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Activation of NFKB-JMJD3 signaling promotes bladder fibrosis *via* boosting bladder smooth muscle cell proliferation and collagen accumulation

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ABSTRACT

Chronic cystitis is characterized by the hyperplasia and fibrosis of the bladder wall as well as attenuated compliance of the bladder. To further unravel its underlying molecular mechanism, the role of NFκB-JMJD3 signaling pathway in cystitis induced bladder fibrosis was investigated. Jmjd3 and Col1/3 expression was detected in a cystitis mouse model that was developed by intraperitoneal injection of cyclophosphamide (CYP). Human bladder smooth muscle cells (hBSMCs) were stimulated *in vitro* with lipopolysaccharide (LPS), and the cell proliferation and collagen accumulation were detected using EdU, CCK8, flow cytometry, qPCR, western blotting and immunofluorescence assays. Furthermore, the effects of NFκB and JMJD3 on cell proliferation and collagen accumulation were investigated using its selective antagonists, JSH23 and GSK-J4, respectively. CYP induced cystitis significantly increased Jmjd3, Col1 and Col3 expression in the bladder muscle cells. Furthermore, LPS stimulation markedly activated NFκB signaling and elevated JMJD3 expression in hBSMCs, and the activation of NFκB-JMJD3 signaling significantly promoted cell proliferation and collagen accumulation by upregulating CCND1 and COL1/3 expression, respectively. Our study reveals the critical role of NFκB-JMJD3 signaling in cystitis induced bladder reconstruction by regulating hBSMC proliferation and extracellular matrix (ECM) deposition, and these findings provide an avenue for effective treatment of patients with cystitis.

1. Introduction

Cystitis is a prevalent disease with high morbidity, and the patients with acute cystitis show the classic symptoms of bladder irritation, including frequent, urgent and painful urination [1]. Although the symptom of chronic cystitis is not representative as acute cystitis, it has the distinctly pathological features such as the thickened bladder wall [2], the decreased bladder compliance and capacity [3], and the shortened voiding interval [4]. Previous studies have demonstrated that cystitis caused bladder hypertrophy and deposition of collagen, which further leads to the dysfunction of bladder storage [5,6]. However, its underlying mechanism is still largely unknown.

Recently, epigenetics was demonstrated to play important roles in the progression of many diseases, including inflammation, cancer, and obesity [7–9]. Lysine-specific demethylase 6B (JMJD3, also known as KDM6B) is a demethylase of H3K27me3, previous studies demonstrated that inflammation could induce the expression of JMJD3 [10]. Chen et al. found that JMJD3 promoted the expression of IL-1β in early sepsis [11]. In addition JMJD3 can regulate the proliferation of neointima following vascular injury [12], and promote joint destruction in rheumatoid arthritis by boosting the proliferation and migration of fibroblast-like synoviocyte [13]. In the urinary system, JMJD3 was postulated to be associated with the etiology of bladder pain syndrome/interstitial cystitis [14]. Thus, JMJD3, an epigenetic regulator, may have crucial effects on pathological and physiological activities of bladder reconstruction.

In addition, the expression of phosphorylated NFkB was elevated in a model of LPS induced cystitis [15], and it was reported that JMJD3, as a downstream target of NFkB, regulated the expression of inflammatory gene in vascular endothelial cells [16]. Therefore, we hypothesized that bladder inflammation could induce the activation of NFkB and the expression of JMJD3, and further result in the proliferation and extracellular matrix deposition of human bladder smooth muscle cells (hBSMCs). To elucidate the role of NFκB-JMJD3 signaling in bladder

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fibrosis, this study plans to explore the underlying mechanism using *in vitro* and *in vivo* models. The findings of our study would unravel the underlying mechanism and pave an avenue for the effective treatment of cystitis.

2. Materials and methods

2.1. hBSMC culture and reagents

Primary hBSMCs (ScienCell, San Diego, CA) were cultured in special SMCM medium (ScienCell), supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/ml) and streptomycin (100μg/ml). Primary hBSMCs were passaged two to eight generation with stable state and normal morphology, and then cells were seeded in six-well plates in a CO_2 incubator (Thermo) at a density of 1×10^5 cells/well. LPS (lipopolysaccharide, L2880) and GSK-J4(JMJD3 inhibitor, SML0701) were obtained from Sigma, and JSH23 (NFkB inhibitor, ab144824) was purchased from Abcam.

2.2. RNA isolation and RT-PCR

Total RNA was extracted from hBSMCs using a RNeasy Mini Kit (Qiagen, German). RNA concentration was measured using a Spectrophotometer (IMPLEN Nanophotometer, Germany). The cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Real-time quantitative PCR (qPCR) was performed using Bio-Rad CFX Manager™ software version 3.1 (Bio-Rad CFX96 instrument) and the specific primers were listed in Table 1. GAPDH was set as an internal control. The PCR reaction was performed using IQ™ SYBR Green Supermix reagent (BIO-RAD), and the profile was as follows: 95 °C for 3 min and 40 cycles of 95 °C for 10 s, 55 °C for 30 s. The real-time data were analyzed using the $2^{-\Delta\Delta Ct}$ method. Data were represented as the mean ± standard deviation, and the experiments were performed in triplicate.

