RETRACTED ARTICLE: Circular RNA Plasmacytoma Variant Translocation 1 (CircPVT1) knockdown ameliorates hypoxia-induced bladder fibrosis by regulating the miR-203/Suppressor of Cytokine Signaling 3 (SOCS3) signaling axis

Teng Li, Yi Xing, Guoxian Zhang, Yan Wang, Yinsheng Wei, Lingang Cui, Shaojin Zhang, and Qingwei Wang

ABSTRACT
The effects of circular RNAs (circRNAs) on bladder outlet obstruction (BOO)-induced hypertrophy and fibrogenesis in rats and hypoxia-induced bladder smooth muscle cell (BSMC) fibrosis remain unclear. This study aimed to determine the regulatory role of circRNAs in the phenotypic changes in BSMCs in BOO-induced rats. circRNA microarray and real-time PCR were used to explore differentiated expressed circRNAs. Bioinformatics analyses and dual-luciferase reporter were performed to identify the targets for circRNA PVT1 (circPVT1). BOO was performed to establish a bladder fibrosis animal model. The circPVT1 and suppressor of cytokine signaling 3 (SOCS3) expression levels were upregulated (p = 0.0061 and 0.0328, respectively), whereas the microRNA-203a (miR-203) level was downregulated in rats with bladder remodeling (p = 0.0085). Bioinformatics analyses and dual-luciferase reporter assay results confirmed that circPVT1 sponges miR-203 and that the latter targets the 3′-untranslated region of SOCS3. Additionally, circPVT1 knockdown alleviated BOO-induced bladder hypertrophy and fibrogenesis. Furthermore, hypoxia was induced in BSMCs to establish a cell model of bladder fibrosis. Hypoxia induction in BSMCs resulted in upregulated circPVT1 and SOCS3 levels (p = 0.0052) and downregulated miR-203 levels. Transfection with circPVT1 and SOCS3 shRNA ameliorated hypoxia-induced transforming growth factor-β (TGF-β), TGFβR1, α-smooth muscle actin, fibrotic growth factor, extracellular matrix subtypes, BSMC proliferation, and apoptosis-associated cell injury, whereas co-transfection with miR-203 inhibitor counteracted the effect of circPVT1 shRNA on these phenotypes. These findings revealed a novel circRNA regulator of BOO-associated bladder wall remodeling and hypoxia-induced phenotypic changes in BSMCs by targeting the miR-203–SOCS3 signaling axis.
Introduction

Bladder outlet obstruction (BOO) is a condition that causes substantial bladder growth in old men with an enlarged prostate and has been demonstrated to increase the volume of the bladder matrix as well as increase detrusor stiffness in the long term [1,2]. Fibrosis, which is characterized by extracellular matrix (ECM) accumulation and bladder smooth muscle cell (BSMC) hyperplasia, plays a vital role in BOO, resulting in impaired urinary retention and bladder emptying [3]. In patients with BOO, BSMCs may transform from a contractile and non-proliferative phenotype to a synthetic one, and interactions between BSMCs and ECM are altered, leading to contractile dysfunction of BSMCs. A study has been reported that alterations in the ECM composition, particularly in type I and type III collagen, contribute to hypertrophic bladder fibrosis [4].

Several cytokines play a role in the progression of BOO. Transforming growth factor-beta (TGF-β) signaling stimulates fibrotic changes in BSMCs [5]. BOO has been demonstrated to play a role in muscle stiffness in a chronic obstructive condition in gene-altered mouse trials through the upregulation of TGF-β mRNA expression [6]. Hypoxia results from the deficiency of available oxygen in the blood and body parts and has been diagnosed in multiple conditions [7]. Hypoxia is a pathological condition which has been found in tumors and cardiology and other disease [8,9]. It is found that hypoxia could promote the fibrosis of liver, cardiology, renal and so on [10,11]. It is also considered to be vital in fibrotic changes in the bladder [12]. A recent study reported its involvement in the progression of bladder dysfunction after BOO and its auxo-action on the degree of inflammatory response, dedifferentiation, promotion of fibrosis, and ECM level in BSMCs [13]. Hypoxia has been found to occur in cases of BOO [14]. It is well established that hypoxia promotes fibrosis in BOO. A hypoxia-inducible factor inhibitor was shown to decrease BOO-induced bladder dysfunction [15,16]; however, due to the short time of BOO, the mechanism is not well known [16]. A previous study induced hypoxia in BSMCs to establish a cell model of BOO [17]. Therefore, BMSCs were treated with hypoxia to establish a BOO cell model in this study.

Circular RNAs (circRNAs) are a class of endogenous noncoding RNA molecules that are generated by reverse splicing of pre-mRNA and are characterized by a single-stranded ring structure and the lack of a 5’ cap and a 3’ tail [18]. Abnormal circRNA regulation commonly functions as a tumor-inhibiting factor or leads to tumor formation, and circRNAs modulate cell multiplication, migration, and invasion; the cell cycle; changes in autophagy; programmed cell death; and other biological processes of cells that occur during tumor formation and development [19]. However, to the best of our knowledge, no study has evaluated the effect of circRNA in bladder fibrosis. Recent studies have linked CircRNA Plasmacytoma Variant Translocation 1 (circPVT1) to the development of lung cancer, liver cancer, osteosarcoma, and bladder cancer [20,21,22,23], but its role in bladder fibrosis remains unclear.

