

Urine-Derived Stem Cells Attenuate Post-Injury Urethral Fibrosis in Association With Reduced Smad3/JunD Signaling Activation, Inflammation, and Extracellular Matrix Remodeling

Liangliang Huang^{1,*}, Yuhui Yao¹, Jingjing Yang¹, Junhong Deng¹, Jianming Liu¹, Zhoua Cai¹, Jianming Lu¹, Xiaobin Wang¹, Yanni Wei¹, Lizhen Tian¹, Haomin Su¹

¹Department of Andrology, Guangzhou First People's Hospital, School of Medicine, South China University of Technology, 510180 Guangzhou, Guangdong, China

*Correspondence: 13824460178@163.com; eyllh@scut.edu.cn (Liangliang Huang)

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Background: Urethral stricture is characterized by persistent inflammation and excessive extracellular matrix deposition, which contribute to progressive urethral fibrosis and narrowing. Urine-derived stem cells (USCs) have shown anti-inflammatory and anti-fibrotic potential, but their role in post-injury urethral fibrosis remains unclear. This study aimed to investigate whether early administration of USCs attenuates post-injury urethral fibrosis and subsequent stricture formation, and whether these effects are associated with changes in Smad3/JunD signaling activation, inflammation, and extracellular matrix remodeling.

Methods: The adipogenic and osteogenic differentiation potential of USCs was evaluated by lineage-specific staining, including Oil Red O staining for lipid droplet formation and Alizarin Red staining for mineralized matrix deposition. A rat model of urethral stricture was established, and the animals were divided into five groups: Sham, US, USCs, US + USCs, and US + USCs + TGF- β 1. Changes in tissue structure and collagen accumulation were determined using hematoxylin and eosin (H&E) staining and Masson's trichrome staining, and urethral diameter was measured. Immunohistochemistry was performed to detect alpha-smooth muscle actin (α -SMA) expression. Urethral scar fibroblasts were isolated and indirectly co-cultured with USCs. Cell proliferation and fibrosis-related protein expression were assessed using the 5-ethynyl-2'-deoxyuridine (EdU) assay and immunofluorescence staining, respectively. Expression levels of key mediators associated with Smad signaling and fibrotic remodeling, including phosphorylated Smad3 (p-Smad3), Smad family member 3 (Smad3), JunD proto-oncogene (JunD), collagen type I (COL-1), collagen type III (COL-3), fibronectin (FN), thrombospondin-1 (TSP-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1), were determined using Western blot analysis. The levels of inflammatory cytokines, namely tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and interleukin-10 (IL-10), were assessed by enzyme-linked immunosorbent assay (ELISA).

Results: USCs exhibited multilineage differentiation potential. At day 14 after urethral injury, rats showed a significant reduction in urethral diameter accompanied by epithelial hyperplasia, inflammatory cell infiltration, and increased collagen deposition. Early administration of USCs significantly increased urethral diameter, reduced collagen accumulation, and decreased α -SMA expression ($p < 0.001$). *In vitro* experiments demonstrated that USCs inhibited the proliferation of urethral scar fibroblasts and reduced the expression of α -SMA, COL-1, and TSP-1 ($p < 0.001$). USC treatment was accompanied by reduced p-Smad3/Smad3 and JunD expression in urethral tissues and fibroblasts. TGF- β 1 treatment partly attenuated the anti-inflammatory and anti-fibrotic effects after USC treatment ($p < 0.001$).

Conclusion: Early administration of USCs attenuates post-injury urethral tissue damage and fibrosis, and these effects may be partly associated with reduced Smad3/JunD signaling activation, decreased inflammation, and lower extracellular matrix deposition. These findings provide preclinical evidence for the early intervention of post-injury urethral fibrosis.

Keywords: USCs; post-injury urethral fibrosis; Smad3/JunD signaling pathway; inflammation; extracellular matrix remodeling

Introduction

Urethral stricture is a common condition among urological patients, characterized by pathological narrowing of the urethral lumen, leading to impaired urinary function. Urethral stricture is mainly caused by urethral injury, infection and inflammation, or iatrogenic procedures [1,2]. The core pathological features of urethral stricture include persistent inflammatory responses and excessive deposition of extracellular matrix (ECM), which ultimately result in urethral tissue fibrosis and structural remodeling [3,4]. Although surgical interventions such as urethroplasty or endoscopic treatment are currently the main clinical approaches, the postoperative recurrence rate remains relatively high due to persistence of inflammation and inadequate control of fibrotic remodeling [5,6]. Therefore, exploring novel therapeutic strategies targeting inflammatory responses and fibrotic processes is of great clinical significance.

During the progression of urethral stricture, abnormal activation and proliferation of fibroblasts represent key events that promote the development of fibrosis [7]. Following activation, fibroblasts acquire a profibrotic myofibroblast phenotype and become major producers of extracellular matrix proteins. The resulting accumulation of collagen, fibronectin, and other matrix components contributes to abnormal tissue reconstruction and fibrosis progression [8]. As a key signaling network involved in tissue remodeling and fibrosis, transforming growth factor beta (TGF- β) exerts its regulatory functions largely through Smad-dependent intracellular signal transduction, which governs the expression of numerous profibrotic genes [9,10]. Smad3, as a key signal transduction molecule, promotes fibroblast activation and enhances extracellular matrix synthesis [11]. Meanwhile, the transcription factor JunD, a member of the AP-1 family, can cooperate with the Smad signaling pathway to regulate the expression of inflammation- and fibrosis-related genes. Therefore, abnormal activation of the Smad3/JunD signaling axis is considered one of the important molecular mechanisms underlying tissue fibrosis and inflammatory remodeling [12].

In recent years, stem cell-based therapies have shown considerable potential in tissue repair and anti-fibrotic treatment [13]. Urine-derived stem cells (USCs) are a type of mesenchymal stem cells isolated from urine and possess several advantages, including noninvasive collection, abundant sources, strong proliferative capacity, and multilineage differentiation potential [14]. USCs have demonstrated substantial differentiation versatility, with the ability to develop into a variety of specialized cellular lineages, including adipocytes and osteoblasts, and exhibit significant anti-inflammatory and anti-fibrotic effects [15]. In addition, USCs can regulate inflammatory responses and extracellular matrix remodeling through the secretion of multiple paracrine factors, demonstrating promising therapeutic

potential in various models of tissue injury and fibrotic diseases [16].

However, the role of USCs in urethral stricture and the underlying molecular mechanisms remain unclear. In particular, whether USCs exert anti-inflammatory and anti-fibrotic effects by regulating the Smad3/JunD signaling pathway requires further investigation.

Therefore, in this study, a rat urethral injury model was established, and USCs were administered immediately after injury to evaluate their early intervention effects on subsequent tissue damage, inflammation, fibroblast proliferation, extracellular matrix remodeling, and urethral fibrosis. Urethral scar fibroblasts were also isolated and cultured for *in vitro* experiments. Furthermore, we investigated whether these effects were associated with reduced activation of Smad3/JunD signaling.

Materials and Methods

Cell Culture

Commercially available urine-derived stem cells (USCs; PRI-H-00207), which were of human origin, were obtained from Zhongqiao Xinzhou Biotechnology Co., Ltd. (Shanghai, China). The cells were expanded in complete DMEM supplemented with fetal bovine serum and antibiotics (PCM-H-129, Zhongqiao Xinzhou). Cultures were maintained in a humidified incubator at 37 °C with 5% CO₂, and the medium was replaced every 72 h. When the cells reached near confluence, they were detached using trypsin-EDTA (T1320, Solarbio, Beijing, China) and passaged at a 1:3 ratio. Third-passage cells were used for subsequent experiments. The purchased USCs had undergone STR authentication and tested negative for mycoplasma. Commercially available cells were selected to provide a standardized cell source and reduce variability associated with primary cell isolation and culture.

Osteogenic Differentiation of USCs

To assess osteogenic differentiation, third-passage cells were cultured in osteogenesis-promoting medium (MM301-01, Transgen, Beijing, China) after reaching the desired confluence. The differentiation medium was replaced periodically during the 21-day induction period. At the end of the induction period, the cells were fixed and subjected to Alizarin Red staining (G8550, Solarbio, Beijing, China) to visualize calcium-rich extracellular deposits. Mineralization was subsequently examined and documented using inverted microscopy (BX53, Olympus, Tokyo, Japan).