2.3. Protein extraction and western blot

Total proteins were extracted using RIPA buffer (Biosharp, China) containing protease inhibitor and phosphatase inhibitor. In total, 20 μg of proteins were separated using 10% polyacrylamide gel electrophoresis (PAGE) gels (Bio-Bad) and transferred onto polyvinylidene fluoride (PVDF) membranes. Then, 5% skimmed milk was used to block the membranes for 1 h at room temperature (RT), and further incubated with primary antibodies at 4 °C overnight. The membranes were incubated with secondary anti-rabbit IgG or anti-mouse IgG (MBL Japan) for 1 h at RT. The following primary antibodies were used in this study (Supplementary Table 1): Anti-Collagen I (abcam, ab34710), Anti-Collagen III (abcam, ab7778), phospho-NFkB (Zen Bioscience, 310,013, phospho Ser536); NFkB (Zen Bioscience, 250,060); JMJD3(abcam, ab169197); CCND1(553, MBL); α-Tubulin (Zen Bioscience, 252,156). Then the protein bands were exposed and the results were analyzed using Gel-pro software.

Table 1
Primer sequences for quantitative-PCR.

Gene	Primer sequence
KDM6B	
Forward	CACCCCAGCAAACCATATTATGC
Reverse	CACACAGCCATGCAGGGATT
COL1A1	
Forward	GTGCGATGACGTGATCTGTGA
Reverse	CGGTGGTTTCTTGGTCGGT
COL3A1	
Forward	TTGAAGGAGGATGTTCCCATCT
Reverse	ACAGACACATATTTGGCATGGTT
CCND1	
Forward	TGGAGCCCGTGAAAAAGAGC
Reverse	TCTCCTTCATCTTAGAGGCCAC
GAPDH	
Forward	ACAACTTTGGTATCGTGGAAGG
Reverse	GCCATCACGCCACAGTTTC

2.4. Cell fluorescence staining

The hBMSCs were cultured in 24-well plates at a density of 2×10^4 cells per well, and then the cells were fixed with 4% paraformaldehyde at RT for 30 min. Next, cells were incubated with PBS containing 5% Bovine Serum Albumin (BSA) and 1% TritonX-100 at RT for 2 h, and then incubated with primary antibodies overnight at 4 °C. After three washes for 30 min, cells were further incubated with Alexa Fluor™ 488 goat anti-rabbit IgG (H + L) for 1 h at RT, and the fluorescence was examined using a fluorescent microscopy (Zeiss Axio observer D1, Germany). The expression of collagen I and collagen III was determined at 100× magnification in 5 different fields of vision using Image-Pro Plus version 6.0.

2.5. Cell cycle detection kit

The cell cycle of hBSMCs was detected using a Cell Cycle Detection Kit (KeyGEN BioTECH, China). The cells cultured in 6-well plates were digested and centrifuged at 300 ×*g* for 5 min. Cells were resuspended with PBS, and hBSMCs were fixed with 70% ethanol and maintained at 4 °C overnight. The hBSMCs were washed with PBS and incubated with 500 μL of staining buffer containing RNases and PI mixture at a ratio of 1:9 for 30 min in dark, and then cells were analyzed using flow cytometry.

2.6. CCK-8 proliferation assay

The effect of GSK-J4 and JSH23 on the proliferation of LPS-induced hBSMCs was assessed using a CCK-8 kit from Dojindo Molecular Technologies, (Rockville, MD, USA). Briefly, hBSMCs were cultured into 96-well plates at a density of 1×10^4 cells/well for 24 h. Next, cells were incubated with GSK-J4 and JSH23 for 1 h, and 10 μg/mL of LPS for another 24 h. Cells were then incubated with CCK-8 solution at 37 °C for 30 min, and the absorbance was detected using a microplate reader (Shanghai Utrao Medical Instrument Co., Ltd., Shanghai, China).

2.7. 5-ethynyl-2′-deoxyuridine (EdU) staining

The Cell-Light™ EdU Kit from Rui Bo Guangzhou Biotechnology (Guangzhou, China) was employed to detect hBSMC proliferation. hBSMCs were seeded into 96-well plates, and 100 μL of EdU (50 μM) was added into each well for 2 h. EdU-positive cells were fixed and stained as per the manufacture's protocol, and images were obtained using fluorescence microscopy (Zeiss Axio observer D1, Germany). The percentage of EdU-positive cells was obtained using 5 different fields at 100× magnification.

2.8. Cystitis mouse model

C57BL/6 female mice were housed with normal diet in a cycle of 12 h light and 12 h dark. Cystitis mouse model was induced by intraperitoneally injection of 200 mg/200 μL /1 kg of cyclophosphamide (CYP) for 24 h [17–19]. The mice in the control (ctrl, hereafter) group (n = 5/group) was injected with PBS. Then the bladder tissue was harvested after mice were sacrificed with CO_2.