MicroRNAs (miRNAs) are a class of small RNA molecules with a single-stranded structure that act complementarily to mRNAs to control protein synthesis via the degradation and suppression of protein translation [1–3,24–27]. Increasing evidence has demonstrated that miRNAs play fundamental roles in almost all biological processes and serve as multifunctional mediators of cell growth and multiplication, cell differentiation, programmed cell death, and organ remodeling [28]. Recent reports have linked fibrosis to miRNAs; in turn, miRNAs have become a promising biomarker and potential therapeutic target for the diagnosis and prognosis of fibrosis because changes in miRNA expression levels are of vital significance for the development of fibrosis [29]. Among miRNAs, the expression of microRNA-203a (miR-203), a bidirectional tumor regulator, is downregulated in various human malignant tumors such as carcinoma of the urinary bladder [30]. Its expression has been associated with the development of chemotherapeutic resistance in multiple cancers. It has been reported as a prognostic indicator of carcinoma of the urinary bladder and increases chemotherapeutic sensitivity to cisplatin through programmed cell death [31]. However, to date, the potential role of miR-203 in BOO-associated bladder fibrosis remains unclear. In the present study, we aimed to demonstrate the function of circPVT1, miR-203, and SOCS3 in
BOO-induced bladder remodeling and hypoxia-induced BSMC fibrosis.

**Materials and methods**

**Animals and grouping**

Twenty-four adult male Sprague Dawley rats were acquired from Shanghai Super-B&K Laboratory Animal Corp. Ltd. (Shanghai, China). The rats had a mean weight of 220 ± 10 g and were randomized into the following groups: control group (n = 6), which was subjected to sham operation for 8 weeks; BOO group (n = 6), which was subjected to surgically induced BOO for 8 weeks; the control in knockdown (KD) group (n = 6), which was subjected to circPVT1 KD before sham operation; and the BOO in KD group (n = 6), which was subjected to circPVT1 KD before BOO induction.

**BOO modeling in rats**

A biochemical analysis of the bladders of all rats was conducted after they had been treated for 8 weeks. All animal procedures were approved by the Animal Ethics Committees of The First Affiliated Hospital of Zhengzhou University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. The rat model of BOO was established by ligating the proximal urethra of the rats [32]. Briefly, the rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally). Subsequently, the rats were placed in the supine position, and an incision approximately 1.5-cm deep was made in the abdomen to retract the bilateral prostates, thereby exposing the proximal urethra. A 4–0 silk thread was sutured around the proximal urethra, and a catheter (1.10 mm OD) was juxtaposed outside the proximal urethra as a calibration stent. The catheter was then constricted with the urethra by tightening the ligature before it was carefully removed. Finally, the incision was sutured in two layers. The proximal urethra of rats subjected to sham operation was not ligated.

**CircPVT1 KD in rats**

The procedure for circPVT1 KD in the bladder tissues of rats has been described previously [33]. Briefly, the rats were intravesically administered ADV vectors expressing circPVT1 short hairpin RNA (shRNA) or control shRNA at three–five sites within the bladder, and each rat was administered 3,000 viral genome particles (approximately 250 μL) biweekly via an insulin syringe with a 30-gauge needle.

**miRNA microarray**

Total RNA was extracted from BOO bladder samples (n = 3) and normal bladder samples (n = 3) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Before the microarray experiment, an Agilent Technologies 2100 Bioanalyzer system was used to determine RNA quality. The rRNA ratio (28S/18S) of all samples was >1.8, and the RNA integrity number was >8.0. Data were analyzed using the Affymetrix® miRNA QC Tool. miRNAs with a 1.5-fold change in their expression levels with statistical difference were considered significantly dysregulated in BOO samples.

**Histological processing**

The bladder samples were fixed using 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm transverse sections. Histopathological diagnosis was performed using both Masson trichrome staining and hematoxylin and eosin (H&E) staining (Boster, Wuhan, China). To determine the percentage of bladder tissues with fibrosis, the collagen-to-smooth muscle ratio was calculated using ImageJ version 1.50, an image analysis software (NIH, Bethesda, MD, USA).

**Primary culture and identification of smooth muscle cells in human bladder tissue samples**

Enzymatic dispersion modification was conducted to harvest human BSMCs [34]. Upon pathological examination, samples were obtained from healthy rat bladders after cystectomy, and peribladder fat and bladder mucosa that adhered to the samples were removed using fine scissors. The rat bladder
tissue samples were then rinsed with sterile phosphate-buffered saline (PBS) and incubated in PBS containing 0.25% trypsin at 37°C for 30 min. Subsequently, the samples were cut into 1-mm sections and incubated in PBS containing 0.1% collagenase solution at 37°C for 4 h. The cell suspension was centrifuged at 800 × g at 4°C for 5 min. BSMCs were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 mg/mL), and glutamine (2 mL) at 37°C in a humidified atmosphere containing 5% CO₂. It was identified in approximately 95% of the cultured cells through α-smooth muscle actin (SMA) immunofluorescence staining. Cells from passages 2–5 that attained approximately 90% confluence were harvested and used for the subsequent procedures.

**Hypoxic cell culture and transfection**

BSMCs were cultured in DMEM containing 10% FBS, antibiotics, and 5% CO₂ at 37°C. In the hypoxia trial, the cell culture was placed in a 37°C incubator with 1% O₂. The cells were plated in DMEM containing 10% FBS at a density of 5 × 10⁵ cells/mL on the day prior to transfection of shRNA-circPVT1 (300 ng/50 μL), shRNA-suppressor of cytokine signaling 3 (SOCS3) (300 ng/50 μL), shRNA-NC (300 ng/50 μL), miR-203 inhibitor (500 ng/50 μL), or NC inhibitor (500 ng/50 μL) (Shanghai GenePharma Co., Ltd., Shanghai, China) in Lipofectamine 2000 reagent according to the manufacturer’s instructions. Six hours after transfection, the medium was replaced with DMEM containing 10% FBS, and transfection continued for an additional 36–48 h.