Adipogenic Differentiation of USCs

P3 cells were thawed and seeded following the same procedures described above. Following replacement of the routine medium with adipogenic differentiation medium (MM201-01, Transgen, Beijing, China), cells underwent

induction for 14 days. Lipid-rich cells were subsequently identified by Oil Red O staining (G1262, Solarbio, Beijing, China), and differentiation outcomes were recorded through microscopic imaging (BX53, Olympus, Tokyo, Japan).

Experimental Animals and Grouping

Fifty male Sprague–Dawley (SD) rats (8–10 weeks old, 250–300 g) were purchased from Jilin Genet-Med Biotechnology Co., Ltd. (A12100_1; Changchun, China). The animals were housed at a room temperature of 25 ± 2 °C under a 12 h light/dark cycle with free access to food and water. After one week of acclimatization, the rats were randomly divided into five groups ($n = 10$ per group). This study was approved by the Experimental Animal Ethics Committee of South China University of Technology (Approval No. 20230905). All animal experiments were conducted and reported in accordance with the ARRIVE Guidelines 2.0.

Sham group: Rats underwent the same anesthesia and surgical exposure procedures as the model group, but no urethral injury was induced.

US group: Rats underwent surgically induced urethral injury without USC administration. The injury subsequently induced inflammation, fibrotic remodeling, and urethral narrowing during the 14-day observation period.

USCs group: Cultured USCs were prepared as a cell suspension (1×10^6 cells/100 μ L PBS, J75889, Chengdu Huachun Technology Co., Ltd., Chengdu, China) and administered to normal rats via intraperitoneal injection as a single dose at the corresponding time point.

US + USCs group: Rats were first subjected to urethral stricture modeling and then treated with USCs. The cultured USCs were prepared as a cell suspension (1×10^6 cells/100 μ L PBS) and injected intraperitoneally into the rats as a single dose immediately after model establishment on day 0.

US + USCs + TGF- β 1 group: Rats were first subjected to urethral stricture modeling and subsequently treated with USCs along with TGF- β 1 (91701ES, Yeasen Biotechnology Co., Ltd., Shanghai, China), which was used to stimulate Smad-related signaling activity. USCs were administered intraperitoneally as a cell suspension (1×10^6 cells/100 μ L PBS) as a single dose immediately after model establishment on day 0, followed by intraperitoneal injection of TGF- β 1 (10 ng/100 μ L PBS) as a single dose at the same time point.

The day of urethral injury was designated as day 0. All interventions were administered once immediately after injury on day 0, and no repeated administration was performed during the 14-day observation period. No immunosuppressive agents were administered during the experiment. Immune responses specifically directed against the administered cells were not assessed. In addition, the cells were not labeled or tracked *in vivo*. Therefore, their

persistence and viability during the 14-day observation period could not be determined. On postoperative day 14, the rats were humanely euthanized by intraperitoneal administration of an overdose of sodium pentobarbital (50 mg/mL, 120 mg/kg) (P-010, Merck KGaA, Darmstadt, Germany), as approved in the institutional animal protocol. Cervical dislocation was subsequently performed as a secondary physical method before tissue collection. Death was then confirmed by the absence of spontaneous respiration, heartbeat, corneal reflex, and pedal withdrawal reflex. Urethral injury tissues were collected for subsequent analysis.

Establishment of a Rat Model of Post-injury Urethral Fibrosis and Stricture Formation

Prior to surgery, rats were administered sodium pentobarbital (40–50 mg/kg, i.p.) to achieve anesthesia and were subsequently secured in a supine posture. The rat urethral stricture model was established with reference to previously reported electrocoagulation-induced rat urethral stricture models [17] and trauma-induced urethral injury models [18], with minor modifications.

The perineal area was shaved and disinfected routinely. A midline incision approximately 1–1.5 cm in length was made in the perineum, and the surrounding tissues were carefully dissected to fully expose the penile urethra. A micro-electrocautery device (Bovie A2350 Surgi-Center PRO, Aspen Surgical Products, Inc., Caledonia, MI, USA) was used to induce urethral mucosal injury. The device was operated in pinpoint coagulation mode, with the maximum output power set at 120 W and an output frequency of $490 \text{ kHz} \pm 4.9 \text{ kHz}$ according to the device specifications. The output current and probe-tip temperature were not displayed as fixed operating parameters and were therefore not separately recorded. Three points around the urethra were selected for electrocauterization, with each point cauterized for approximately 3 s to create controlled mucosal injury. This injury could induce local inflammatory responses and fibroblast activation, promoting collagen deposition and ultimately leading to urethral fibrosis and stricture. After model establishment, the tissues and skin were sutured layer by layer. Once the rats recovered from anesthesia, they were returned to their cages and provided with routine postoperative care. Two weeks after surgery, post-injury urethral fibrosis and stricture formation were evaluated by histological examination and measurement of the urethral lumen diameter. The endpoint criteria included obvious urethral lumen narrowing accompanied by epithelial hyperplasia, inflammatory cell infiltration, and collagen deposition.

Hematoxylin–Eosin (H&E) Staining

Following euthanasia, urethral specimens from the stricture site were harvested and fixed in 4% paraformaldehyde (YX-DS00705, Zhejiang Yuxiang Biotechnology Co., Ltd., Hangzhou, China) for 24 h. Samples were sub-

sequently processed through graded ethanol dehydration, paraffin embedding, and sectioning into 4–5 μm -thick slices for histological analysis. H&E staining (Hematoxylin and Eosin; ab245880, Abcam, Cambridge, UK) was performed according to standard procedures.

The stained tissue sections were observed and photographed under a light microscope (BX53, Olympus Corporation, Tokyo, Japan). ImageJ software (version 1.54, National Institutes of Health, Bethesda, MD, USA) was used to measure the urethral cross-sections, and the maximum transverse diameter of the urethral lumen was recorded as the urethral diameter. For each specimen, three random visual fields were measured, and the mean value was used as the final result. In addition, the degree of urethral tissue injury was semi-quantitatively evaluated based on epithelial structural integrity, inflammatory cell infiltration, edema, and fibrosis using a histopathological injury score. The histopathological injury scoring system was adapted from previous histopathological evaluations of rat urethral injury and urethral stricture models [17,19]. Four parameters were assessed, including epithelial structural damage, inflammatory cell infiltration, tissue edema, and fibrotic remodeling. Each parameter was scored on a 0–3 scale: 0, absent or normal; 1, mild; 2, moderate; and 3, severe. The total injury score ranged from 0 to 12, with higher scores indicating more severe urethral tissue injury. All sections were coded and evaluated in a random order by two qualified pathologists who were blinded to the experimental grouping. When discrepancies occurred, the final score was determined by joint review and consensus.

Masson Staining

To assess fibrotic remodeling, urethral tissue sections prepared from paraffin blocks were subjected to Masson's trichrome staining (C0189, Beyotime Biotechnology, Shanghai, China) following routine histological processing. The staining procedure allowed clear visualization of collagen fibers, muscle structures, and nuclei. Histopathological evaluation was performed under light microscopy, with collagen accumulation serving as a marker of fibrosis severity. Collagen volume fraction (CVF) was quantified using ImageJ software (version 1.54). Three randomly selected non-overlapping fields were analyzed for each section. The periurethral tissue was selected as the analysis area, excluding the urethral lumen and obvious artifacts. A fixed HSB color threshold (Hue, 135–195; Saturation, 25–255; Brightness, 20–255) was applied to identify blue-stained collagen. CVF was calculated as: $\text{CVF (\%)} = \frac{\text{collagen-positive area}}{\text{total tissue area}} \times 100\%$.