2.9. Immunofluoresence staining of mouse bladder

Mouse bladders were fixed in 4% paraformaldehyde at 4 °C overnight, and then dehydrated sequentially in 10%- 30% of sucrose

solution each at 4 °C overnight. The samples were embedded into the optimal cutting temperature compound (O.C.T.) (Sakura Finetek, Torrance CA, USA), and five micron-thick sections were simultaneously blocked and permeabilized with 0.5% Triton X-100 and 5% goat serum in 1× PBS at RT for 2 h. The sections were incubated with primary antibodies against Col1 (1:100 diluted, cat # ab34710, Abcam, Cambridge MA, USA), Col3 (1:100 diluted, cat # ab7778, Abcam, Cambridge MA, USA) and Jmjd3 (1:100 diluted, cat # ab169197, Abcam, Cambridge MA, USA) at 4 °C overnight, and further incubated with secondary antibodies (Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 647 and Goat anti-Mouse IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 647, Waltham, MA USA, Life Technologies) at RT for 1 h in the dark. Finally, the slides were mounted using the VECTASHIELD mounting medium (Vector Laboratories, Burlingame CA, USA). Slides were photographed using a fluorescence microscope, and the exposure time for the fluorescence of Jmjd3, Col1, Col3 and DAPI was set as 500, 500, 300 and 10 milliseconds (ms), respectively. The intensities of Jmjd3, Col1 and Col3 were obtained using Image J software and normalized against DAPI intensities [20].

2.10. Statistical analysis

The experiments were performed in triplicate and all data were presented as means ± Standard Error of Mean (SEM) or means ± the standard deviation (SD). Statistical significances for comparisons among multiple groups (> 3) and between groups were determined using analysis of variance (ANOVA) and a Student's paired *t*-test, respectively, in Prism 7.0.

3. Results

3.1. Jmjd3, Col1 and Col3 expression is upregulated in mice with cystitis

As previously reported, cystitis can result in bladder fibrosis, therefore, the Col1 and Col3 expression pattern in bladder of CYP induced cystitis model mice was detected using immunofluorescence. To further investigate the epigenetic alteration in bladder with cystitis, Jmjd3 expression pattern was also detected. As shown in Fig. 1, these three proteins presented higher expression in the cystitis group than those in the control group, suggesting the positive association between the cystitis and Jmjd3, Col1 and Col3 expression, and the dysregulation of Jmjd3, Col1 and Col3 may be associated with the progression of cystitis.

3.2. LPS upregulates JMJD3 expression of hBSMCs

To further investigate the underlying mechanism involved in the dysregulation of JMJD3 expression in cystitis, hBSMCs were incubated with 10 and 50 μg/mL of LPS, a classical reagent for induction of inflammation, for 24 h. No significant difference of JMJD3 expression was observed between groups of 10 or 50 μg/mL of LPS (supplementary Fig. 1). However, the mRNA and protein levels of JMJD3 in the LPS treated group were significant higher than that in the control group (Fig. 2). Thus, we concluded that LPS can promote the expression of JMJD3 in hBSMCs.

3.3. LPS promotes hBSMC proliferation by regulating JMJD3 activity

To investigate the role of JMJD3 in cell cycle, we firstly evaluated the cell viability of hBSMCs using CCK-8 assay. The data showed that LPS significantly increased the cell viability, and the inhibition of JMJD3 markedly decrease the cell viability (Fig. 3A). Furthermore, the cell cycle of hBSMCs were detected using flow cytometry, and the results indicated that LPS significantly increased and GSK-J4 decreased the proliferative index (Fig. 3B and C). Therefore, as an inhibitor of JMJD3, GSK-J4 showed remarkable effect on the cell viability and cell cycle. Furthermore, the effect of GSK-J4 on hBSMC proliferation was explored using EdU assay, and the results showed that the cell proliferation induced by LPS can be inhibited by GSK-J4 (Fig. 4A and B). To uncover its underlying mechanism, the mRNA and protein levels of Cyclin D1 (CCND1), a key factor for regulating cell cycle were detected. As shown in Fig. 5, the mRNA and protein levels of CCND1 was significantly decreased in the LPS + GSK-J4 group when compared to the LPS group. These findings suggested that JMJD3 influenced cell proliferation by regulating CCND1 expression.

3.4. LPS modulates JMJD3 expression by regulating NFκB activity

To uncover the upstream regulator of JMJD3, the activity of NFκB, a canonical effector of LPS, was tested. Changes to JMJD3 mRNA and protein expression were further detected using a NFκB inhibitor, JSH. As illustrated in Fig. 6, JMJD3 mRNA and protein expression was significantly decreased in hBSMCs treated with LPS + JSH when compared to cells treated with LPS. Moreover, the level of NFκB phosphorylation was also critically repressed in the LPS + JSH treated