**Bioinformatics**

Two algorithms-starBase (http://starbase.sysu.edu.cn/) and TargetScan (http://www.targetscan.org)-were used to predict the targets of circPVT1 and miR-203, respectively. The results were listed according to the prediction efficacy. An alternative list was prepared based on the probability of conserved targeting [35].

**Dual-luciferase reporter assay**

A dual-luciferase reporter assay was performed to identify regulatory interactions among circPVT1, miR-203, and SOCS3. The wild-type (WT) and mutant 3'-untranslated regions (UTRs) of circPVT1 and SOCS3 were utilized. Renilla luciferase was used as an internal normalization standard. The cells were incubated for 36 h before transfection of miR-203 mimic or NC mimic and a luminescence vector.

**Western blot (WB) analysis**

Whole-cell lysate was prepared using a protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland) and a radiolabeled precipitation buffer (pH 8.0). A bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) was used to determine the protein content. Following protein separation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The cells were rinsed with Tris-buffered saline containing Tween 20 (TBST) after the unoccupied sites on the cell membrane were blocked following overnight incubation with primary antibodies at 4°C. Subsequently, cells were incubated with a secondary antibody for 1 h at room temperature to detect the immunoblot. The information of antibodies used here was showed as follows: anti-SOCS3 (1:1000, ab16030, Abcam), anti-alpha-MA (1:1000, ab5694, Abcam), anti-CTGF (1:1000, ab92486, Abcam), anti-TGF-beta 1 (1:2000, ab92160, Abcam), anti-TGFBR1 (1:500, ab31013, Abcam), anti-Collagen I (1:2500, ab34710, Abcam), anti-Elastin (1:2500, ab7778, Abcam), anti-h-caldesmon (1:2500, ab8247, Abcam), anti-Tubulin (1:5000, ab6046, Abcam), HRP-conjugated Goat anti-rabbit (1:5000, ab6721, Abcam), and HRP-conjugated Goat anti-mouse (1:5000, ab6789, Abcam). After washing several times in TBST, the bands were visualized using a SuperSignal™ West Femto Maximum Sensitivity Substrate kit (ThermoFisher, Waltham, MA, USA).
RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Following RNA extraction from cells or tissues (10 mg) using TRIzol reagent, the RNA concentrations were assessed using a Nanodrop 2000 instrument (OD$_{260}$). The M-MLV First-Strand Kit (Invitrogen, Carlsbad, CA, USA) and Oligo (dT) 20 primer were used to prepare complementary DNA via reverse transcription. qPCR was performed using the SYBR Select Master Mix (Invitrogen, Carlsbad, CA, USA) with the corresponding kits, and all procedures were performed according to the manufacturer’s instructions. GAPDH mRNA and U6 expression were used as internal references. The reaction conditions were as follows: denaturation for 10 min at 95°C, 40 cycles of amplification for 15 s at 95°C, and extension for 40 s at 60°C. The target mRNA levels were quantitatively analyzed using the $2^{-\Delta\Delta CT}$ method. All tests were conducted in triplicate.

Cell viability

A Cell Counting Kit-8 (CCK-8) assay was performed to assess cell viability. The cells were cultured in 96-well plates for 2 h at 37°C after adding the CCK-8 reagent (10 μL) (Dojindo, Kumamoto, Japan). An automatic microplate reader (Tecan Infinite M200; Mannedorf, Switzerland) was used to measure the optical density at 450 nm.

Flow cytometry

After 48 h of culture, the cells were digested with trypsin. An apoptosis assay was performed using an Annexin V-FITC/PI Assay Kit (Beyotime, Shanghai, China) to determine the apoptosis rate of the cells according to the manufacturer’s instructions. The cell suspension (100 μL) was obtained using 1× Annexin V binding buffer and mixed with Annexin V (5 μL) and PI (1 μL). The resulting mixture was incubated for 15 min in the dark at room temperature, and the reaction was terminated by adding 1× Annexin V binding buffer (400 μL). The apoptosis rate was determined using a Becton Dickinson FACSCalibur flow cytometer (Franklin Lakes, NJ, USA).

Statistical analysis

Data were analyzed using Statistical Product and Service Solutions (version 18.0; IBM Corp., Armonk, NY, USA). The measured variables were expressed as the mean ± standard error of the mean. A t-test was performed for between-group comparisons, and comparisons among more than two groups were performed using one-way analysis of variance. Data with a $p$-value of $>0.05$ were considered statistically significant.

Results

**circPVT1 expression is upregulated in the bladder tissues of BOO rats and hypoxia-treated BSMCs**

To assess the role of circRNAs in the development of BOO-induced bladder remodeling, dysregulated circRNAs were screened in bladder samples of BOO rats using microarray experiments. The expressions of 21 circRNAs, particularly circPVT1, were remarkably upregulated, whereas those of 19 circRNAs were downregulated in BOO-induced rat bladder tissues compared with those in the control group (Figure 1(a)). To confirm this result, circPVT1 expression in the bladder tissues of BOO and control rats was determined using qPCR. circPVT1 expression was upregulated in BOO samples compared with that in the control group (Figure 1(b)). Hypoxia was then induced in human BSMCs to establish a fibrotic cell model. Hypoxia treatment upregulated circPVT1 expression compared with that in the control group (Figure 1(c)). Seven clinical samples from bladder biopsies of BOO patients and 3 healthy volunteers were used to determine circPVT1 in clinical samples. The data showed that circPVT1 was statistically upregulated in BOO samples, compared with healthy controls (Figure 1(d)). Collectively, these findings demonstrated that circPVT1 was upregulated during the development of bladder fibrosis.