Immunohistochemistry

Paraffin-embedded urethral tissues were sectioned at 4–5 μm thickness. The sections were deparaffinized in xylene and rehydrated through a graded ethanol series. Antigen retrieval was performed in 10 mM citrate buffer (pH

6.0) at 95–100 °C for 15 min. After cooling to room temperature, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min. The sections were washed with phosphate-buffered saline (PBS) and blocked with 5% bovine serum albumin for 30 min at room temperature. The sections were incubated with an anti- α -SMA primary antibody (KBR-hlk1311, Shanghai Keorui Biotechnology Co., Ltd., Shanghai, China; 1:200) overnight at 4 °C. After washing with PBS, the sections were incubated with an enzyme-linked secondary antibody (EL258, Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China; 1:500) for 30 min at room temperature. Chromogenic visualization was performed using DAB (9405008, Fushiman Technology, Beijing, China), followed by hematoxylin counterstaining (YB152C, Shanghai Yubo Biotechnology Co., Ltd., Shanghai, China). Brown-yellow cytoplasmic staining was considered positive. The relative positive staining area was quantified using ImageJ software.

Isolation and Culture of Rat Urethral Scar-Derived Cells

Urethral scar tissues were collected from rats with urethral stricture under sterile conditions and washed three times with phosphate-buffered saline containing antibiotics. The tissues were cut into approximately 1 mm³ fragments and digested with 0.2% collagenase (S10053, Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China) at 37 °C for 60 min with gentle agitation. Digestion was terminated by adding an equal volume of complete culture medium containing fetal bovine serum. The resulting cell suspension was filtered through a 70 μm cell strainer and centrifuged at 300 $\times g$ for 5 min. The cell pellet was resuspended in complete growth medium and cultured at 37 °C in a humidified incubator containing 5% CO₂. The medium was changed after 24 h to remove non-adherent cells and subsequently replaced every 2–3 days. Adherent cells were passaged when they reached approximately 80% confluence. Cell morphology was observed using an inverted phase-contrast microscope. Third-passage cells showing stable adherent growth and a relatively uniform spindle-shaped morphology were used for subsequent experiments. Mycoplasma testing was performed before downstream experiments, and only mycoplasma-negative cells were used.

Cell Co-culture

A Transwell chamber system was used to establish an indirect co-culture model of urethral scar fibroblasts and urine-derived stem cells (USCs). An initial population of urethral scar-derived fibroblasts (1×10^5 cells/well) was established in the lower section of 6-well culture plates and maintained in DMEM supplemented with 10% fetal bovine serum under standard culture conditions. When the cells reached 70%–80% confluence, the co-culture experiment was initiated.

For indirect co-culture, USCs were placed in the upper section of a Transwell culture system equipped with a 0.4 μm semipermeable membrane (Corning, Cat. No. 3450, Corning Inc., Corning, NY, USA), with approximately 100,000 cells added to each insert. The Transwell inserts containing USCs were then placed into the 6-well plates seeded with urethral scar fibroblasts, allowing the two cell types to interact through soluble factors in the culture medium without direct cell-to-cell contact.

The co-culture system was maintained in an incubator at 37 °C with 5% CO₂ for 48 h. After co-culture, urethral scar fibroblasts in the lower chamber were collected for subsequent experiments.

Cell Immunofluorescence Staining

Cultured urethral scar fibroblasts grown on coverslips were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature. Because cultured cells rather than paraffin-embedded tissue sections were used, no deparaffinization step was required. The cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin for 30 min at room temperature. The cells were incubated with an anti- α -SMA primary antibody (KBR-hlk1311, Shanghai Keborui Biotechnology Co., Ltd., Shanghai, China; 1:200) or an anti-JunD primary antibody (AF7744, Jiangsu Qinke Biological Research Center Co., Ltd., Changzhou, China; 1:200) overnight at 4 °C. After washing with PBS, the cells were incubated with a fluorescent secondary antibody (ab150077, Abcam, Cambridge, UK; 1:500) for 1 h at room temperature in the dark. Cell nuclei were stained with DAPI (D609734, Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) for 5 min. The cells were mounted using an antifade mounting medium (PR01194, Shanghai Chuangsai Technology Co., Ltd., Shanghai, China) and examined under a fluorescence microscope.

EdU Assay

The proliferative capacity of urethral scar fibroblasts was evaluated by monitoring DNA replication using an EdU assay kit (ab222421, Abcam, Cambridge, UK). Cells cultured in 96-well plates were exposed to EdU at a final working concentration of 10 μM and incubated for 2 h at 37 °C, allowing its integration into newly synthesized DNA strands. After fluorescent labeling and nuclear staining, microscopic images were acquired, and the percentage of proliferating cells was determined by calculating the proportion of EdU-positive nuclei among the total number of DAPI-labeled nuclei.

Western Blotting

Total protein was extracted from urethral tissues and cultured fibroblasts using RIPA lysis buffer containing protease and phosphatase inhibitors. Protein concentrations

were determined using a bicinchoninic acid protein assay. Equal amounts of protein, 30 μg per lane, were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. The membranes were incubated overnight at 4 °C with primary antibodies against phospho-Smad3 (Ser423/425; 9520, Cell Signaling Technology, Inc., USA; rabbit monoclonal; 1:1000), total Smad3 (9513, Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1000), JunD (AF7744, Affinity Biosciences, USA; rabbit polyclonal; 1:1000), COL-1 (sc-59772, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; purchased from Shanghai Lianmai Bioengineering Co., Ltd., Shanghai, China; 1:1000), COL-3 (ab7778, Abcam plc, Cambridge, UK; rabbit polyclonal; 1:1000), FN (63779, Cell Signaling Technology, Inc., Danvers, MA, USA; 1:1000), TSP-1 (3283-100, BioVision, Inc., Milpitas, CA, USA; 1:1000), TIMP-1 (32669-1-AP, Proteintech Group, Inc., Rosemont, IL, USA; 1:1000), α -SMA (E-AB-34268, Elabscience Biotechnology Co., Ltd., Wuhan, China; 1:1000), and GAPDH (FI01101S, FitGene Biotechnology Co., Ltd., Guangzhou, China; mouse monoclonal; 1:5000). After washing with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (H+L) (AS014, ABclonal Technology Co., Ltd., Wuhan, China; 1:5000) or HRP-conjugated goat anti-mouse IgG (H+L) (AS003, ABclonal Technology Co., Ltd., Wuhan, China; 1:5000), as appropriate, for 1 h at room temperature.

ELISA

Approximately 100 mg of urethral tissue from each rat was weighed and homogenized on ice in 900 μL of ice-cold PBS at a tissue-to-buffer ratio of 1:9 (w/v). The homogenates were centrifuged at 12,000 $\times g$ for 10 min at 4 °C, and the supernatants were collected for subsequent analysis. Total protein concentrations were determined before cytokine measurement. TNF- α (ER1393, Fine Biotech Co., Ltd., Wuhan, China), IL-6 (ER0042, Fine Biotech Co., Ltd., Wuhan, China), and IL-10 (ER0033, Fine Biotech Co., Ltd., Wuhan, China) were measured using rat-specific ELISA kits according to the manufacturer's instructions. These kits are designed for rat samples and are suitable for measuring the corresponding cytokines in cell or tissue lysates. The supernatants were initially assayed without dilution. Samples with concentrations above the upper limit of the standard curve were serially diluted twofold using the sample diluent supplied with the corresponding kit and re-assayed. The final concentrations were corrected for the dilution factor and normalized to the total protein content of each sample.

Statistical Analysis

Data processing and statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). The normality of continuous data was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene’s test. The histopathological injury scores were normally distributed according to the Shapiro–Wilk test and showed homogeneity of variance according to Levene’s test. Data that met the assumptions of normality and homogeneity of variance are presented as mean $\pm$ SD. Comparisons between two groups were performed using Student’s *t*-test, whereas comparisons among multiple groups were performed using one-way analysis of variance followed by Tukey’s post hoc test. A two-sided *p* value < 0.05 was considered statistically significant.

Results

Results of Differentiation Assays of Urine-Derived Stem Cells (USCs)

Adipogenic and osteogenic differentiation assays were performed to evaluate the multilineage differentiation potential of USCs. After 14 days of adipogenic induction, intracellular lipid droplets appeared in the cells, presenting a granular or bead-like morphology. Oil Red O staining showed red lipid droplets, indicating that the cultured cells possessed the ability to differentiate into adipocytes (Fig. 1A,B). Quantitative analysis showed that the relative Oil Red O staining intensity was significantly increased after adipogenic induction compared with day 0 ($p < 0.001$). After 21 days of osteogenic induction, Alizarin Red staining revealed red calcified nodules, indicating that the cultured cells had the capacity to differentiate into osteoblasts (Fig. 1C,D). Quantitative analysis showed that the relative Alizarin Red staining intensity was significantly increased after osteogenic induction compared with day 0 ($p < 0.001$).

