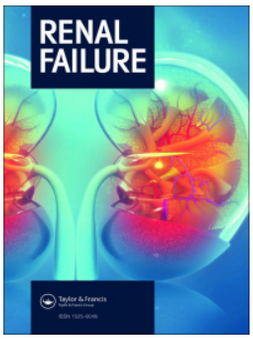

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LABORATORY STUDY

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Inhibition of matrix metalloproteinase-9 attenuates kidney fibrosis and cellular senescence in the transition from acute kidney injury to chronic kidney disease

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ABSTRACT

Background: The mechanisms underlying the progression of chronic kidney disease (CKD) following acute kidney injury (AKI) remain poorly understood. Senescent cells induce maladaptive repair have been identified as a significant contributor to CKD subsequent to AKI.

Purpose: The purpose of this study was to investigate the correlations between kidney fibrosis after unilateral ischemia injury and cellular senescence and explored the potential therapeutic effect of GM6001 on AKI to CKD transition.

Results: The study revealed a progressive increase in both fibrosis and matrix metalloproteinase 9 (MMP9) expression, peaking approximately 14 days following unilateral ischemic injury, indicating a pivotal role of MMP9 in the pathogenesis of renal fibrosis. Additionally, the research identified an elevation in markers of renal senescence over time, including SA-β-gal, P53, P21, and P16. Treatment with GM6001 demonstrated a significant reduction in both fibrosis and senescence, evidenced by decreased MMP9 expression and associated fibrotic markers, alongside improvements in cellular senescence indicators. *In vitro* studies further substantiated these findings, as GM6001 effectively inhibited MMP9 expression in TGF-β-stimulated HK-2 cells, reinforcing its antifibrotic and antisenescent properties and highlighting its potential as a therapeutic intervention for renal fibrosis.

Conclusion: The study provided compelling evidence on the role of MMP9 in the progression of renal fibrosis and the transition from AKI to CKD. The early activation of MMP9 and its association with fibrosis highlighted its potential as a therapeutic target. GM6001, through its inhibition of MMP9, offered a promising avenue for reducing both fibrosis and cellular senescence, suggesting its potential utility in ameliorating CKD.

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Acute kidney injury; AKI-to-CKD transition; cell senescence; fibrosis

Introduction

Acute kidney injury (AKI), especially ischemic AKI, is a common critical condition in clinical practice. Despite the rapid development of critical care interventions, the long-term and renal prognosis of survivors remains unfavorable [1]. It was previously believed that kidney tissue structure could fully recover after AKI but recent studies have found that even if serum creatinine levels return to baseline after AKI, chronic renal pathological damage may persist and lead to chronic kidney disease (CKD) [2,3]. In recent years, the focus of AKI research has shifted from the pathogenesis of AKI to the molecular mechanisms of AKI to CKD but there are currently no clinically feasible prevention and treatment measures [4].

Senescent cells, characterized by permanent growth arrest and altered phenotype, accumulate in the body due to aging, tissue damage, and genetic toxic insults [5,6]. Senescence is associated with exacerbated tissue damage and diminished regenerative capacity following injury. Studies have shown an increase in senescent cell numbers in the kidneys of humans and rodents with aging, CKD, and nephrotoxic drugs [7–10].

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The potential role of senescent cells in promoting increased fibrosis and reduced regenerative capacity in aged and damaged kidneys is a topic of significant clinical interest and research focus [10,11].

Matrix metalloproteinases (MMPs) are a group of zinc-dependent proteolytic metalloenzymes involved in various pathological conditions, including kidney disease [12]. MMP9 is a member of the MMP family and belongs to the gelatinase B subgroup [12]. Its participation in extracellular matrix (ECM) remodeling and renal fibrosis underscores its significance in the onset and progression of kidney disease [13,14]. MMP9 is more closely correlated with kidney disease compared to other MMPs and can serve as a biomarker for various kidney diseases [15]. Previous research has demonstrated that upregulation of the MMP9 gene can induce epithelial-mesenchymal transition (EMT) in tubular cells, leading to renal interstitial fibrosis and subsequent kidney damage. Previous study also predicts MMP9 as a potential cellular senescence biomarker in liver fibrosis [16].

In our previous investigation, it was demonstrated that the MMP inhibitor GM6001 exerts a protective effect against ischemic AKI by preserving the polarity of tubular epithelial cells [17,18]. Nevertheless, the precise mechanism underlying role of MMP9 inhibition in the transition from AKI to CKD, particularly its potential modulation of cell senescence, remains to be elucidated.

