATGCCACAGGATTTCCATACC-3'.

### 2.7 | Cell transfection

According to the manufacturer’s instructions, Lipofectamine™ 2000 reagent (cat. no. 11668019; Thermo Fisher Scientific, Inc.), scrambled siRNAs (1 μl; cat. no. 1337; Shanghai GenePharma Co., Ltd.) and siRNA targeting SGK1 (1 μl; cat. no. 8081; Shanghai GenePharma Co., Ltd.) were used. Mouse BSMCs at 80% density were transfected twice with SGK1 siRNA at 24 h intervals, and subsequent experiments were performed 48 h after transfection. The siRNA sequences used were as follows: scrambled siRNA, sense: 5'-UUCUCCGAACGUGUCACGU-3' and antisense: 5'-ACGUGACACGUUCGGAGAAT-3'; SGK1 siRNA#1, sense: 5'-GCCAAUAACUCCUGUGUGCAUTT-3' and antisense: 5'-AUGCAUGCAG-3'; and SGK1 siRNA#2, sense: 5'-GGUUUCUUCAGCAAGCCACTT-3' and antisense: 5'-GUGCCUUGCUAGAAGACCTT-3'.

### 2.8 | Assessment of MBSMC proliferation

MBSMC proliferation was assessed by the cell counting kit 8 (CCK-8) method, the 5-ethynyl-2'-deoxyuridine (EdU) incorporation test and measuring PCNA expression. First, a CCK-8 kit (K009, ZETA LIFE Inc) was used according to the manufacturer’s instructions to determine the MBSMC proliferation rate. A microplate reader (VLBL0TD1, Thermo Fisher Scientific) was used to measure the absorbance at 450 nm. Second, EdU (EdU Cell Proliferation Kit with Alexa Fluor 488, Shanghai Ya Enzyme Biology) was incorporated to detect DNA synthesis and evaluate MBSMC proliferation. The number of EdU-positive cells was counted and normalized to the total number of Hoechst 33342-stained cells. Finally, the proliferation of MBSMCs was assessed by measuring PCNA expression, which is a DNA clamp that acts on chromatin, and chromatin serves as a platform for various proteins to participate in DNA replication-related processes.

### 2.9 | Coimmunoprecipitation

The interaction between SGK1 and TRPV1 was analyzed by Coimmunoprecipitation (CoIP). According to the manufacturer’s instructions, Servicebio IP buffer (G2038, Servicebio) was used to collect untreated MBSMCs, and...
protease and phosphatase inhibitors were added. The lysate was incubated with SGK1 antibodies (ab43606; 1:50; Abcam), TRPV1 antibodies (ab6166; 1:50; Abcam), and mouse IgG (GB23301, 1:100, Servicebio) at 4°C overnight. Then, the cells were incubated with MedChem ExpressTM Protein A magnetic beads (HY-K0203, MedChem Express) at room temperature for 30 min. The immunoprecipitate was washed with PBS containing 0.1% Tween-20 and then immunoblotted with the specified antibody.

2.10 | Statistical analysis

The data are presented as the mean ± SEM. Comparisons among multiple groups were performed using one-way ANOVA followed by the least significant difference post hoc test. Statistical analyses were performed using SPSS software version 22 (IBM Corp.). p < 0.05 was considered to indicate a statistically significant difference.

3 | RESULTS

3.1 | Changes in bladder weight and morphology in the mouse model

Compared with that in the CTR group and sham group, the bladder weight in the BOO group increased significantly after 2 weeks of obstruction (p = 0.003 and p = 0.004), but there was no significant difference in bladder weight between the CTR group and the sham group (p = 0.858) (Figure 1B). Tissue staining analysis showed that the bladder smooth muscle fibers of mice in the CTR group (257.86 ± 7.91 μm) and sham group (276.31 ± 9.12 μm) were neatly arranged without obvious damage, and there was no difference in the thickness of the muscle layer between the two groups (p = 0.321), while in the BOO group (360.24 ± 17.03 μm), the smooth muscle layer of the bladder was obviously thickened after 2 weeks of obstruction and was accompanied by disordered muscle fiber distribution (p = 0.001 and p = 0.003) (Figure 1A,C). Masson staining was used to evaluate the degree of bladder fibrosis induced by BOO. In the CTR group (15.89 ± 0.77%) and sham group (18.30 ± 1.23%), collagen deposition (blue staining) was occasionally observed in the smooth muscle layer of the bladder (p = 0.212). In the BOO 2W group (30.74 ± 3.35%), bladder smooth muscle layer collagen deposition was relatively increased (p < 0.05 and p < 0.05) (Figure 1A,D).