Early Administration of USCs Attenuates Urethral Narrowing, Tissue Injury, and Fibrotic Remodeling After Urethral Injury

Fourteen days after urethral injury, hematoxylin and eosin (H&E) staining was performed to evaluate post-injury tissue changes and urethral narrowing. As shown in Fig. 2A, the urethral diameter in the US group was significantly decreased compared with the Sham group ($p < 0.001$). All injured rats included in the final analysis met the predefined endpoint criteria for urethral fibrosis and narrowing. Compared with the Sham group, there was no significant difference in urethral diameter in normal rats treated with USCs ($p > 0.05$). However, compared with the US group, the urethral diameter in the US + USCs group was significantly increased ($p < 0.001$).

H&E staining further confirmed that, compared with the Sham group, rats in the US group exhibited marked pro-

liferation of urethral epithelial cells, significant narrowing of the urethral lumen, increased inflammatory cell infiltration in the surrounding tissues, and disordered cellular arrangement ($p < 0.001$). In contrast, compared with the US group, rats in the US + USCs group showed an enlarged urethral lumen, accompanied by mild epithelial cell proliferation, with only slight inflammatory cell infiltration in the periurethral tissues and relatively regular cellular arrangement. Compared with the Sham group, no obvious tissue injury or inflammatory response was observed in the urethra of normal rats treated with USCs (Fig. 2B,C).

Masson’s trichrome staining showed that, compared with the Sham group, prominent blue-stained collagen fiber proliferation was observed in the urethral tissues of the US group ($p < 0.001$). Compared with the US group, the collagen content in the urethral tissues of the US + USCs group was significantly reduced, and the collagen fibers were more regularly and orderly arranged ($p < 0.001$). Compared with the Sham group, no obvious increase in blue-stained collagen fibers was observed in the urethral tissues of rats treated with USCs alone (Fig. 2D,E).

Meanwhile, immunohistochemistry (IHC) results showed that α -SMA expression in urethral tissues was significantly increased in the US group compared with the Sham group ($p < 0.001$). Compared with the US group, α -SMA expression in urethral tissues of the US + USCs group was significantly decreased ($p < 0.001$). In addition, compared with the Sham group, no significant change in α -SMA expression was observed in the urethral tissues of normal rats treated with USCs ($p > 0.05$) (Fig. 2F,G).

Morphological Characterization of Rat Urethral Scar-Derived Cells

Primary adherent cells were successfully isolated from urethral scar tissues of rats with urethral stricture. After passaging, the cells gradually showed a relatively uniform morphology. Under phase-contrast microscopy, third-passage cells were predominantly elongated and spindle-shaped and exhibited adherent growth, with locally parallel or whorl-like arrangements (**Supplementary Fig. 1**). These cells were used in subsequent co-culture experiments.

USCs Inhibit the Proliferation and Fibrosis of Urethral Scar Fibroblasts

Subsequently, urethral scar fibroblasts were indirectly co-cultured with USCs. Immunofluorescence staining showed that, compared with the urethral scar fibroblasts cultured alone group, the expression level of α -SMA in urethral scar fibroblasts was significantly decreased in the urethral scar fibroblasts + USCs co-culture group ($p < 0.001$) (Fig. 3A,B). EdU staining demonstrated that, compared with the urethral scar fibroblasts cultured alone group, the proliferative capacity of urethral scar fibroblasts in the urethral scar fibroblasts + USCs group was markedly reduced ($p < 0.001$) (Fig. 3C,D). As shown in Fig. 3E–G, compared

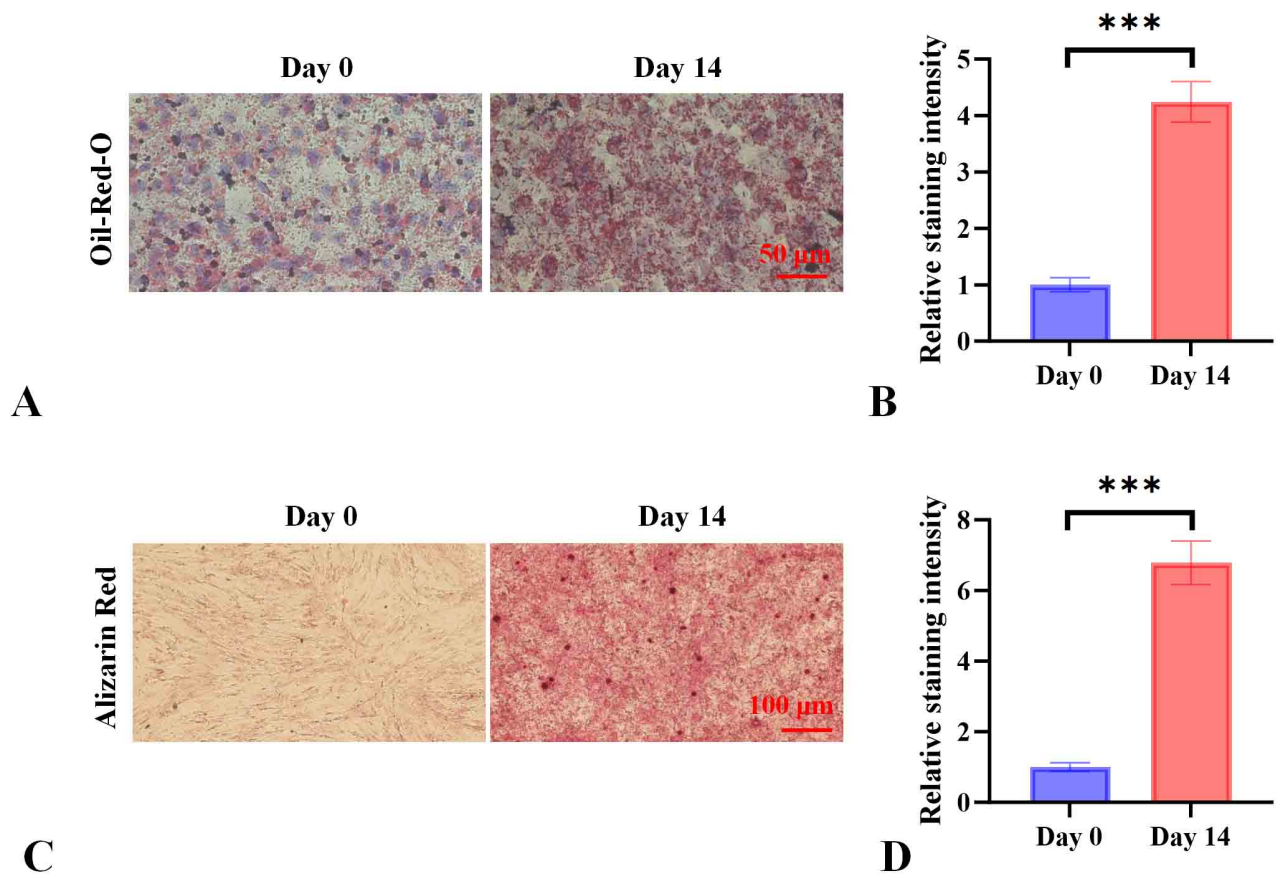


Fig. 1. Identification of the multidirectional differentiation potential of USC. (A) Representative images of Oil Red O staining after adipogenic induction. (B) Quantitative analysis of lipid droplet formation. (C) Representative images of Alizarin Red staining after osteogenic induction. (D) Quantitative analysis of calcified nodule formation. Data are presented as mean $\pm$ SD; $n = 3$ independent experiments. *** $p < 0.001$. USC, urine-derived stem cells.

with the urethral scar fibroblasts cultured alone group, the protein expression levels of COL-1 and TSP-1 in urethral scar fibroblasts were significantly decreased in the urethral scar fibroblasts + USC group ($p < 0.001$).