Materials and methods

Animals

Male C57BL/6 mice (8–10 weeks old) were maintained in the Laboratory Animal Center of Zhongshan Hospital, Fudan University under controlled circumstances (22 ± 2 °C, 12 h light and dark cycle, 50% humidity) and had full access to food and water. Mice were anesthetized using 1% pentobarbital and, once anesthetized, the fur on the left dorsal side was shaved and disinfected. Sterilized surgical instruments were used to make an incision in the skin, exposing the kidney. A microvascular clamp was then applied to the renal pedicle for 30 min. During this time, a heating pad was used to maintain the mouse's body temperature between 36 and 37 °C. In the sham surgery group, after the renal pedicle was exposed, no clamp was applied, and the incision was simply sutured. The success of the clamping was confirmed by observing the kidney color change from red to black. After 30 min, the microvascular clamp was removed, and successful reperfusion was indicated if the kidney color gradually returned from black to red. Mice were euthanized at 3, 7, and 14 days post-surgery for tissue collection and analysis. Additionally, mice treated with intraperitoneal injection of GM6001 (10 mg/kg/day, Selleck, Shanghai) were sacrificed 14 days after surgery. GM6001 was dissolved in DMSO, and the solvent control group received an equal volume of DMSO via intraperitoneal injection [19]. This work was approved by Zhongshan Hospital and carried out in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Cell culture and treatment

The human kidney proximal tubular epithelial cell line (HK-2) were purchased from ATCC. Cells were cultured in DMEM/F12 with 10% FBS and maintained in incubators at 37 °C with 5% CO_2. When cells achieved approximately 80% confluence, 10 ng/ml TGF-β (Abcam, China) with or without 50 μM GM6001 was added into the medium for 24 h.

Histopathologic evaluation

Formalin-fixed kidney tissues were sectioned (4 μm) and stained with Masson staining to evaluate fibrosis. To measure collagen deposition, a semi-quantitative study was performed by randomly picking 10 sites per sample (200× magnification).

Western blot analysis

Proteins were extracted using RIPA buffer (Millipore, USA), then isolated on 8 to 12% SDS-PAGE gel, transferred to PVDF membrane, which was sealed with 5% milk. The membrane is then incubated with the primary antibody overnight. including β-actin (1: 5000, Beyotime, China), Fibronectin (1:1000, Abcam, USA), α-SMA (1:1000, Abcam, USA), MMP9 (1:1000, Biodragon, China), P53 (1:1000, Proteintech, China), P21 (1:1000, Proteintech, China), P16 (1:1000, Proteintech, China), Lamin B (1:1000, Proteintech, China), γH2A.X (1:1000, Abcam, USA). After being incubated with HRP-conjugated secondary antibodies, the samples were properly washed to eliminate any unbound antibodies and then exposed to ECL detection.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from mice kidneys or cells using Trizol (Sigma-Aldrich, USA) according to the manufacturer's instructions. The Prime Script RT reagent Kit (TaKaRa, Japan) was used to generate first-strand cDNA. Quantitative real-time PCR was carried out in accordance with the manufacturer's instructions: 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 34 s. The primer sequences used are listed in the following.

Genes	Forward	Reverse
β-actin	CATTGCTGACAGGATGCAGAAGG	TGCTGGAAGGTGGACAGTGAGG
TGF-β	TGATACGCCTGAGTGGCTGTCT	CACAAGAGCAGTGAGCGCTGAA
SNAIL	TGTCTGCACGACCTGTGGAAAG	CTTCACATCCGAGTGGGTTTGG
SLUG	TCTGTGGCAAGGCTTTCTCCAG	TGCAGATGTGCCCTCAGGTTTG
TWIST	GATTCAGACCCTCAAACTGGCG	AGACGGAGAAGGCGTAGCTGAG
P21	CCTGGTGATGTCCGACCTG	CCATGAGCGCATCGCAATC
P16	CGCAGGTTCTTGGTCACTGT	TGTTCACGAAAGCCAGAGCG
Fibronectin	ACAAGGTTCGGGAAGAGGTT	CCGTGTAAGGGTCAAAGCAT
α-SMA	CTGACAGAGGCACCACTGAA	AGAGGCATAGAGGGACAGCA

Cell senescence β-galactosidase staining

The senescence β-Galactosidase staining kit (Beyotime, China) was used to detect senescent cells or tissues based on the

upregulation of the senescence-associated β-galactosidase (SA-β-Gal) activity during aging. After staining and fixing, the cells in 12-well plates or frozen slides were washed with PBS for 3 min for 3 times and then incubated with working buffer at 37 °C overnight. Finally, cells or slices were observed by microscopes.