**circPVT1 serves as a sponge of miR-203, which in turn regulates SOCS3 expression**

A bioinformatics analysis was performed to predict the targets of circPVT1. We found that circPVT1 targeted miR-203 and the latter targeted the 3'UTR
of SOCS3, as reported previously [36,37] (Figure 2(a)). We then performed two independent dual-luciferase reporter assays to investigate the correlation between the circPVT1–miR-203 and miR-203–SOCS3 interactions. The luciferase activity was inhibited by 50% and 65% in BSMCs transfected using miR-203 mimic fused to WT circPVT1 and WT SOCS3, respectively, compared with those in the control group (Figure 2(b,c)). We next evaluated the expressions of miR-203 and SOCS3 in the bladder samples of BOO rats and control rats. The mRNA levels of miR-203 and SOCS3 were downregulated and upregulated, respectively, in BOO bladder samples compared with those in the control group (Figure 2(d,e)). Furthermore, hypoxia treatment downregulated miR-203 mRNA levels and upregulated SOCS3 mRNA levels in BSMCs (Figure 2(f,g)). The WB results showed that SOCS3 expression was clearly upregulated in BOO rats (Figure 2(h)) and hypoxia conditioned cells (Figure 2(i)). These findings indicate the presence of an inverse correlation among circPVT1, miR-203, and SOCS3.

**circPVT1 KD ameliorated bladder hypertrophy and fibrosis in BOO rats**

The potential relationships between circPVT1 expression and bladder hypertrophy and between circPVT1 expression and fibrosis were assessed by establishing a BOO rat model in both WT and circPVT1 KD rats, respectively. All the rats were maintained in suitable conditions up to the experimental end points. As determined using qPCR, circPVT1 expression in KD rats was significantly downregulated compared with that in

---

**Figure 1.** circPVT1 expression in bladder tissues and BSMCs. (a) microarray showing differentially expressed circRNAs between the bladder tissues of BOO rats (n = 4) and control bladder tissues (n = 4). (b) circPVT1 expression in bladder tissues of BOO and control rats was determined using qPCR (c) isolated BSMCs were conditioned with hypoxia for 24 h. circPVT1 expression was determined using qPCR. (d) qPCR detected circPVT1 expression in BOO clinical samples and normal healthy control. The results are expressed as the mean ± SEM. **p < 0.01 and ***p < 0.001 compared with the control group.
WT rats. circPVT1 KD also upregulated miR-203 expression and downregulated the protein and mRNA levels of SOCS3 (Figure 3(a-d)). Following BOO modeling, BOO surgery led to the upregulation of circPVT1 and SOCS3 levels and downregulation of miR-203 expression in bladder tissues compared with those in the sham control group. However, circPVT1 KD abolished the effects of BOO surgery on the expression levels of these genes (Figure 3(a-d)).

We then determined the bladder and body weights of the rats and calculated their ratio. The mean bladder weight/body weight ratio of BOO-induced WT and KD rats was significantly higher than that of sham-operated WT and KD rats (Figure 4(a)). However, compared with that of BOO-operated WT rats, the bladder weight/body weight ratio of BOO-operated KD rats was significantly reduced (Figure 4(a)), suggesting that circPVT1 repressed BOO-induced bladder hypertrophy.

To determine whether circPVT1 KD ameliorated BOO-induced bladder hypertrophy and fibrosis, histological examination and morphometric analysis were performed using H&E staining and Masson trichrome staining. Eight weeks after BOO induction, WT rats showed apparent infiltration of inflammatory cells in their bladder walls on H&E staining compared with the control rats, whereas KD rats showed weaker infiltration of inflammatory cells in their bladder walls (Figure 4(b)). Masson trichrome staining revealed that they exhibited higher degrees of fibrotic lesions and smooth muscle hypertrophy than the control rats (Figure 4(c)). Following circPVT1 KD, BOO-induced rats had

Figure 2. circPVT1 targeted miR-203, and miR-203 targeted SOCS3. (a) graph of the conserved circPVT1-binding motifs in miR-203 and miR-203-binding motifs in the 3'UTR of SOCS3. (B & C) luciferase activity was measured with the dual-luciferase reporter assay using a WT or MU copy of human circPVT1 and SOCS3 following transfection of miR-203/NC mimic in BSMCs. Luciferase function was normalized to renilla luciferase levels. (d, e) qPCR revealed the miR-203 and SOCS3 mRNA expression levels in bladder samples of BOO rats and sham-operated rats. (f, g) BSMCs were conditioned with hypoxia for 24 h. miR-203 and SOCS3 mRNA expression levels in BSMCs were determined using qPCR. (h, i) The SOCS3 level in (h) bladder tissues and (i) BSMCs was determined using WB. The results are expressed as the mean ± SEM. *p < 0.05 and **p < 0.01 compared with the indicated group.
less severe detrusor hypertrophy in the interfascicular region than the BOO-operated circPVT1 WT rats (Figure 4(c)). In addition, collagen deposition was more severe in BOO-treated WT rats, and the collagen-to-smooth muscle ratio was significantly higher than that in BOO-treated KD rats (Figure 4(d)).

We then evaluated whether circPVT1 regulated the expression levels of fibrotic genes, including α-SMA, connective tissue growth factor (CTGF), and transforming growth factor beta 1 (TGF-β1), as well as the gene encoding TGFBR1 [4–6]. As determined using qPCR, the BOO groups showed significant upregulation of these four genes compared with the control groups (Figure 5(a-d)). However, circPVT1 KD ameliorated the α-SMA, CTGF, TGF-β1, and TGFBR1 mRNA levels in the bladder tissues of KD rats compared with that in WT rats (Figure 5(a-d)). The WB results were consistent with the qPCR results (Figure 5(e)). These findings suggest that circPVT1 upregulation is essential for the development of bladder hypertrophy and fibrosis in BOO-induced rats.