USCs Reduce p-Smad3/Smad3 and JunD Expression in Urethral Tissues and Urethral Scar Fibroblasts

As shown in Fig. 4A–C, compared with the US group, the protein expression levels of p-Smad3/Smad3 and JunD in urethral tissues were significantly decreased in the US + USC group ($p < 0.001$). As shown in Fig. 4D–F, compared with the urethral scar fibroblasts cultured alone group, the protein expression levels of p-Smad3/Smad3 and JunD in urethral scar fibroblasts were markedly reduced in the urethral scar fibroblasts + USC co-culture group ($p < 0.001$). As shown in Fig. 4G,H, immunofluorescence staining was performed to detect JunD expression in the nuclei of urethral scar fibroblasts. The results showed that, compared with the urethral scar fibroblasts cultured alone group, the protein expression level of nuclear JunD in urethral scar fibroblasts was significantly decreased in the urethral scar fibroblasts + USC co-culture group ($p < 0.001$).

Early Administration of USC Inhibits Inflammation and Extracellular Matrix Remodeling After Urethral Injury With Reduced Smad/JunD Signaling Activation

As shown in Fig. 5A–C, compared with the US group, the protein expression levels of p-Smad3/Smad3 and JunD in urethral tissues were significantly decreased in the US + USC group ($p < 0.001$). However, compared with the US + USC group, the protein expression levels of p-Smad3/Smad3 and JunD were markedly increased in the US + USC + TGF- β 1 group ($p < 0.001$).

ELISA results showed that, compared with the US group, the levels of TNF- α and IL-6 in urethral tissues were significantly decreased, whereas the level of IL-10 was significantly increased in the US + USC group ($p < 0.001$). In contrast, compared with the US + USC group, the levels of TNF- α and IL-6 in urethral tissues were significantly elevated, while the level of IL-10 was significantly reduced in the US + USC + TGF- β 1 group ($p < 0.001$) (Fig. 5D–F).

As shown in Fig. 5G–I, compared with the US group, the protein expression levels of COL-1, COL-3, FN, α -SMA, and TIMP-1 in urethral tissues were significantly de-

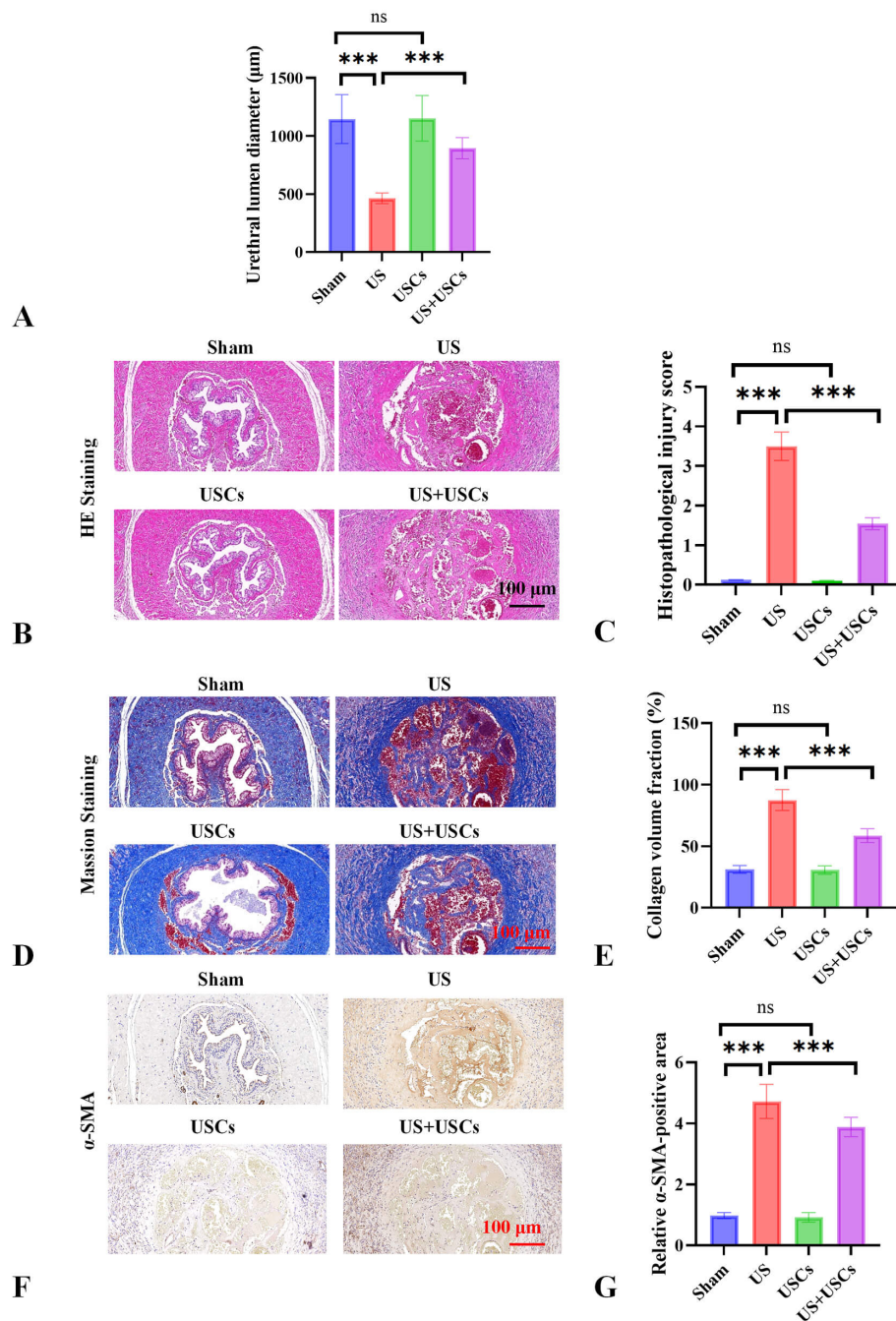


Fig. 2. Early administration of USC attenuates urethral narrowing, histological injury, and fibrotic remodeling after urethral injury. (A) Quantitative analysis of urethral lumen diameter. (B) Representative H&E staining images of urethral tissues. (C) Quantitative analysis of histopathological injury scores. (D) Representative Masson's trichrome staining images of urethral tissues. (E) Quantitative analysis of collagen volume fraction (CVF). (F) Representative immunohistochemical staining images of alpha-smooth muscle actin (α -SMA). (G) Quantitative analysis of the relative α -SMA-positive area. Data are presented as mean $\pm$ SD; $n = 10$ rats per group. *** $p < 0.001$; ns, not significant.

creased in the US + USC group ($p < 0.001$). However, compared with the US + USC group, the protein expression levels of COL-1, COL-3, FN, α -SMA, and TIMP-1 were significantly increased in the US + USC + TGF- β 1 group ($p < 0.001$).

Early Administration of USC Attenuates Post-injury Urethral Tissue Damage and Fibrosis in Association With Reduced Smad/JunD Signaling Activation

Hematoxylin and eosin (H&E) staining showed that, compared with the US group, rats in the US + USC group