Immunofluorescence staining

Paraffin-embedded tissue sections are dewaxed and rehydrated and then incubated with MMP9 antibody overnight at 4 °C. Alexa Fluor 488-labeled Goat Anti-Rabbit IgG(H + L) (1:200, Beyotime, China) was used as the secondary antibody. Immunofluorescence was visualized and captured using OLYMPUS microscopes.

Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 10.0 software. For comparisons between multiple groups, a one-way analysis of variance (ANOVA) was conducted, followed by Tukey's post-hoc test to assess pairwise differences. The level of statistical significance was determined using a p value threshold of less than 0.05 ($p < 0.05$).

Results

Ischemia-induced MMP9 overexpression and AKI-CKD transition in mice

In the present study, we first established the unilateral ischemia/reperfusion (UIR) kidney injury models to observe the natural course of AKI to CKD transition. A schematic diagram depicting the animal treatment procedure was demonstrated in Figure 1A. Our results demonstrated a progressive rise in the quantity of blue collagen fibers in Masson's trichrome staining with delayed molding time, suggesting the progression of fibrosis. Furthermore, immuno-histological staining and western blot analysis showed a progressive increase in fibrotic-related proteins such as fibronectin and α-SMA in the UIR group (Figure 1(B–F)). Additionally, we observed an early elevation in renal abundance of MMP9 on day 3 and peaked on day 14 (Figure 1(F–H)), suggesting that activation of MMP9 is closely associated with the development of renal fibrosis. These results provide valuable insights into the molecular mechanisms underlying AKI to CKD transition and highlight the potential role of MMP9 in driving renal fibrosis progression.

Markers of renal senescence were increased in UIR mice kidneys

It has been acknowledged that accelerated cellular senescence contributes to the pathogenesis of renal fibrosis. This process involves the accumulation of senescent cells, which are characterized by an increase in activity of the senescence-associated β-galactosidase (SA-β-gal) enzyme. To detect these senescent cells in renal tissue, we stained renal sections for SA-β-gal and observed a time-dependent increase in its activity on day 3, day 7, and day 14 (Figure 2(A)). Additionally, we found that the levels of senescence-related mRNA markers such as P53, P21, and P16 were elevated as early as day 3 but did not reach statistical significance until day 14 (Figure 2(B)).

Furthermore, our analysis revealed a reduction in the abundance of Lamin B protein, along with an increase in the levels of P53, P16, and γH2A.X on day 14 (Figure 2(C–F)), indicating significant renal cell senescence as AKI progresses to CKD. These findings provided valuable insights into the molecular mechanisms underlying renal fibrosis and suggest potential targets for therapeutic intervention aimed at preventing or reversing this pathological process.

GM6001 treatment attenuated renal tubulointerstitial fibrosis in the UIR mice

GM6001 has demonstrated potential protective effects in animal models of AKI, but its role in the transition from AKI to CKD has not been fully characterized. In order to elucidate the possible pathway, C57BL/6 mice underwent UIR surgery and were treated with GM6001. Figure 3(A) displays the pathological staining results of UIR kidneys treated with GM6001. The pathological analysis of the UIR kidneys showed signs of tubular expansion, collagen deposition, and interstitial fibrosis. However, treatment with GM6001 effectively alleviated these pathological changes (Figure 3 (A)). Furthermore, inhibition of MMP9 expression by GM6001 led to a significant decrease in the expression of fibrosis-related genes such as Fibronectin, α-SMA, TGF-β, Snail, Slug, and Twist (Figure 3(B–D)). Additionally, compared to the vehicle-treated UIR group, the GM6001-treated UIR group exhibited reduced protein expression levels of Fibronectin and α-SMA (Figure 3(E–F)). These findings suggested a potential antifibrotic role for GM6001.