**Effect of circPVT1–miR-203 cross-talk and SOCS3 silencing on the expression of contractile proteins, growth factors, and ECM subtypes in hypoxia-induced BSMCs**

To monitor the effect of circPVT1–miR-203 cross-talk and SOCS3 silencing in an in vitro cell model of bladder fibrosis, hypoxia-induced BSMCs were transfected with shRNA-NC, shRNA-circPVT1, or shRNA-SOCS3 or co-transfected with miR-203/NC inhibitor and shRNA-circPVT1. First, the transfection efficiency of shRNA-SOCS3 was assessed in untreated cells as the SOCS3 expression was significantly downregulated (Supplementary Figure 1). qPCR and WB were then performed to assess gene expression following transfection in hypoxia-induced BMSCs. Hypoxia induction significantly upregulated circPVT1 and SOCS3 expression, whereas miR-203 expression was downregulated, a trend that was similar to that observed in the in vivo BOO model. After transfection with shRNA-circPVT1, the circPVT1 and SOCS3 expressions were clearly downregulated, whereas the miR-203 expression was upregulated. To clarify the involvement of...

![Figure 3](image-url)
miR-203 in the function of circPVT1, the cells were also transfected with miR-203 inhibitor. miR-203 inhibition resulted in the significant downregulation of miR-203 expression and upregulation of the SOCS3 mRNA level. Furthermore, to elucidate the role of SOCS3 in hypoxia-induced BSMCs, SOCS3 expression was silenced after transfection with shRNA-SOCS3 (Figure 6(a-d), Supplementary Figure 2a, 2b, 2c).

Regarding the expression levels of ECM subtypes, growth factors, and contractile proteins, exposure of BSMCs to hypoxia resulted in the upregulation of α-SMA, type I collagen, type III collagen, elastin, and CTGF levels and the downregulation of contractile protein h-caldesmon level (Figure 7(a-g), Supplementary Figures 2d, 2e). Silencing of both circPVT1 and SOCS3 in hypoxia-induced BSMCs led to downregulation of the α-SMA, type I collagen, type III collagen, elastin, and CTGF levels and upregulation of the h-caldesmon level. Furthermore, co-inhibition of miR-203 counteracted the effect of circPVT1 silencing on the expression of these genes (Figure 7(a-g)). These data suggest that hypoxia induction led to fibrotic phenotype changes in BMSCs, as evidenced by the increased levels of ECM subtypes and growth factors and dysregulated contractile proteins; however, silencing of circPVT1 and downstream SOCS3 ameliorated these changes.

To determine the effects of circPVT1–miR-203 cross-talk and SOCS3 silencing on the regulation of hypoxia-induced proliferation of BSMCs, we performed CCK-8 assay and flow cytometry to assess the viability and apoptosis of BSMCs. Exposure to hypoxia significantly reduced the cell viability, whereas circPVT1 and SOCS3 silencing
partially reversed this effect. Furthermore, co-inhibition of miR-203 abolished the protective role of circPVT1 silencing on the viability of BSMCs (Figure 8(a), Supplementary figure 2f). Hypoxia induced robust apoptosis in BMSCs, whereas circPVT1 and SOCS3 silencing suppressed this effect. Finally, miR-203 inhibition restored the proportion of apoptotic cells in hypoxia-induced BMSCs following circPVT1 silencing (Figure 8(b,c)). According to these findings, circPVT1 and SOCS3 play roles in hypoxia-induced apoptosis of BSMCs, whereas miR-203 has an inhibitory effect on cell apoptosis.

Discussion

The role of circPVT1 in bladder fibrosis is unclear. The bladder could adapt to BOO-induced workload via remodeling and hypertrophic changes. This remodeling is characterized by the synthesis and deposition of ECM in the bladder wall and proliferation of BSMCs; however, the mechanism by which circRNAs function in these biological processes remains mostly undiscovered. The expression levels of circPVT1, miR-203, and SOCS3 in BOO-induced bladder remodeling and hypoxia-induced BSMC fibrosis were evaluated in this study. Using a rat model of BOO, we confirmed the involvement of circPVT1 knockdown (KD) in BOO-induced bladder hypertrophy and fibrogenesis. In the hypoxia-induced BSMC fibrosis model, the effect of circPVT1, miR-203, and SOCS3 dysregulation on the expression of ECM genes and BSMC proliferation and apoptosis were assessed. Furthermore, a bioinformatics analysis predicted the cross-talk between circPVT1–miR-203 and miR-203–SOCS3, which was verified using a dual-luciferase reporter assay. We then observed that circPVT1 and SOCS3 expression was upregulated and miR-203 expression was downregulated in the bladder tissues of rats with BOO and hypoxia-induced BSMCs. We validated the association between the circPVT1–miR-203–SOCS3 axis. The increase in the levels of fibrosis-
related growth factors and α-SMA, which is a biochemical marker of smooth muscle differentiation, was repressed after circPVT1 KD. Meanwhile, BOO-induced bladder hypertrophy and fibrosis in the rats were also ameliorated following circPVT1 KD. We also evaluated the role of circPVT1, miR-203, and SOCS3 in fibrosis translation in hypoxia-induced BSMCs. circPVT1 participated in the process of BSMC fibrosis by mediating miR-203–SOCS3 expression. These data suggest that circPVT1 is essential for BOO-induced fibrosis and hypertrophy in the bladder walls and indicate the possibility of targeting circPVT1 as a promising antifibrosis treatment strategy for BOO-induced bladder remodeling.