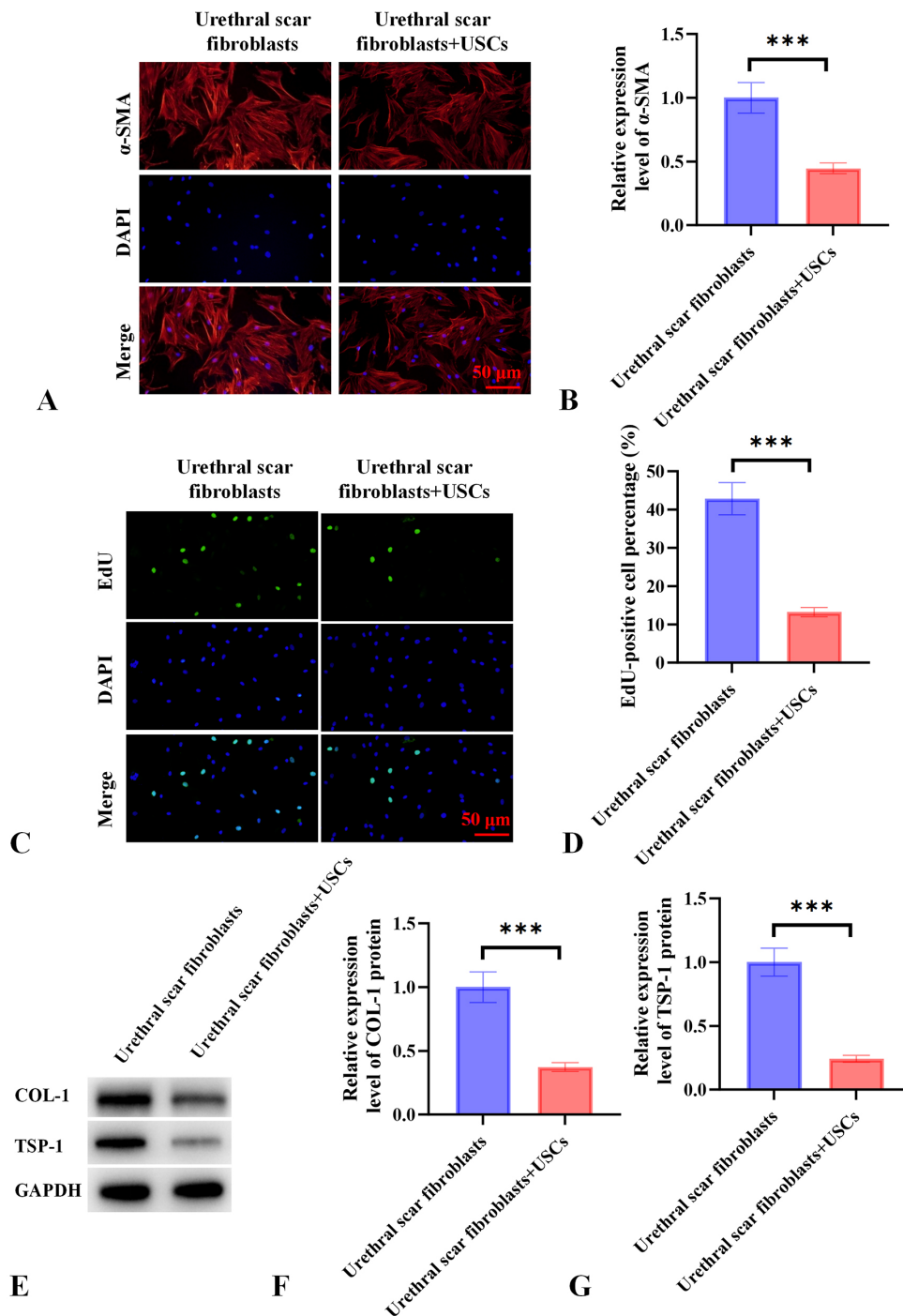


Fig. 3. USCs inhibit proliferation and fibrosis of urethral scar fibroblasts. (A) Representative immunofluorescence staining images of α -SMA in urethral scar fibroblasts. (B) Quantification of α -SMA fluorescence intensity. (C) Representative EdU staining images. (D) Quantification of EdU-positive cells. (E) Representative Western blot bands of collagen type I (COL-1) and TSP-1. (F,G) Quantitative analysis of COL-1 and TSP-1 protein expression. Data are presented as mean $\pm$ SD; $n = 3$ independent experiments. *** $p < 0.001$.

exhibited an enlarged urethral lumen, accompanied by mild epithelial cell proliferation. Only a small number of inflammatory cells were observed in the periurethral tissues, and the cellular arrangement remained relatively regular. After TGF- β 1 administration, the degree of urethral tissue injury in the US + USCs + TGF- β 1 group was significantly in-

creased compared with that in the US + USCs group ($p < 0.001$) (Fig. 6A,B).

Masson's trichrome staining results showed that, compared with the US group, the collagen content in urethral tissues of the US + USCs group was significantly reduced ($p < 0.001$). However, compared with the US + USCs group,

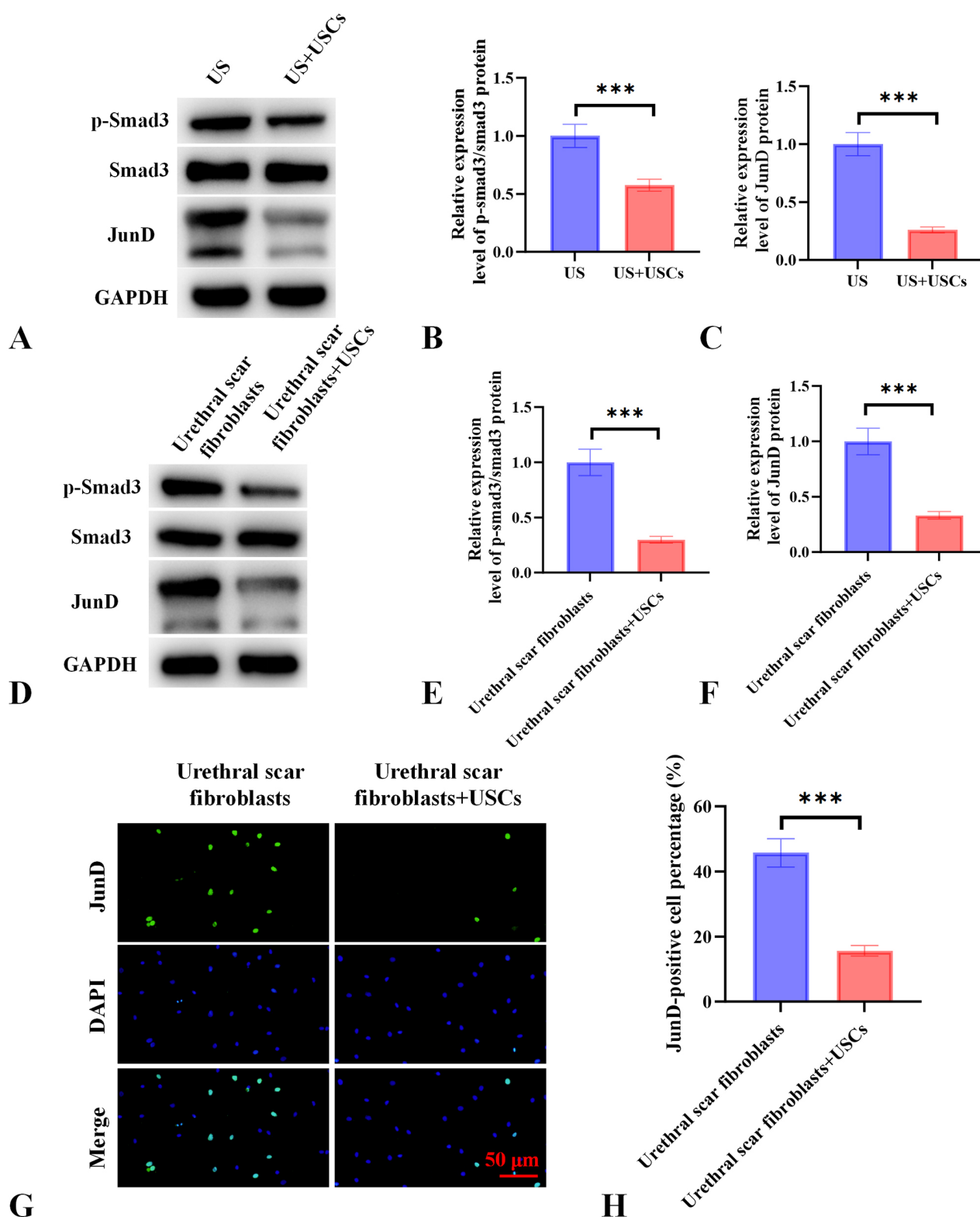


Fig. 4. USCs reduce p-Smad3/Smad3 and JunD expression in urethral tissues and urethral scar fibroblasts. (A) Representative Western blot bands of p-Smad3, Smad3, and JunD in urethral tissues. (B,C) Quantitative analysis of p-Smad3/Smad3 and JunD expression. (D) Representative Western blot bands of p-Smad3, Smad3, and JunD in urethral scar fibroblasts. (E,F) Quantitative analysis of p-Smad3/Smad3 and JunD expression. (G) Representative immunofluorescence staining images of nuclear JunD. (H) Quantification of nuclear JunD fluorescence intensity. For tissue experiments, $n = 10$ rats per group; for cell experiments, $n = 3$ independent experiments. Data are presented as mean $\pm$ SD. *** $p < 0.001$.