3.2 | Changes in bladder cytokine levels during BOO

Compared with those in the sham group, the protein expression levels of SGK1, TRPV1, NFAT2, and PCNA in the bladder tissue in the CTR group were not significantly different, while the expression levels of these four proteins increased after 2 weeks of BOO (p < 0.05) (Figure 2A,B);
the PCNA expression level increased significantly, indicating that the bladder smooth muscle cells of mice had compensatory proliferation after 2 weeks of BOO. Immunohistochemical analysis showed that SGK1, TRPV1, and NFAT2 in the BOO 2W group were more evenly distributed in the bladder tissue than in the CTR or sham group. This finding is consistent with the trend in the expression levels of these three molecules after 2 weeks of BOO ($p < 0.05$) (Figure 2C,D).

### 3.3 | Comparison of different Dex concentrations and times on SGK1 expression

When the treatment time was 6 h, the SGK1 protein expression level did not change significantly with increasing Dex concentrations (Figure 3A,B); when the treatment time was 12 h, the SGK1 protein expression level changed significantly with increasing Dex concentrations. Compared with $0 \mu M$, $1 \mu M$ Dex induced no significant changes in SGK1 protein expression ($p = 0.164$); at $5 \mu M$, the protein expression began to increase ($p < 0.05$); and at $10 \mu M$ and $15 \mu M$, SGK1 protein expression was significantly higher than at $0 \mu M$ ($p < 0.05$) and slightly higher at $15 \mu M$, but there is no significant difference between the two. At $20 \mu M$, the protein expression of SGK1 showed a downward trend but was still higher than that at $0 \mu M$ ($p < 0.05$) (Figure 3A,C). Due to the principle of the minimum dose of experimental drugs, it was determined that the Dex concentration used in subsequent experiments was $10 \mu M$ and the time was $12 \text{ h}$.

### 3.4 | SGK1 overexpression promotes the expression of downstream molecules, and the TRPV1 inhibitor SB705498 can reverse this trend

Compared with those in the CTR group, SGK1, TRPV1, NFAT2, PCNA protein and gene expression levels increased significantly in the Dex-10 $\mu M$ group ($p < 0.05$),

![Figure 2](image)

**FIGURE 2** Changes in cytokine levels in bladder tissue during bladder outlet obstruction (BOO). The protein expression of serum/glucocorticoid-regulated kinase 1 (SGK1), transient receptor potential oxalate subtype 1 (TRPV1), activated T cell nuclear factor transcription factor 2 (NFAT2), and proliferating cell nuclear antigen (PCNA) in mouse bladder tissue in the CTR group, sham group, and BOO 2W group was (A) determined by Western blotting and (B) quantified. The distribution of SGK1, TRPV1, NFAT2, and PCNA in mouse bladder tissue (C) was determined by immunohistochemistry (magnification: $\times 200$) and (D) quantified. The data are expressed as the mean $\pm$ SEM, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.
and only the protein and gene expression of SGK1 increased in the Dex-10 μM + SB705498 group (p < 0.05), while TRPV1, NFAT2, PCNA did not change significantly (Figure 4A–C). Compared with the Dex-10 μM group, the Dex-10 μM + SB705498 group had no significant difference in SGK1 protein or gene expression, while TRPV1, NFAT2, PCNA protein and gene expression were all decreased (p < 0.05) (Figure 4A–C), which indicates that TRPV1 is a downstream molecule that acts as a link between SGK1 and NFAT2. The trend in the downstream molecules after SGK1 activation was consistent with the trend in bladder molecules after 2 weeks of BOO, which again confirmed that SGK1 may transmit cell proliferation signals to NFAT2 through TRPV1.

3.5 | After silencing SGK1, downstream protein and gene expression levels decreased, and TRPV1 was the direct target of SGK1

Compared with the scrambled siRNA group, SGK1, TRPV1, NFAT2, PCNA protein and gene expression levels were significantly reduced in the SGK1 siRNA#2 group (p < 0.05), while SGK1, TRPV1, NFAT2, PCNA protein and gene expression were slightly decreased in the SGK1 siRNA#1 group, but the difference was not statistically significant (Figure 5A–C). To verify that TRPV1 may be a direct target of SGK1, we confirmed that signal transduction from SGK1 to NFAT2 occurred through TRPV1 by activating or silencing SGK1 and inhibiting TRPV1. However, whether there is a direct interaction between SGK1 and TRPV1 requires further CoIP analysis. Therefore, for CoIP, we collected proteins from MBSMCs, used SGK1 and TRPV1 antibodies to IP these factors, and then used SGK1 and TRPV1 antibodies to determine whether the proteins were associated with one another. After activating or silencing SGK1, TRPV1 showed a trend, indicating that TRPV1 was the direct target of SGK1 (Figure 5D).