Previous studies reported that upregulation of miR-203 expression leads to the inhibition of fibrosis in myocardial and hepatic tissues [38–40]. In a study on miRNA expression levels in the bladder tissues of BOO-induced rats, Ekman et al. [6] observed the deregulation of multiple miRNAs during fibrosis. Here, miR-203 expression was evaluated in an in vivo model of BOO and an in vitro model of hypoxia characterized by obstructed bladder disease and upregulated expression of fibrotic genes. We also clearly demonstrated that miR-203 expression was negatively regulated by circPVT1 in fibrotic bladder tissues and hypoxia-induced BSMCs. In the in vivo study, upregulated miR-203 expression was associated with poor bladder wall hypertrophy and fibrogenesis and downregulated expression of fibrotic growth factors. The in vitro experiments showed that inhibition of miR-203 expression in hypoxia-induced BSMCs following circPVT1 KD restored cell apoptosis and repressed cell viability and the expression of growth factors, contractile proteins, and ECM subtypes. This suggests that
Figure 7. Gene expression levels of ECM subtypes, h-caldesmon, growth factors, and α-SMA in hypoxia-induced BSMCs. The cultured BSMCs were transfected with shRNA-NC, shRNA-circPVT1, or shRNA-SOCS3 for 24 h or co-transfected with miR-203/NC inhibitor and shRNA-circPVT1 for 24 h, followed by 24 h of hypoxia conditioning. (a, b, c, d, e, f) The expression levels of α-SMA, type I collagen, type III collagen, elastin, CTGF, and h-caldesmon mRNAs in BSMCs were determined using qPCR. (g) The expression levels of α-SMA, type I collagen, type III collagen, elastin, CTGF, and h-caldesmon in BSMCs were determined using WB. The results are presented as the mean ± SEM. **p < 0.01, ***p < 0.001 in the Hypoxia group compared with the control group; &&p < 0.01 and &&&p < 0.001 compared with the Hypoxia + shNC group; $p < 0.01 and $$$p < 0.001 compared with the Hypoxia + shPVT1 + NC inhibitor group.
miR-203 plays an inhibitory role in BOO-induced bladder wall remodeling. These findings demonstrate the intricate roles of miR-203 in the development and progression of bladder fibrosis.

The SOCS family comprises eight members. Among them, SOCS3 is composed of eight structurally interacting SOCS proteins and plays a role in cell activation and multiplication [41]. Recent studies confirmed that SOCS3 expression plays a positive regulatory role in hepatic fibrosis [42,43] and cardiac fibrosis [44]. The present study provides evidence of the high expression of SOCS3 in BOO and hypoxia-induced BSMCs. The downregulation of SOCS3 expression caused by circPVT1 KD was associated with ameliorated bladder hypertrophy and fibrogenesis in BOO-induced bladder tissues, whereas silencing of SOCS3 expression led to downregulated fibrotic gene expression and ECM deposition in hypoxia-induced BSMCs.

TGF-β1, a crucial protein for normal cell growth and differentiation, is a strong mediator of fibrosis. TGF-β1 has also been linked to myofibroblast activation, epithelial–mesenchymal transition, epithelial cell apoptosis, ECM production, and several other conditions [45]. Fibrosis could be inhibited by downregulating TGF-β expression and suppressing signaling pathways and functions. Upregulation of TGF-β1 expression is physiologically correlated with the severity of BOO in humans and rats, and its inhibition was shown to result in the alleviation of fibrotic changes [46–49]. The present study revealed the significant downregulation of TGFβ1 in BOO-induced bladder tissues and hypoxia-induced BSMCs after downregulation of circPVT1 expression and silencing of SOCS3 expression.

However, we still admitted that circPVT1-SOCS3-miR-203 axis-caused modest changes of

\[\text{Figure 8. Cell viability and apoptosis in hypoxia-induced BSMCs. (a) The viability of BSMCs was assessed using the CCK-8 assay. (b) Programmed cell death of BSMCs was detected using flow cytometry with Annexin V-FITC and PI dual staining. (c) Quantitative and statistical analyses of the apoptotic cell proportion of BSMCs. The results are expressed as the mean ± SEM. **}p < 0.01 \text{ and ***}p < 0.001 \text{ in the Hypoxia group compared with the control group; **}p < 0.01 \text{ and } &&p < 0.001 \text{ compared with the Hypoxia + shNC group; } &&p < 0.01 \text{ and } $$$p < 0.001 \text{ compared with the Hypoxia + shPVT1 + NC inhibitor group.} \]
downstream effectors, including α-SMA, Collagen I, III, and so on, might be the attribution for the diminished response in vivo, although the difference was still statistically significant. This is one of limitations of the present study. In another aspect, BOO surgery used in this study caused bladder wall remodeling in rats, including hypertrophy and fibrosis, which is also the major characters of human BOO [50], meanwhile, this model has been applied in many similar reports [51,52]. We have demonstrated a role of circPVT1 on BOO development in animal model and cell-based model, but its application to human BOO were still obscure. To elucidate its effect on human BOO, human samples should be used in future study.

**Conclusions**

In conclusion, the expression levels of circPVT1 and SOCS3 were upregulated, whereas that of miR-203 was downregulated in the bladder tissues of a rat model of BOO. circPVT1 expression was positively correlated with BOO- and hypoxia-induced fibrosis and regulated the miR-203–SOCS3 axis. Our results also indicate that miR-203 and SOCS3 serve as antifibrotic and profibrotic genes, respectively, in bladder remodeling after BOO.