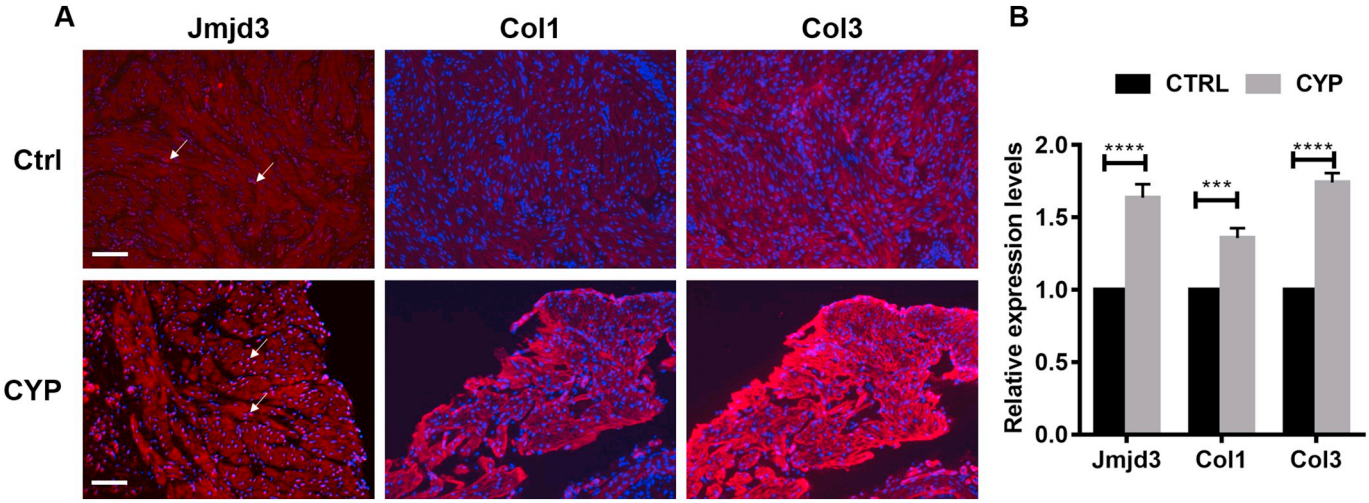

Fig. 1. Jmjd3, Col1 and Col3 expression is significantly upregulated in mice with cystitis induced by CYP. A. The moue bladders were harvested 24 h after intraperitoneal injection of CYP, Jmjd3, Col1 and Col3 expression was analyzed using immunofluorescence. The arrows indicate the positive staining of Jmjd3. B. The relative expression of Jmjd3, Col1 and Col3 was obtained by measuring the dentities of fluorescence. Ctrl, control (hereafter); CYP, cyclophosphamide.

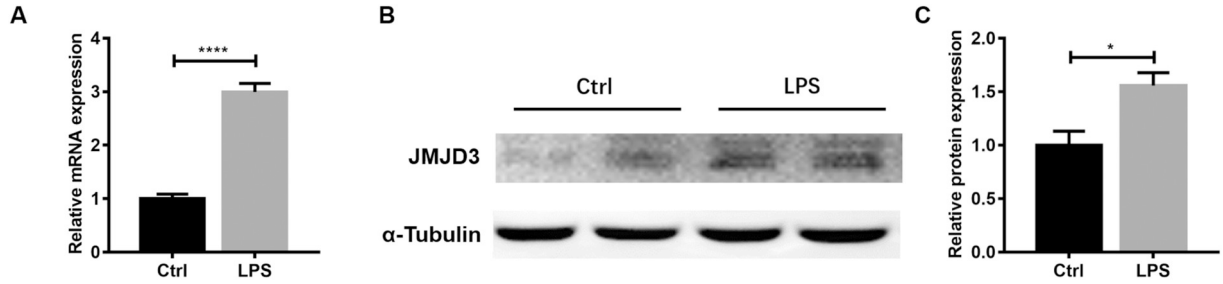

Fig. 2. LPS promotes JMJD3 expression of hBMSCs. The mRNA (A) and protein (B—C) levels of JMJD3 significantly increased in the LPS treated group when compared to the control group. * $P < 0.05$, **** $P < 0.0001$.

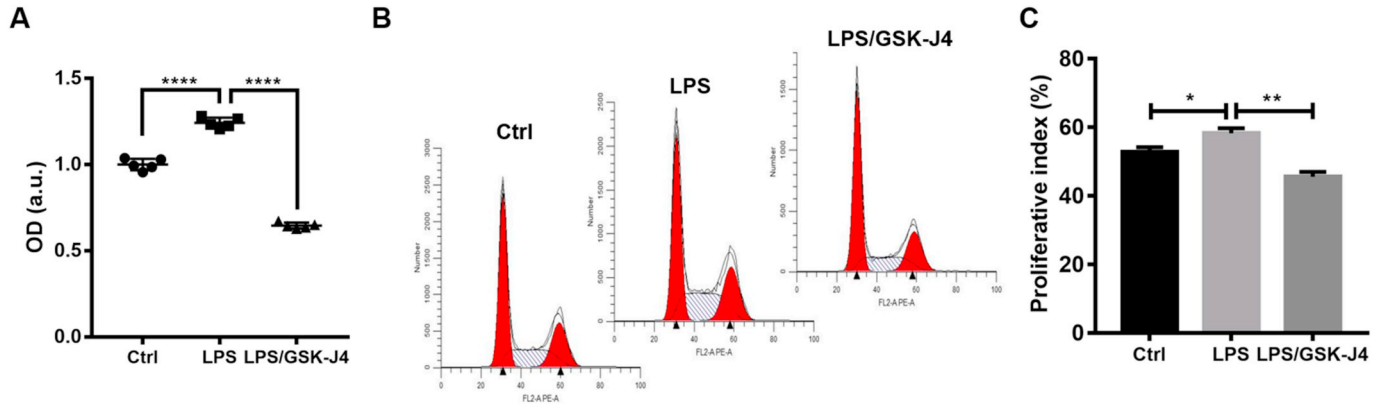

Fig. 3. LPS induces hBSMC proliferation by affecting JMJD3 activity. A. hBSMC cell viability was assessed by CCK-8 assay. B—C. Cell proliferation was detected using flow cytometry, and the proliferative index was calculated as follows: proliferative index (%) = $(S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100$. * P < 0.05, ** P < 0.01, **** P < 0.0001.

group. The results indicated that LPS regulated JMJD3 expression and activity by influencing NFκB activity.