GM6001 treatment inhibited tubular senescence after UIR

Senescent cells have been recognized to contribute to the pathogenesis of renal fibrosis. To investigate whether GM6001 could reduce renal fibrosis by decreasing senescence, we performed SA-β-Gal staining after GM6001 treatment. The results demonstrated that GM6001 effectively inhibited renal senescence following UIR insult (Figure 4(A)). Additionally, GM6001 suppressed the mRNA expression of P21 and P16, as well as proinflammatory factors associated with the secretory phenotype (SASP) such as TNF-α and IL-6 (Figure 4(B)). Furthermore, we observed a significant downregulation of Lamin B and an upregulation of senescence-associated genes P53, P16, and γH2A.X in the Veh + UIR group, which were notably reversed after GM6001 treatment (Figure 4(C–F)).

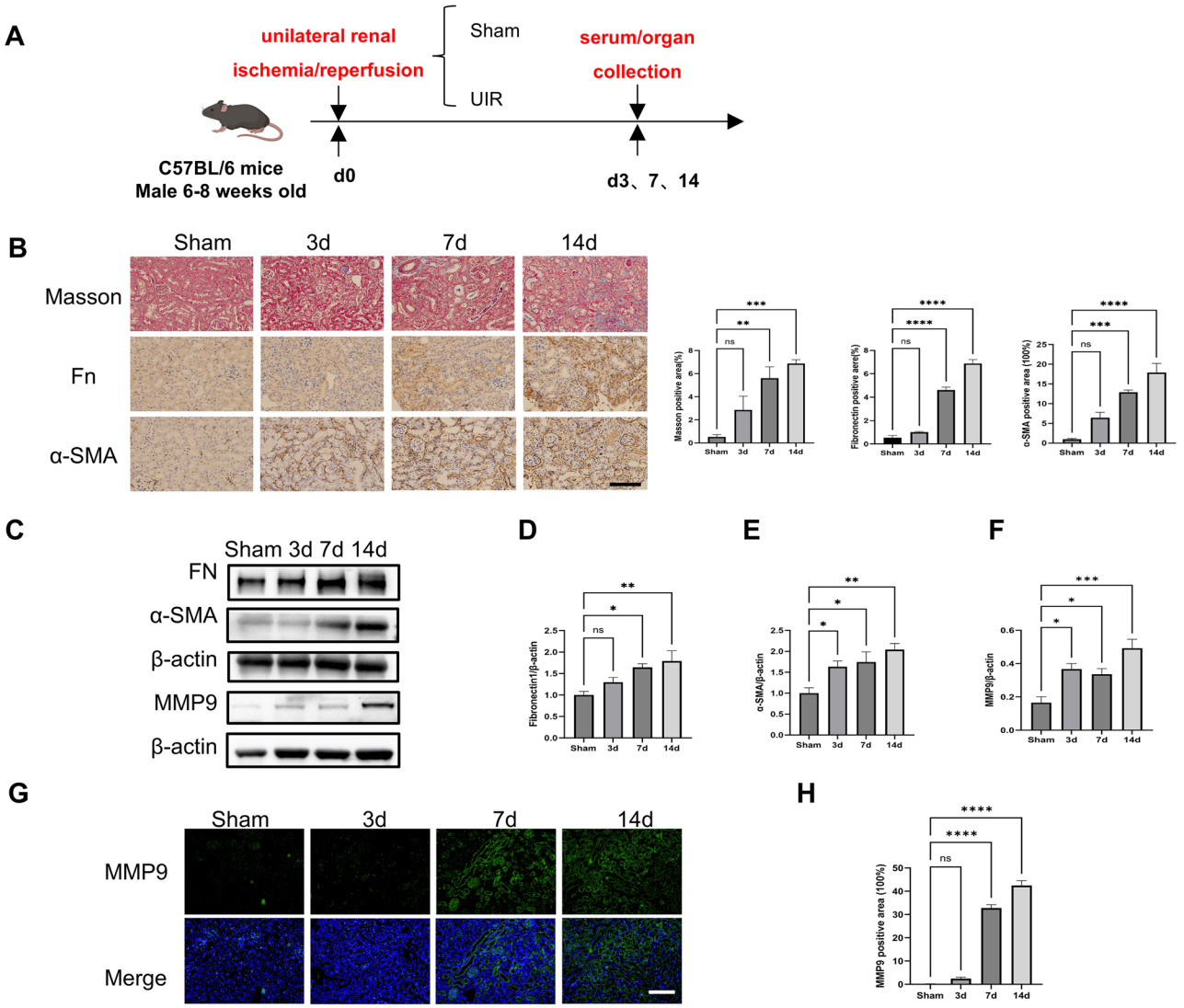

Figure 1. UIR-induced MMP9 expression and development of AKI to CKD in mice. (A) Schematic diagram of unilateral URI surgery. (B) Staining of Masson, Fibronectin, and α-SMA after different UIR surgery days. (C–F) Western blot images and quantitative analysis of Fibronectin, α-SMA, and MMP9 in mouse kidneys after UIR surgery. (G) Immunofluorescence of MMP9 in mouse kidneys after UIR. Scale bar, 100 μm. (H) Quantitative analysis of MMP9 fluorescence. All $n = 4$ to 6 per group unless stated, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: no significant difference.

Inhibition of MMP9 alleviated senescence and fibrosis in TGF-β-stimulated tubular epithelial HK-2 cells