**Highlights**

1. CircPVT1 is upregulated in the bladder tissues of BOO rats and hypoxia-treated BSMCs
2. CircPVT1 serves as a sponge of miR-203, which in turn regulates SOCS3 expression
3. CircPVT1 KD ameliorated bladder hypertrophy and fibrosis in BOO rats
4. Effect of circPVT1-miR-203 cross-talk and SOCS3 silencing on the expression of contractile proteins, growth factors, and ECM subtypes in hypoxia-induced BSMCs.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

**Funding**

This work was supported by The First Affiliated Hospital of Zhengzhou University.

**Data Availability Statement**

My manuscript has no associated data.

**Ethical approval**

This study was approved by the Animal Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

**References**

[1] Damaser MS, Arner A, Uvelius B. Partial outlet obstruction induces chronic distension and increased stiffness of rat urinary bladder. Neurourol Urodyn. 1996;15(6):650–665.
[2] Maciejewski CC, Honardoust D, Tredget EE, et al. Differential expression of class I small leucine-rich proteoglycans in an animal model of partial bladder outlet obstruction. J Urol; 2012;188(4S):1543–1548.
[3] Fusco F, Creta M, De Nunzio C, et al. Progressive bladder remodeling due to bladder outlet obstruction: a systematic review of morphological and molecular evidences in humans. Bmc Urol. 2018;18(1):15.
[4] Bellucci C, Ribeiro WO, Hemerly TS, et al. Increased detrusor collagen is associated with detrusor overactivity and decreased bladder compliance in men with benign prostatic obstruction. Prostate Int. 2017;5(2):70–74.
[5] Koeck I, Burkhard FC, Monastyrskaya K. Activation of common signaling pathways during remodeling of the heart and the bladder. Biochem Pharmacol. 2016;102:7–19.
[6] Ekman M, Bhattachariya A, Dahan D, et al. Mir-29 repression in bladder outlet obstruction contributes to matrix remodeling and altered stiffness. Plos One. 2013;8(12):e82308.
[7] Rankin EB, Giaccia AJ. Hypoxic control of metastasis. Science. 2016;352(6282):175–180.
[8] Nakada Y, Canseco DC, Thet S, et al. Hypoxia induces heart regeneration in adult mice. Nature. 2017;541(7636):222–227.
[9] Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. Nat Rev Cancer. 2011;11(6):393–410.
[10] Liu M, Liu L, Bai M, et al. Hypoxia-induced activation of Twist/miR-214/E-cadherin axis promotes renal tubular epithelial cell mesenchymal transition and renal fibrosis. Biochem Biophys Res Commun. 2018;495:2324–2330.
[11] Watson CJ, Collier P, Tea I, et al. Hypoxia-induced epigenetic modifications are associated with cardiac tissue fibrosis and the development of a myofibroblast-like phenotype. Hum Mol Genet. 2014;23(8):2176–2188.
[12] Xiong A, Liu Y. Targeting hypoxia inducible factors-1alpha as a novel therapy in Fibrosis. Front Pharmacol. 2017;8:326.
[13] Wiafe B, Adesida A, Churchill T, et al. Hypoxia-increased expression of genes involved in inflammation, dedifferentiation, pro-fibrosis, and extracellular matrix remodeling of human bladder smooth muscle cells. Vitro Cell Dev Biol Anim. 2017;53(1):58–66.

[14] Drzewiecki BA, Anumanthan G, Penn HA, et al. Modulation of the hypoxic response following partial bladder outlet obstruction. J Urol. 2012;188(4S):1549–1554.

[15] Iguchi N, Donmez MI, Malykhina AP, et al. Preventative effects of a HIF inhibitor, 17-DMAG, on partial bladder outlet obstruction-induced bladder dysfunction. Am J Physiol Renal Physiol. 2017;313(5):F1149–F1160.

[16] Iguchi N, Malykhina AP, Wilcox DT. Inhibition of HIF reduces bladder hypertrophy and improves bladder function in murine model of partial bladder outlet obstruction. J Urol. 2016;195(4 Part 2):1250–1256.

[17] Wang N, Duan L, Ding J, et al. MicroRNA-101 protects bladder of BOO from hypoxia-induced fibrosis by attenuating TGF-beta-smad2/3 signaling. Iubmb Life. 2019;71:235–243.

[18] Memczak S, Jens M, Elefsinioti A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. Nature. 2013;495(7441):333–338.

[19] Yang CY, Wang J, Zhang JQ, et al. Human circular RNA hsa_circRNA_101705 (circTXNDC11) regulates renal cancer progression by regulating MAPK/ERK pathway. Bioengineered. 2021;12(1):4342–4441.

[20] He Y, Zhang H, Deng J, et al. The functions of fluoxetine and identification of fluoxetine-mediated circular RNAs and messenger RNAs in cerebral ischemic stroke. Bioengineered. 2021;12(1):2364–2376.

[21] Kristensen LS, Andersen MS, Stagsted L, et al. The biogenesis, biology and characterization of circular RNAs. Nat Rev Genet. 2019;20(11):675–691.

[22] Adhikary J, Chakraborty S, Dalal S, et al. Circular PVT1: an oncogenic non-coding RNA with emerging clinical importance. J Clin Pathol. 2019;72(8):513–519.

[23] Liu YP, Wan J, Long F, et al. circPVT1 facilitates invasion and metastasis by regulating miR-205-5p/c-FLIP axis in osteosarcoma. Cancer Manag Res. 2020;12:1229–1240.

[24] Liu G, Lei Y, Luo S, et al. MicroRNA expression profile and identification of novel microRNA biomarkers for metabolic syndrome. Bioengineered. 2021;12(1):3864–3872.