3.5. NFκB-JMJD3 signaling regulates LPS-induced hBSMC proliferation

To further investigate the effect NFκB-JMJD3 signaling on LPS-induced hBSMC proliferation, the cells were treated with LPS and LPS + JSH. Data of CCK-8 assay showed that NFκB inhibition significantly reduced cell proliferation (Fig. 7A), and the CCND1 expression was also markedly decreased by JSH treatment (Fig. 7B-D), suggesting LPS influenced cell proliferation by regulating NFκB activity.

3.6. LPS-induced hBSMC collagen deposition by regulating NFκB-JMJD3 signaling

Besides the cell proliferation, collagen deposition is another characteristic of cystitis induced fibrosis. To investigate the relationship between NFκB-JMJD3 signaling and collagen accumulation, the expression of two main collagen components, COL1 and COL3, were detected after hBSMCs were treated with inhibitors of NFκB or JMJD3. As shown in Fig. 8, the mRNA and protein expression of COL1 and COL3 was significantly decreased in the LPS + JSH23 or LPS + GSK-J4 groups when compared to the LPS group, suggesting that LPS promotes collagen deposition by regulating NFκB-JMJD3 signaling. The

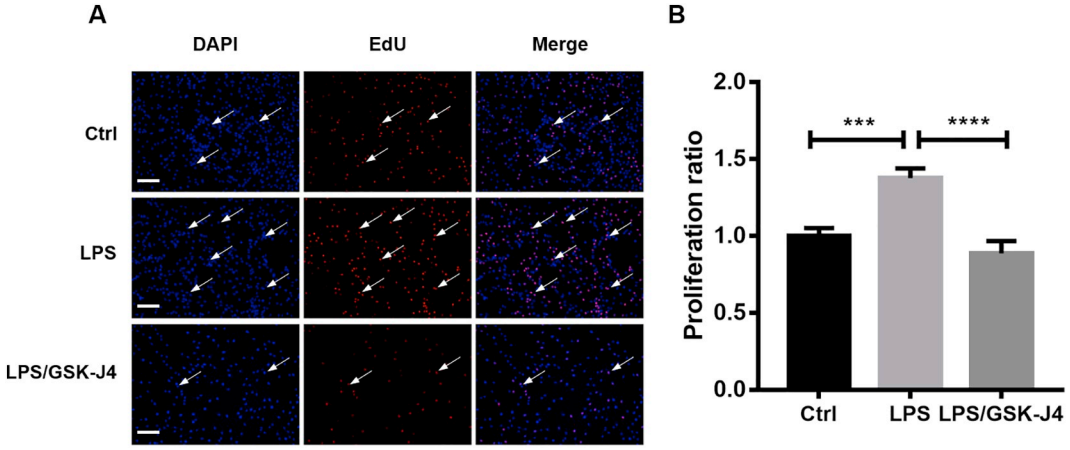

Fig. 4. LPS promotes hBSMC proliferation by regulating JMJD3 activity. A-B. EdU assay was used to evaluate the role of JMJD3 in regulating LPS induced hBSMC proliferation. As an inhibitor of JMJD3, GSK-J4 treatment significantly decreased the number of EdU positive cells. The white arrows were used to indicate co-localization of EdU and DAPI fluorescence. *** $P < 0.001$, **** P < 0.0001.

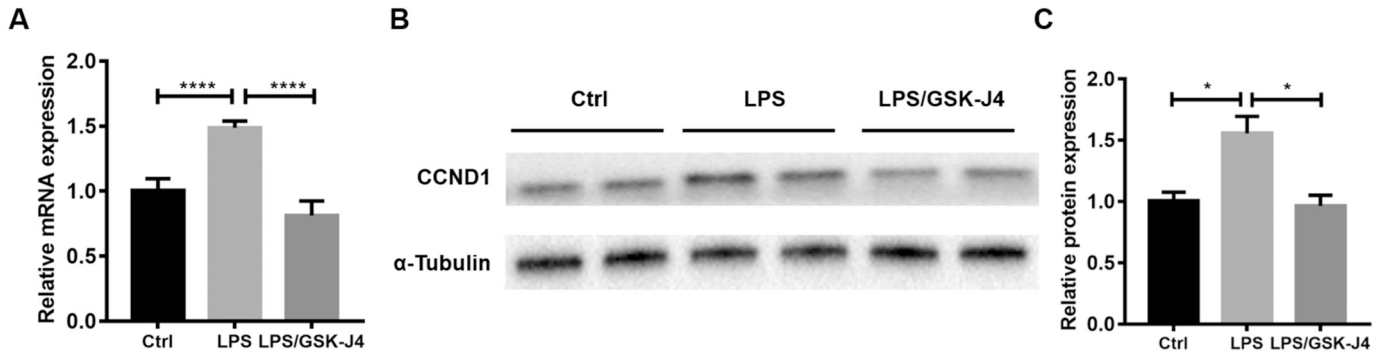

Fig. 5. JMJD3 regulates CCND1 mRNA and protein expression. A. LPS and GSK-J4 significantly increased and decreased the mRNA expression of CCND1, respectively. B–C. the protein level of CCND1 were determined by western blotting. * $P < 0.05$, **** $P < 0.0001$.

expression of inflammatory mediators, including IFNγ, IL11, IL17, IL1β, IL6, IL8 and TNFα, was also detected, and the results showed that the expression of IL11 and IL8 were significantly upregulated, and it is consistent with JMJD3 expression (Supplementary Fig. 2). In addition, the expression of tissue inhibitors of metalloprotease 1(TIMP1) and TIMP2 was significantly upregulated after 24 h of LPS stimulation (Supplementary Fig. 3). These results indicated that NFκB-JMJD3 signaling play an important role in cystitis caused fibrosis by regulating the expression of collagens, inflammatory mediators and TIMPs, and this could provide potential targets for effective treatment of cystitis clinically.