3.6 | The effect of SGK1 overexpression, TRPV1 inhibition and SGK1 silencing on the proliferation of MBSMCs

MBSMC proliferation was evaluated by a CCK-8 kit, EdU staining, and PCNA mRNA and protein expression measurement. Compared with that in the CTR group, PCNA gene and protein expression in the Dex-10 μM group was significantly increased (p < 0.05), while the Dex-10 μM + SB705498 group was blocked by TRPV1, and the PCNA gene and protein expression was higher than that in the Dex group. There was a decrease in the 10 μM group (p < 0.05) (Figure 4A–C). After silencing SGK1, the gene and protein expression of PCNA in the SGK1 siRNA#2 group was significantly lower than that in the scrambled siRNA group (p < 0.05), while there was no significant difference in the SGK1 siRNA#1 group (Figure 5A–C). Cell proliferation was measured by EdU and CCK-8 assays and compared with those in the CTR group, the number, proportion and absorbance of EdU-positive cells in the Dex-10 μM group increased (p < 0.05); the number of EdU-positive cells, the ratio and the absorbance decreased (p < 0.05); and the number, proportion and absorbance of EdU-positive cells in the SGK1 siRNA#2 group were significantly lower than those in the scrambled siRNA group (p < 0.05) (Figure 6A–D). These results indicate that high expression of SGK1 can promote the proliferation of MBSMCs. Blocking TRPV1 reversed the effect of high SGK1 expression on MBSMC proliferation. Silencing SGK1 also inhibited the proliferation of MBSMCs. Therefore, it can be concluded that SGK1 targets TRPV1 and regulates MBSMC proliferation through NFAT2.
FIGURE 4  The effect of dexamethasone (Dex) and SB705498 on serum/glucocorticoid-regulated kinase 1 (SGK1) and downstream gene and protein expression. The protein expression of SGK1, transient receptor potential oxalate subtype 1 (TRPV1), activated T cell nuclear factor transcription factor 2 (NFAT2), and proliferating cell nuclear antigen (PCNA) in the CTR group, Dex-10 μM group and Dex-10 μM + SB705498 group was determined and quantified by Western blotting. The mRNA expression of SGK1, TRPV1, NFAT2, and PCNA was determined by RT-PCR. The data are expressed as the mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001.

FIGURE 5  Comparison of the gene and protein expression of serum/glucocorticoid-regulated kinase 1 (SGK1) and downstream molecules after silencing SGK1 and the relationship between SGK1 and transient receptor potential oxalate subtype 1 (TRPV1). The protein expression of SGK1, TRPV1, activated T cell nuclear factor transcription factor 2 (NFAT2), and proliferating cell nuclear antigen (PCNA) in the scrambled siRNA group, SGK1 siRNA#1 group and SGK1 siRNA#2 group was determined and quantified by Western blotting. The mRNA expression of SGK1, TRPV1, NFAT2, and PCNA was determined by RT-PCR. The targeting of SGK1 to TRPV1 was determined by coimmunoprecipitation. The data are expressed as the mean ± SEM, *p < 0.05, **p < 0.01, and ***p < 0.001.
DISCUSSION

BOO is a common chronic disease of the urinary system that is caused by various bladder outflow tract obstructions and causes related lower urinary tract symptoms. Long-term obstruction can lead to progressive bladder tissue remodeling and may even severely impair kidney function. A large number of studies have shown that in the pathophysiological process of bladder remodeling, increased intravesical pressure, which is mechanical stress, is an important stimulator of hypertrophy and the proliferation of bladder wall muscle layer cells. Our previous studies have shown that a hydrostatic pressure of 200 cm H₂O can promote the proliferation of MBSMCs, which is achieved through the SGK1-NFAT2 signaling pathway, but unfortunately, no specific connection between these two factors has been found. Therefore, this study further explored the specific mechanism by which the SGK1-NFAT2 signaling pathway mediates MBSMC proliferation in vivo and in vitro.

After BOO occurs, it usually leads to an increase in the weight and volume of the bladder. In vivo, we simulated the pathophysiological process of human BOO as much as possible and found that the weight of the bladder increased in mice after 2 weeks of BOO. The bladder tissue was removed and sliced for staining analysis. The