Subsequently, GM6001 was incubated with HK-2 cells to investigate the potential antisenescent and antifibrotic effects of MMP9 inhibition *in vitro*. Consistent with *in vivo* results, TGF-β upregulated MMP9 expression in a time-dependent manner (Figure 5(A)). As in our previous studies [20], 50 μM GM6001 effectively inhibited MMP9 expression (Figure 5(B)). Downregulation of MMP9 by GM6001 treatment also significantly alleviated fibrosis in HK-2 cells. The increase in Fibronectin and α-SMA protein expression following TGF-β stimulation was mitigated by GM6001 treatment (Figure 5(C–D)). Blockage of MMP9 suppressed SA-β-Gal activity induced by TGF-β as shown in Figure 5(E). Furthermore, Western blot analysis demonstrated that pretreatment with GM6001 largely blocked the TGFβ-induced overexpression of P53, P21, P16, and γH2A.X, while restoring Lamin B levels (Figure 5(F–H)).

Discussion

AKI transitioning to CKD represents a significant global health concern. The core mechanism of kidney fibrosis includes the abnormal deposition of ECM and excessive renal interstitial fibrosis, which leads to the gradual loss of renal function [3,21]. Recent studies have shown that cellular senescence plays an important role in this process, especially the promotion of fibrosis by senescent cells has attracted widespread attention [11,22]. Our investigation revealed a correlation between renal fibrosis progression and increased renal senescence. Utilizing the UIR model, our team has elucidated the influence of renal senescence on advancing renal fibrosis. Inhibition of MMP9 could effectively alleviate kidney fibrosis after ischemic insult through reducing cellular senescence.

Matrix metalloproteinases, particularly MMP9, play a crucial role in ECM remodeling and the pathogenesis of fibrosis [19]. The current study demonstrated a progressive increase in both fibrosis and MMP9 expression following unilateral