4. Discussion

JMJD3 is an important epigenetic factor which functions as a histone demethylase, and it can reduce the trimethyl level rather than di- or monomethyl levels of H3K27 [21]. Previous studies showed that JMJD3 could be associated with various carcinomas, including metastatic prostate [22] and breast cancer [23]. Other studies showed that JMJD3 was involved in different types of cellular processes [21]. Our study firstly verified that the Jmjd3, Col1 and Col3 expression was upregulated in bladder of CYP induced cystitis mouse model, indicating that Col1 and Col3 dysregulation could be related to Jmjd3. To further unravel the underlying mechanism, we employed LPS to simulate an inflammatory environment *in vitro*. Interestingly, Haldar et al. reported that the CYP-induced cystitis is associated with the activation of apoptotic pathways in the detrusor cells followed by proliferation [24], and the differential effects of CYP and LPS on cell proliferation presumably caused by their different receptors on cell membrane and the downstream pathways.

Importantly, we demonstrated that the expression of JMJD3 and phospho-NFκB increased in LPS-induced hBSMCs. The upstream NFκB signaling regulated JMJD3 expression and activity, and the findings of our study implied that NFκB-JMJD3 signaling play critical roles in

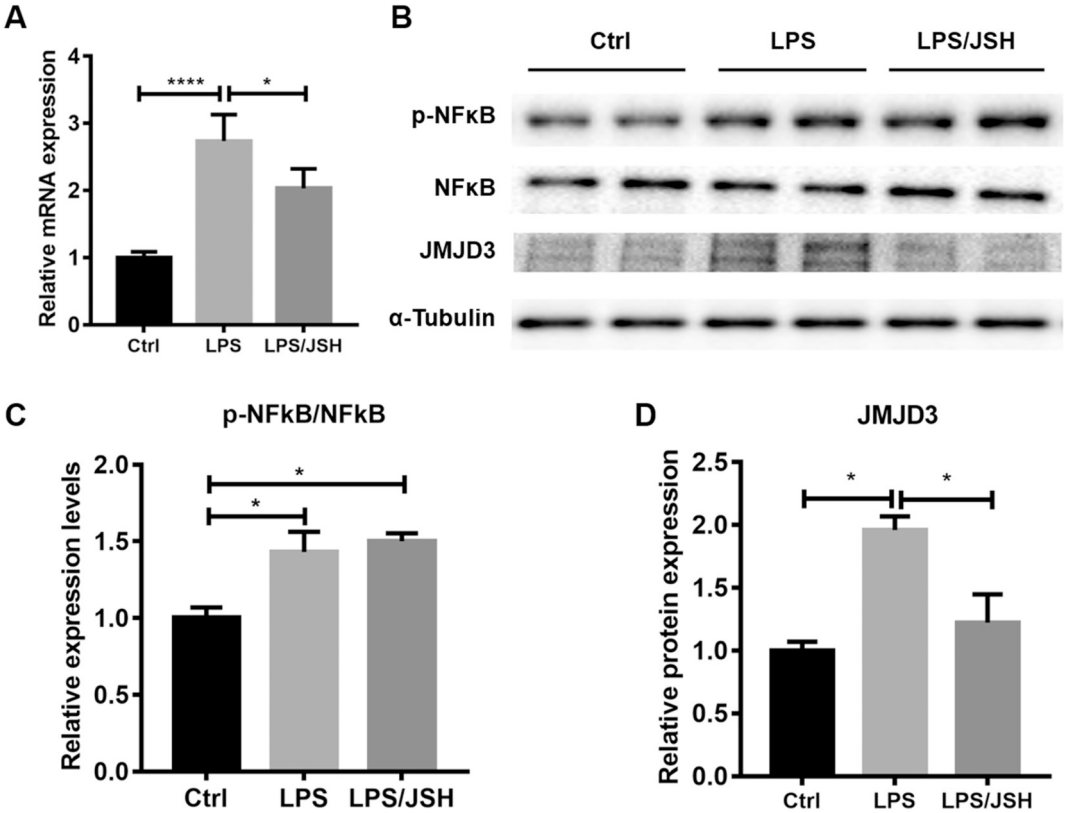

Fig. 6. LPS regulates JMJD3 by affecting NFκB activity in hBSMCs. A. JSH23 significantly decreased LPS induced upregulation of JMJD3 mRNA level. B-D. JSH23 significantly decreased JMJD3 protein expression by inhibiting NFκB activity. * $P < 0.05$, **** $P < 0.0001$.