FIGURE 6 The effect on the proliferation of bladder smooth muscle cells in each group. 5-ethynyl-2'-deoxyuridine (EdU)-positive cells in the CTR group, dexamethasone (Dex)-10 μM group, Dex-10 μM+ SB705498 group, Scrambled siRNA group, serum/glucocorticoid-regulated kinase 1 (SGK1) siRNA#1 group, SGK1 siRNA#2 group were detected by the EdU method (A) and quantified (B). The absorbance value (OD value) at 450 nm in the CTR group, CTR group, Dex-10 μM group, Dex-10 μM+ SB705498 was quantified by the CCK-8 method (C). The absorbance value (OD value) at 450 nm in the scrambled siRNA group, SGK1 siRNA#1 group, SGK1 siRNA#2 group was quantified by the CCK-8 method (D). The data are expressed as the mean ± SEM, **p < 0.01, and ***p < 0.001
thickness of the bladder muscle layer of mice after 2 weeks of BOO was significantly increased compared with that of the control group. This effect may be related to the smooth muscle of the bladder overcoming resistance to urination. Moreover, collagen deposition in the bladder tissue also increased compared with that in the control group, as indicated by Masson staining, and the distribution of muscle fibers was disordered, suggesting that the compliance of the bladder may be reduced, the structure and function of the bladder wall may be problematic, and the bladder may be decompensated. At the molecular level, the protein expression levels of SGK1 and the downstream molecules TRPV1, NFAT2, and PCNA in the bladder tissue in the BOO 2W group were significantly higher than those in the control group. In addition, the immunohistochemical results also indicated that these molecules in the BOO 2W group were distributed in the bladder tissue in addition to PCNA. These results indicate that the SGK1-TRPV1-NFAT2 signaling pathway may be involved in the proliferation of bladder smooth muscle cells during BOO in mice, but this conclusion still needs to be confirmed in vitro.

Due to the increased expression of the upstream molecule SGK1 in mouse bladder tissue during BOO, we first determined the appropriate concentration and intervention time for the SGK1 agonist Dex by Western blotting in vitro. Then, MBSMCs were divided into the CTR group, Dex-10 μM group, and Dex-10 μM + SB705498 group to verify the expression of TRPV1, NFAT2, and PCNA under Dex and SB705498 intervention and to determine whether TRPV1 was a downstream molecule of SGK1 and the influence on MBSMC proliferation. First, when Dex induced high expression of SGK1, TRPV1, NFAT2, PCNA protein and gene expression levels were significantly increased compared to those in the control group. CCK-8 and EdU assays showed that MBSMCs proliferated significantly, which was similar to the molecular changes in the bladder tissue of mice after 2 weeks of BOO. The trend was consistent. Thus, after using the TRPV1 inhibitor SB705498, the protein and gene expression levels of TRPV1, NFAT2, and PCNA decreased significantly compared with those in the Dex-10 μM group, and the proliferation of MBSMCs also decreased, but the expression of SGK1 was not affected. After silencing SGK1, the protein and gene expression levels of TRPV1, NFAT2, and PCNA also decreased significantly compared with those in the scrambled siRNA group, and the proliferation of MBSMCs decreased. Therefore, it can be concluded that TRPV1, as a downstream molecule of SGK1, acts as an intermediate bridge in the SGK1-NFAT2 signaling pathway to mediate the proliferation of MBSMCs.

However, the relationship between TRPV1 and SGK1 needs further verification. The immunoprecipitation results showed that SGK1 has a direct relationship with TRPV1. Combined with the previous experimental results, it can be inferred that SGK1 targets TRPV1. Regarding the relationship between TRPV1 and NFAT2, we consulted the literature and hypothesize that TRPV1 is a unique type of ion channel. When TRPV1 is activated, its conformation changes, and its permeability to cations (mainly Ca2+) increases. Ca2+ entry into the cell activates calcineurin (CaN), and then CaN binds with NFAT2 to dephosphorylate and transfer into the nucleus to regulate cell proliferation-related proteins and factors, which in turn has a certain effect on cell proliferation. Of course, this hypothesis can be further demonstrated in future research. However, this study also has some limitations. The molecular changes in the bladder tissue have not been verified in gene knockout mice. This is because we tried to knock out the SGK1 gene in mice, but unfortunately, we did not obtain the desired effect. Second, Dex is not a specific agonist of SGK1, but there is no specific agonist of SGK1 available, and Dex is still the most classic drug for inducing SGK1 transcription, so Dex was used in this experiment. Therefore, in future studies, we will optimize and improve the shortcomings of these experiments as much as possible.

In summary, we have shown that SGK1 targets TRPV1 and regulates MBSMC proliferation after BOO in mice through NFAT2. Therefore, our research results provide convincing evidence for further study of the pathogenesis of bladder remodeling, and this may provide some help for the screening of BOO therapeutic drug targets.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
Jiangshu He, Pinglin He, and Jia Li conceived and designed the study protocol, collected data, and performed data analysis. Lin Chen, Jin Yang, Xun Liu, Kai Wang, Taotao Dong, Xudong Ma, Amend Bastian, and Stenzl Arnulf designed the study. Lin Chen and Jin Yang were involved in designing the study and editing the manuscript. All authors read and approved the final manuscript.
ETHICS APPROVAL
The present study was approved by the Animal Ethics Committee of the Affiliated Hospital of Chengdu University.

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