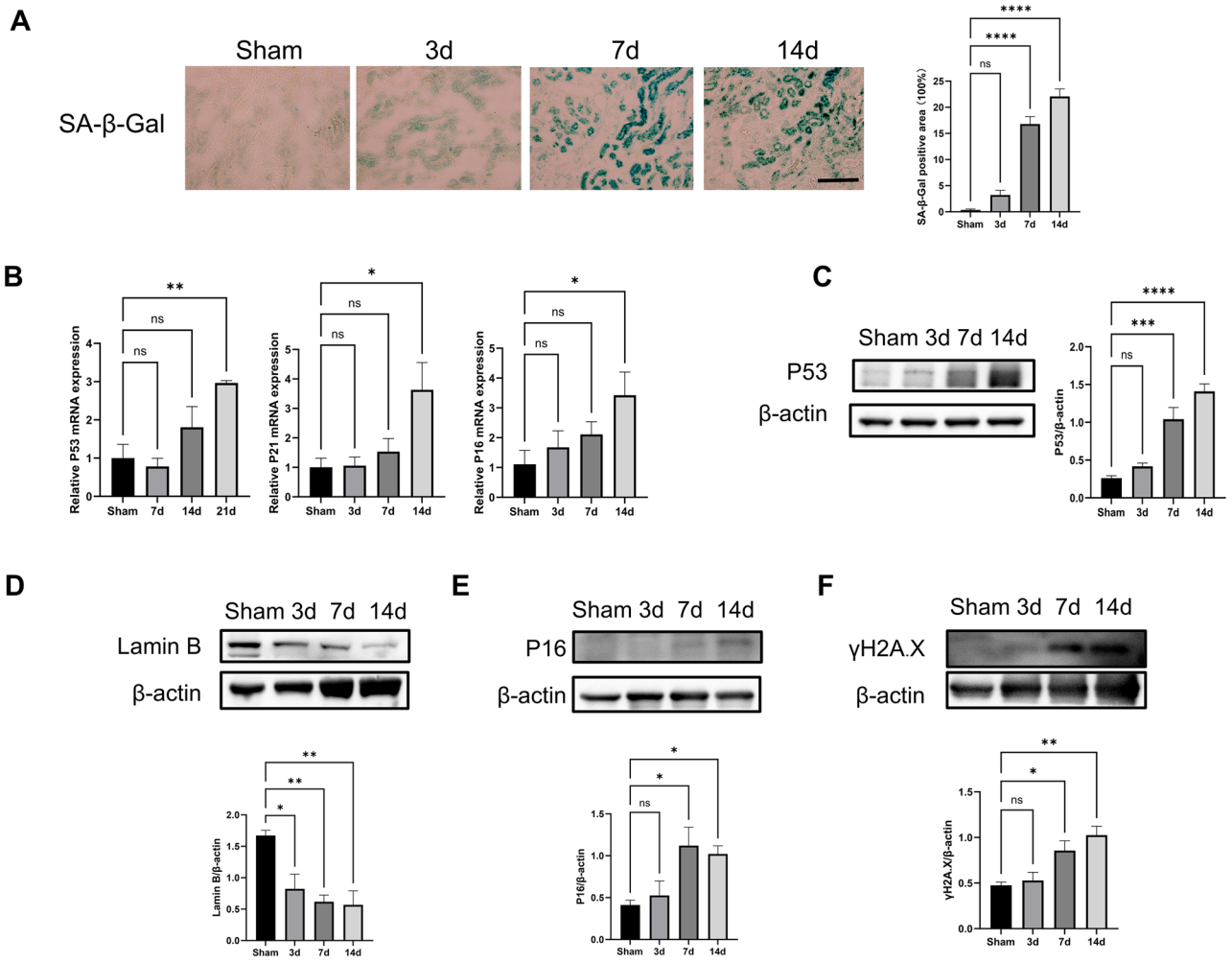

Figure 2. UIRI-induced renal senescence during the development of AKI to CKD. (A) Representative images and quantitative analysis of SA-β-Gal staining after different ischemia duration. Scale bar, 100 μm, $n = 3$. (B) Quantitative analysis of mRNA expression of P53, P21, and P16 in the kidney. (C) Western blot images and quantitative analysis of P53. (D) Western blot images and quantitative analysis of Lamin B. (E) Western blot images and quantitative analysis of P16. (F) Western blot images and quantitative analysis of γH2A.X. All $n = 4$ to 6 per group unless stated, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ns: no significant difference.

ischemic injury, with a peak at approximately 14 days post-injury. This finding was consistent with previous studies suggesting that MMP9 is involved in the early stages of kidney injury [18], promoting ECM remodeling and contributing to fibrotic scarring. The upregulation of MMP9 observed in this study supports its role in both the initiation and propagation of fibrotic processes in the kidney following ischemic injury. Furthermore, the relationship between MMP9 and fibrosis is not merely a consequence of ECM degradation but also reflects a complex interplay with inflammatory and cellular stress responses [23,24]. MMP9 can activate various cytokines and growth factors, including transforming growth factor-β (TGF-β), a key mediator of fibrosis. This study's observation that GM6001 treatment reduced both MMP9 expression and fibrotic markers provides compelling evidence for the critical role of MMP9 in the progression of renal fibrosis and suggests that targeting MMP9 could be an effective strategy for preventing or mitigating CKD progression after AKI.

Senescent cells undergo a state of irreversible growth arrest as a consequence of prolonged internal and external environmental stressors, including oxidative stress, inflammatory responses, and DNA damage [8,25]. These cells not only lose their capacity for normal proliferation but also secrete an array of pro-inflammatory and pro-fibrotic factors collectively referred to as the SASP [26]. In alignment with prior research, our study demonstrated a time-dependent elevation in SASP following ischemic injury, encompassing cytokines such as TNF-α, IL-6, and TGF-β. Within the kidney context, the accumulation of senescent cells is intricately linked to the progression of fibrosis. SASP factors can provoke inflammatory responses and fibrotic processes in adjacent cells, thereby exacerbating tissue damage and functional decline. Notably, studies have indicated that senescent renal tubular epithelial cells can facilitate the transdifferentiation of fibroblasts into myofibroblasts [27,28]. MMP9 has been suggested as a potential biomarker of cellular senescence, with prior studies showing its upregulation in senescent cells. As a key component of the SASP, MMP9 contributes to the inflammatory microenvironment typical of aging tissues [29]. Excessive activation of MMP9 plays a critical role in the