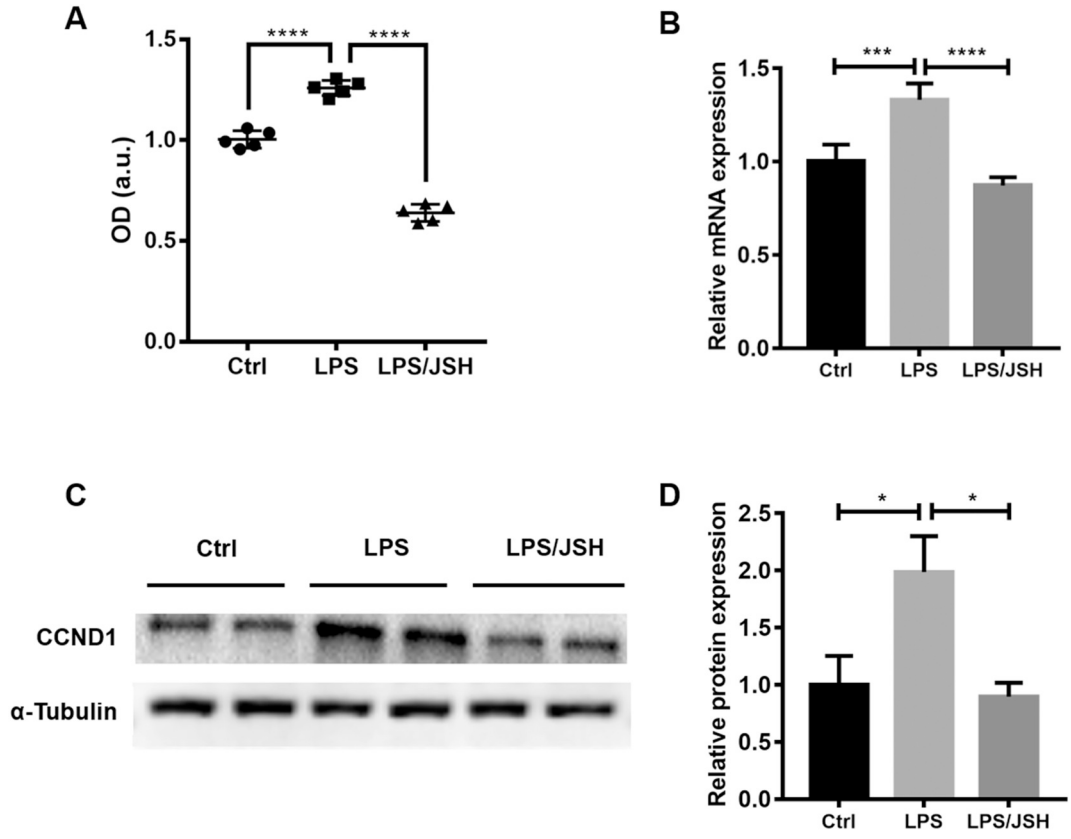

Fig. 7. NFκB-JMJD3 signaling mediates LPS induced hBSMC proliferation. A. JSH23 treatment significantly repressed LPS induced cell proliferation. B. the mRNA expression of CCND1 decreased markedly in hBSMCs that were pretreated with JSH23. C-D. the protein level of CCND1 is consistent with its mRNA level. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$.

regulating cell proliferation and collagen deposition of hBSMCs simultaneously.

To confirm the role of JMJD3 in regulating LPS-induced hBSMC proliferation, CCK8, flow cytometry and EdU assays were employed in this study, as well as CCND1 expression was investigated. A previous study has proved that CCND1 is critically associated with the cell cycle and proliferation [25]. The higher expression of CCND1 implied more cells at S and G_2 - M phases [26]. Additionally, as an upstream regulator of JMJD3, NFκB also regulated the proliferation of LPS-induced hBSMCs. These findings confirmed the role of NFκB-JMJD3-CCND1 signaling in the proliferation of LPS-induced hBSMCs.

Our study further investigated the effect of JMJD3 on collagen deposition of hBSMCs, COL1 and COL3. The results showed that JMJD3 could regulate the expression of COL1 and COL3 in LPS-induced hBSMCs. This is the first time to directly prove the correlation of JMJD3 and collagen expression. Meanwhile, NFκB regulated the expression of collagen *via* JMJD3, however, the regulatory effect of NFkB seems to be weaker than JMJD3, suggesting that NFκB regulated Col1 and Col3 expression through JMJD3. In particular, JMJD3 may also regulate collagen expression by affecting the expression of cytokines, such as IL8, IL11 and so on [27]. It is worthy to evaluate the roles of these cytokines in regulating collagen expression further. Our data demonstrated that increased IL-11 and IL-8 expression was correlated with JMJD3 upregulation (Supplemental Fig. 2), indicating that IL-11 and IL-8 may act as potential regulators of collagen synthesis/deposition.

Interestingly, as a canonical pathway, the downstream inflammatory pathways of NFkB could be also involved in regulating cell cycle and ECM accumulation, hence, further study needs to be conducted to reveal its underlying mechanism. Based on previous studies and our data, NFkB affected downstream gene expression, *i.e. Ccnd1*, *Col1* and *Col3*, by regulating the expression of JMJD3, a critical epigenetic factor for the modification of histone H3. This finding suggested that NFkB plays a role in regulating gene expression by influencing epigenetic factors in cystitis, and it is quite different from the canonical NFkB-inflammation signaling.

Clinically, cystitis is common in patients with recurrent urinary tract infection. Cystitis causes the proliferation of bladder smooth muscle cells [28] and deposition of collagen [29], which would thicken the bladder wall, increase the mass of the bladder [30], impair bladder function, and finally result in symptoms of urinary frequency and urgency. Hence, as a crucial factor for regulating cell proliferation and collagen deposition of hBSMCs, NFκB-JMJD3 signaling provides a novel therapeutic target for cystitis.