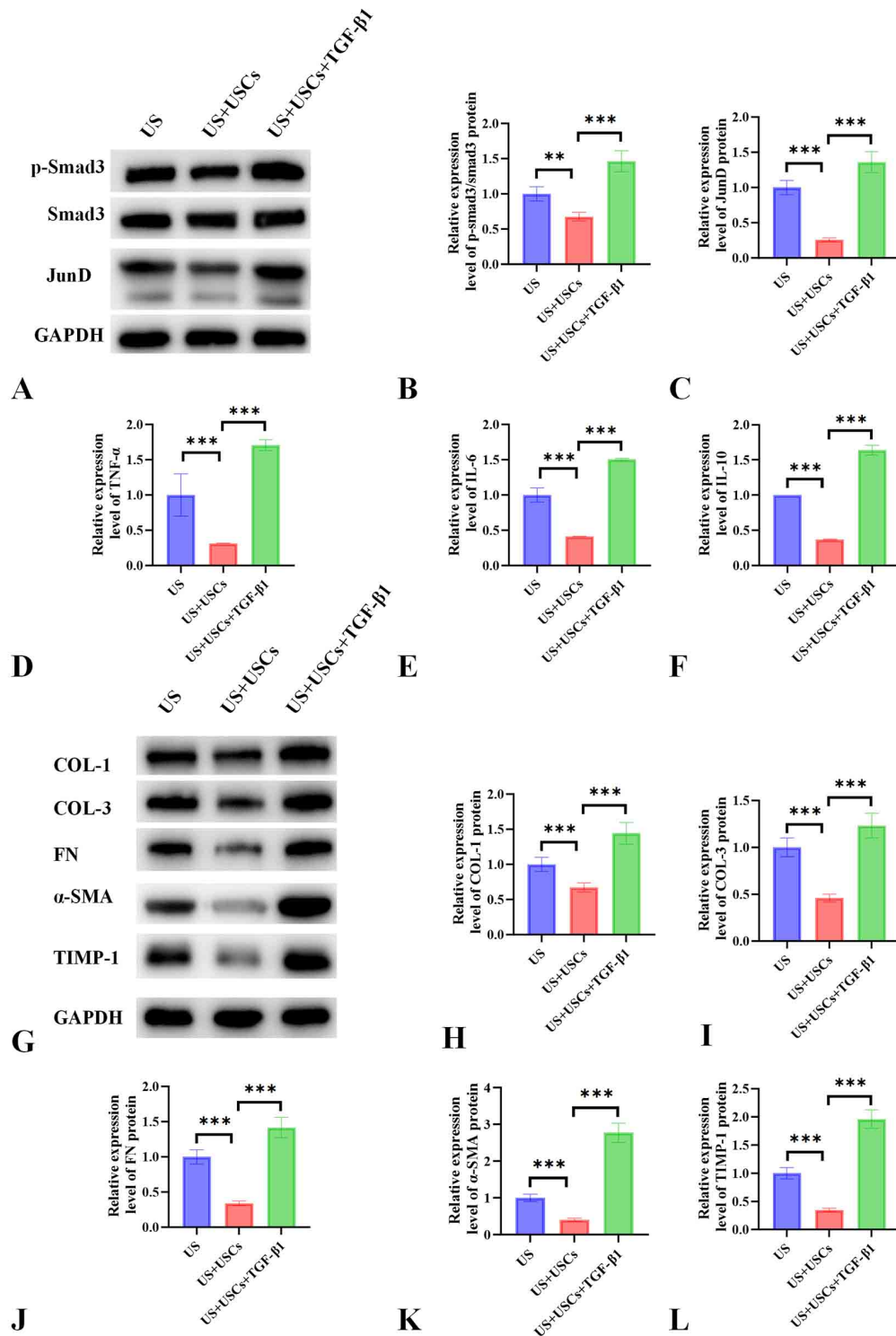


Fig. 5. USCs inhibit inflammation and extracellular matrix remodeling in association with reduced Smad/JunD signaling activation. (A) Representative Western blot bands of p-Smad3, Smad3, and JunD in urethral tissues. (B,C) Quantitative analysis of p-Smad3/Smad3 and JunD expression. (D–F) ELISA analysis of TNF-α, IL-6, and IL-10 levels in urethral tissues. (G) Representative Western blot bands of COL-1, COL-3, FN, α-SMA, and TIMP-1. (H–L) Quantitative analysis of COL-1, COL-3, FN, α-SMA, and TIMP-1 protein expression. Data are presented as mean ± SD; n = 10 rats per group. ***p* < 0.01, ****p* < 0.001. TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-10, interleukin-10; COL-3, collagen type III; FN, fibronectin; TIMP-1, tissue inhibitor of metalloproteinase-1.

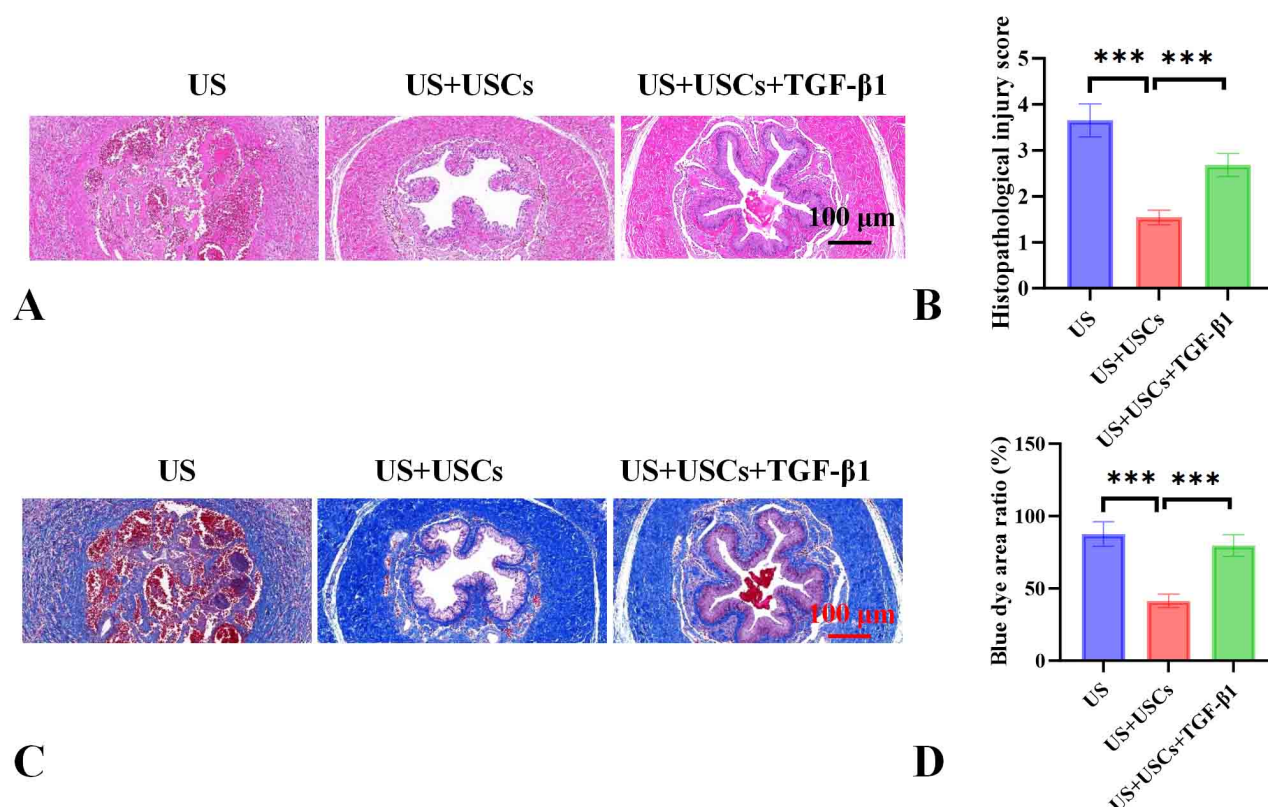


Fig. 6. Early administration of USC attenuates post-injury urethral tissue damage and fibrosis in association with reduced Smad/JunD signaling activation. (A) Representative H&E staining images of urethral tissues. (B) Quantitative analysis of urethral injury scores. (C) Representative Masson's trichrome staining images of urethral tissues. (D) Quantitative analysis of collagen volume fraction (CVF). Abbreviations: US, urethral stricture; USC, urine-derived stem cells; CVF, collagen volume fraction; FN, fibronectin; TIMP-1, tissue inhibitor of metalloproteinase-1. Data are presented as mean ± SD; n = 10 rats per group. *** $p < 0.001$.

the proliferation of blue-stained collagen fibers in urethral tissues was markedly increased in the US + USC + TGF-β1 group ($p < 0.001$) (Fig. 6C,D).