[25] Rahbarghazi R, Keyhanmanesh R, Rezaie J, et al. c-kit+ cells offer hopes in ameliorating asthmatic pathologies via regulation of miRNA-133 and miRNA-126. Iran J Basic Med Sci. 2021;24:369–376.

[26] Alamdari AF, Rahmehayan S, Rajabi H, et al. Melatonin as a promising modulator of aging related neurodegenerative disorders: role of microRNAs. Pharmacol Res. 2021;173:105839.

[27] Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008;9(2):102–114.

[28] Catto JW, Alcaraz A, Bjartell AS, et al. MicroRNA in prostate, bladder, and kidney cancer: a systematic review. Eur Urol. 2011;59(5):671–681.

[29] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215–233.

[30] Saini S, Arora S, Majid S, et al. Circumventing the TGF-beta-activation of p38-MAPK signaling. Mol Cancer Ther. 2011;10(10):1698–1709.

[31] Zhang X, Zhang Y, Liu X, et al. MicroRNA-203 is a prognostic indicator in bladder cancer and enhances chemosensitivity to cisplatin via apoptosis by targeting Bcl-w and survivin. Plos One. 2015;10:e143441.

[32] Liu F, Yao L, Yuan J, et al. Protective effects of inosine on urinary bladder function in rats with partial bladder outlet obstruction. Urology. 2011;78(6):1417–1422.

[33] Huang S, Li X, Zheng H, et al. MicroRNA-203 increases proliferation of bladder tumor cells and promotes bladder cancer progression by regulating MAPK/ERK pathway. Oncotarget. 2019;10(97):2857–2876.

[34] Ma F, Higashira H, Ukai Y, et al. A new enzymic method for the isolation and culture of human bladder body smooth muscle cells. Neurourology. 2002;21(1):71–79.

[35] Friedman RC, Farh KK, Burge CB, et al. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92–105.

[36] Lin XM, Chen H, Zhan XL. Mir-203 regulates JAK-STAT pathway in affecting pancreatic cancer cells proliferation and apoptosis by targeting SOCS3. Eur Rev Med Pharmacol Sci. 2019;23:6906–6913.

[37] Zhu Y, Liu Y, Xiao B, et al. The circular RNA PVT1/miR-203/HOXD3 pathway promotes the progression of human hepatocellular carcinoma. Biol Open. 2019;8:bio403687.

[38] He Q, Wang CM, Qin JY, et al. Effect of miR-203 expression on myocardial fibrosis. Eur Rev Med Pharmacol Sci. 2017;21:837–842.

[39] Liu W, Feng R, Li X, et al. TGF-beta- and lipopolysaccharide-induced upregulation of circular RNA PWWP2A promotes hepatic fibrosis via sponging miR-203 and miR-223. Aging (Albany NY). 2019;11(21):9569–9580.

[40] Xiao C, Hong H, Yu H, et al. Mir-340 affects gastric cancer cell proliferation, cycle, and apoptosis through regulating SOCS3/JAK-STAT signaling pathway. Immunopharmacol Immunotoxicol. 2018;40(4):278–283.

[41] Jadid FZ, Chilab H, Alj HS, et al. Control of progression of liver fibrosis and hepatocellular carcinoma by SOCS3 polymorphisms in chronic HCV-infected patients. Infect Genet Evol. 2018;66:1–8.

[42] Ogata H, Chinen T, Yoshida T, et al. Loss of SOCS3 in the liver promotes fibrosis by enhancing STAT3-mediated TGF-beta1 production. Oncogene. 2006;25(17):2520–2530.
Dees C, Potter S, Zhang Y, et al. TGF-beta-induced epigenetic deregulation of SOCS3 facilitates STAT3 signaling to promote fibrosis. J Clin Invest. 2020;130(5):2347–2363.

Varga J, Pasche B. Antitransforming growth factor-beta therapy in fibrosis: recent progress and implications for systemic sclerosis. Curr Opin Rheumatol. 2008;20(6):720–728.

Anumanthan G, Tanaka ST, Adams CM, et al. Bladder stromal loss of transforming growth factor receptor II decreases fibrosis after bladder obstruction. J Urol. 2009;182(4S):1775–1780.

MacRae DK, Hoffman BB, Leonard MB, et al. Increased urinary transforming growth factor-beta(1) excretion in children with posterior urethral valves. Urology. 2000;56(2):311–314.

Sager C, Lopez JC, Duran V, et al. Transforming growth factor-beta1 in congenital ureteropelvic junction obstruction: diagnosis and follow-up. Int Braz J Urol. 2009;35(3):323–325.

Wiafe B, Adesida A, Churchill T, et al. Mesenchymal stem cells inhibit hypoxia-induced inflammatory and fibrotic pathways in bladder smooth muscle cells. World J Urol. 2018;36(7):1157–1165.

Buttyan R, Chen MW, Levin RM. Animal models of bladder outlet obstruction and molecular insights into the basis for the development of bladder dysfunction. Eur Urol. 1997;32(Suppl 1):32–39.

Chen L, Lv L, Zhang L, et al. Metformin ameliorates bladder dysfunction in a rat model of partial bladder outlet obstruction. Am J Physiol Renal Physiol. 2021;320(5):F838–F858.

Lee JY, Park JM, Na YG, et al. Expression of bladder α1-adrenoceptor subtype after relief of partial bladder outlet obstruction in a rat model. Investig Clin Urol. 2020;61(3):297–303.

Niemczyk G, Fux L, Czarzasta K, et al. Expression of toll-like receptors in the animal model of bladder outlet obstruction. Biomed Res Int. 2020:2020:6632359.