Taken together, LPS induced hBSMCs proliferation and collagen deposition through regulating NFκB-JMJD3 signaling, accordingly, the bladder detrusor hyperplasia and low compliance caused by cystitis could be improved by inhibiting the activity of NFκB-JMJD3 signaling. Our study paves an avenue for effective treatment of patients with cystitis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.05.008.

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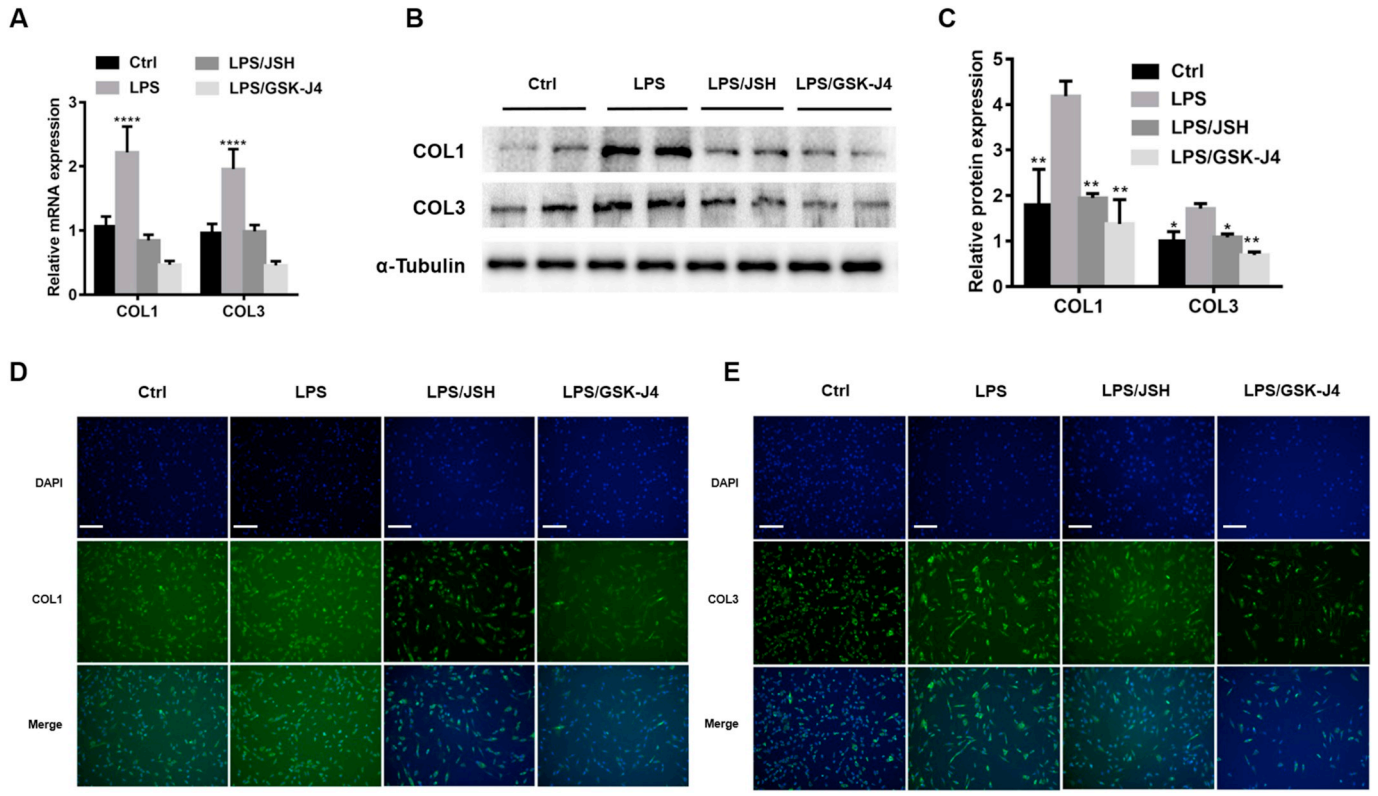

Fig. 8. NFκB-JMJD3 signaling regulates COL1 and COL3 expression in LPS-induced hBSMCs. A. The mRNA expression of *COL1* and *COL3* were increased in hBSMCs treated with LPS and decreased in hBSMCs treated with LPS + JSH23 or LPS + GSK-J4. B—C. COL1 and COL3 protein level were determined by western blotting and the grey intensity of the bands were shown. D-E. The fluorescence images of COL1 and COL3 in hBSMCs that were treated with control, LPS, LPS + JSH23 and LPS + GSK-J4. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

Author contributions

Junyu Lai and Manqing Ge and Sikui Shen performed experiments; Junyu Lai, Manqing Ge, Sikui Shen, Tao Jin collected and analyzed data; Jianzhong Ai conceived this study, Hang Xu, Xiaonan Zheng and Shi Qiu performed expperiments for supplementary figures, Jianzhong Ai, Lu Yang, Hong Li and Kunjie Wang drafted and polished the manuscript. Jianzhong Ai, Dehong Cao and Qiang Wei revised the manuscript. All authors have read and approved the final manuscript.

Transparency Document

The Transparency document associated with this article can be found, in online version.

Declaration of Competing Interest

None.

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