Discussion

The results of this study demonstrated that administration of urine-derived stem cells (USCs) immediately after urethral injury attenuated subsequent tissue damage, inflammation, fibrotic remodeling, and urethral narrowing in rats. Because USC were administered on day 0 and urethral fibrosis and narrowing were evaluated on day 14, the present findings reflect an early intervention effect during the development of post-injury fibrosis rather than a therapeutic effect on established urethral stricture. The observed effects may be associated with reduced Smad3/JunD signaling activation. USC are a type of mesenchymal stem cell derived from urine and possess several advantages, including non-invasive collection, abundant sources, strong proliferative capacity, and multidirectional differentiation potential. In this study, the multipotent differentiation ability of USC was first verified through adipogenic and osteogenic induction experiments. Oil Red

O staining revealed prominent intracellular lipid droplets, whereas Alizarin Red staining showed prominent calcified nodules, indicating that the cultured cells possessed the ability to differentiate into adipocytes and osteoblasts. These results are consistent with previous studies, indicating that USC possess biological characteristics similar to those of bone marrow-derived mesenchymal stem cells and adipose-derived stem cells, particularly their multipotent differentiation potential. Moreover, USC offer advantages such as ease of collection and fewer ethical limitations. Therefore, they have considerable potential for application in the fields of tissue repair and regenerative medicine [20,21].

Inflammatory responses and fibroblast activation play critical roles in the development and progression of urethral stricture [22]. Activated fibroblasts can differentiate into myofibroblasts and secrete large amounts of extracellular matrix (ECM) components, such as COL-1, COL-3, FN, and α-SMA, leading to abnormal tissue remodeling and narrowing of the urethral lumen [23]. In this study, H&E staining and Masson's trichrome staining revealed that rats in the urethral stricture model group exhibited marked epithelial cell proliferation, inflammatory cell infiltration, and

substantial collagen fiber deposition in urethral tissues, accompanied by a significant reduction in urethral diameter. However, early administration of USCs following urethral injury significantly reduced subsequent urethral narrowing, inflammatory cell infiltration, collagen deposition, and α -SMA expression. These findings suggest that early administration of USCs can reduce myofibroblast activation and attenuate post-injury urethral fibrosis. This finding is consistent with previous studies reporting that mesenchymal stem cells possess anti-inflammatory and anti-fibrotic properties [24,25,26,27].

In *in vitro* experiments, this study further revealed that USCs could significantly inhibit the proliferation and fibrotic phenotype of urethral scar fibroblasts. EdU staining showed that, compared with scar fibroblasts cultured alone, the proliferative capacity of the cells was markedly reduced after co-culture with USCs. Meanwhile, immunofluorescence and Western blot analyses demonstrated that the expression levels of fibrosis-related proteins, including α -SMA, COL-1, and TSP-1, were significantly decreased. Previous studies have shown that mesenchymal stem cells can secrete various anti-inflammatory and anti-fibrotic factors through paracrine mechanisms, thereby inhibiting fibroblast proliferation and collagen synthesis and ultimately alleviating tissue fibrosis [28,29,30]. The findings of this study further support this view.

The TGF- β /Smad signaling pathway is a classical pathway that plays a crucial role in the regulation of tissue fibrosis [31]. Among its components, Smad3 serves as a key downstream signaling molecule that promotes fibroblast activation and extracellular matrix synthesis [32,33]. In addition, JunD, an important member of the AP-1 family, can cooperate with the Smad signaling pathway to regulate the expression of genes associated with inflammation and fibrosis [12]. Previous studies have shown that abnormal activation of the Smad3 signaling pathway plays an important role in various diseases, including liver fibrosis, pulmonary fibrosis, and renal fibrosis [34,35,36]. In the present study, we found that the expression levels of p-Smad3/Smad3 and JunD were significantly increased in both urethral tissues of rats with urethral stricture and urethral scar fibroblasts, suggesting increased Smad3/JunD-related signaling activity during urethral fibrosis.

Further investigations revealed that after treatment with USCs, the expression levels of p-Smad3/Smad3 and JunD in urethral tissues and scar fibroblasts were significantly decreased. Meanwhile, immunofluorescence results showed that the nuclear expression of JunD was markedly reduced, suggesting that USC treatment was associated with reduced activation of Smad3/JunD signaling. To further explore the involvement of this pathway in USC-related effects, TGF- β 1 was added to stimulate Smad-related signaling activity. The results showed that TGF- β 1 treatment markedly attenuated the protective effects observed after USC treatment. Specifically, the ex-

pression levels of p-Smad3/Smad3 and JunD were elevated again. The levels of TNF- α and IL-6 increased, whereas the level of IL-10 decreased, and the expression of fibrosis-related proteins, including COL-1, COL-3, FN, α -SMA, and TIMP-1, was significantly upregulated. Histological analysis also showed that urethral tissue injury and collagen deposition were further aggravated. These findings provide supportive evidence that the anti-inflammatory and anti-fibrotic effects observed after USC treatment were associated with reduced activation of Smad3/JunD signaling. Given that TGF- β 1 can affect multiple signaling pathways and that a US + TGF- β 1 control group was not included, the TGF- β 1 intervention should be interpreted only as supportive pharmacological evidence for this association rather than as a formal rescue experiment or pathway-specific mechanistic evidence.

Although this study provides preliminary evidence, several limitations should be considered. First, because USCs were administered immediately after urethral injury, the present study primarily reflects an early intervention effect on post-injury fibrosis rather than the treatment of established urethral stricture. The findings were obtained from an animal model, and their clinical relevance requires further validation. Second, the observation period was limited to 14 days, and the effects were evaluated mainly through histological changes and urethral diameter. Long-term recurrence, sustained urethral patency, and urinary function were not assessed. Third, the urethral scar-derived cells used in *in vitro* experiments were identified primarily based on adherent growth and spindle-shaped morphology. Therefore, the possible presence of other stromal or smooth muscle cells cannot be excluded. Fourth, after intraperitoneal administration, the survival, distribution, local homing, and xenogeneic immune responses of USCs were not evaluated, and their exact site and mode of action remain unclear. Fifth, α -SMA can also be expressed in urethral smooth muscle and vascular structures, which limits its specificity as an indicator of myofibroblast activation. Finally, this study primarily evaluated changes in p-Smad3/Smad3 and JunD expression and used TGF- β 1 to stimulate Smad-related signaling activity. Smad3- or JunD-specific interventions were not performed, and a US + TGF- β 1 control group was not included. Therefore, the independent effect of TGF- β 1 in the urethral injury model could not be determined, and the TGF- β 1 intervention should be interpreted only as supportive pharmacological evidence rather than as a formal rescue experiment. Accordingly, the observed effects should be interpreted as being associated with reduced Smad3/JunD signaling activity, while the potential involvement of other signaling pathways cannot be excluded.

Conclusion

In conclusion, early administration of USCs after urethral injury attenuated subsequent tissue damage, inflammation, fibroblast proliferation, extracellular matrix deposition, urethral fibrosis, and lumen narrowing. These effects may be partly associated with reduced activation of Smad3/JunD signaling. The present findings support the early intervention effect of USCs on post-injury urethral fibrosis rather than its therapeutic effect on established urethral stricture.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

LLH, YHY, JJY, JHD, JMLiu, ZDC, JMLu, XBW, YNW, LZT and HMS designed the research study. LLH performed the research. LLH, YHY, JJY, JHD, JMLiu, ZDC, JMLu, XBW, YNW, LZT and HMS contributed to the experiments. LLH analyzed the data. LLH has been involved in drafting the manuscript and all authors contributed to important editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was approved by the Experimental Animal Ethics Committee of South China University of Technology (Approval No. 20230905). All animal experiments were conducted and reported in accordance with the ARRIVE Guidelines 2.0.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.24976/Discover.Med.202638213.259>.

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