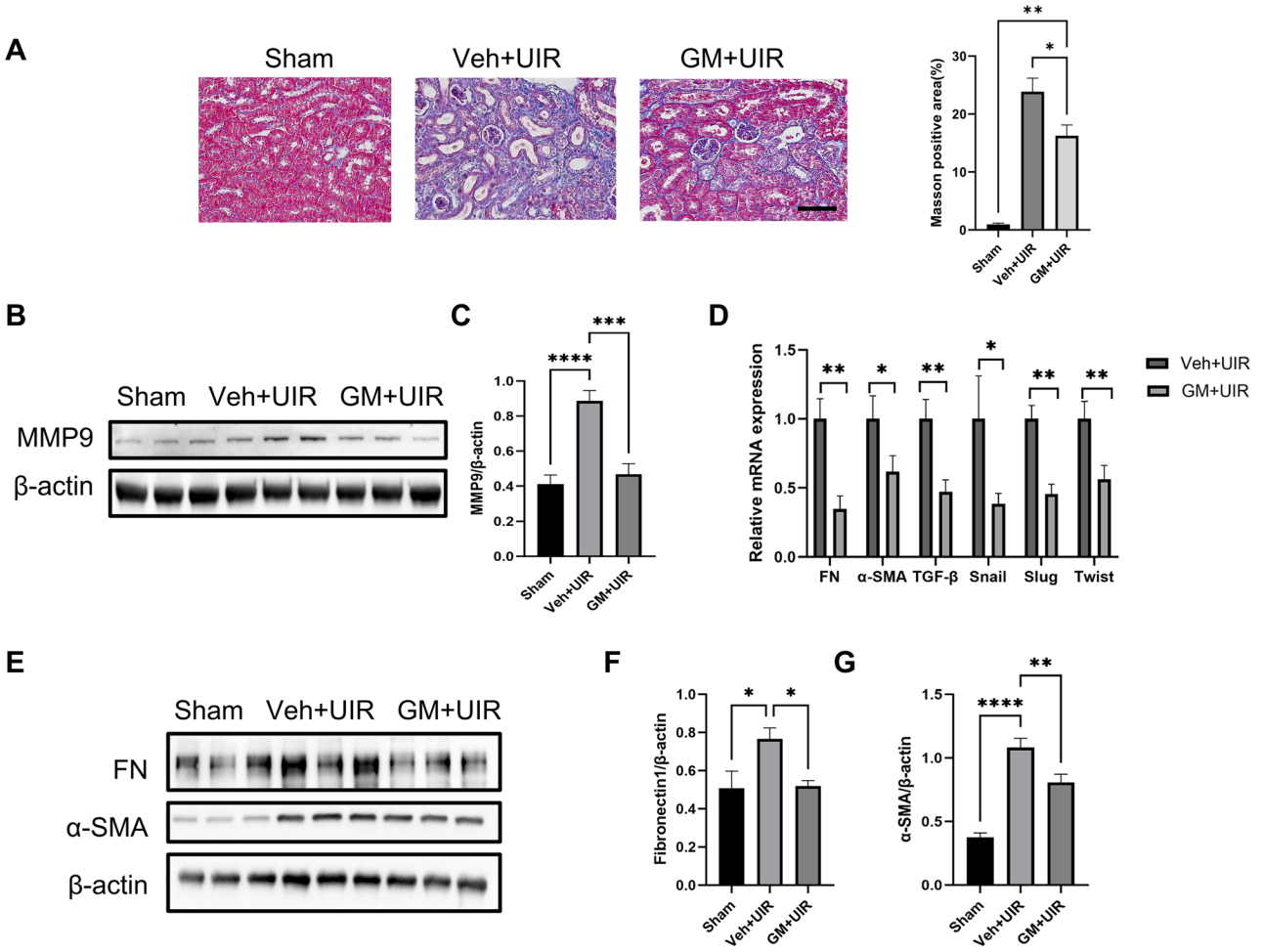

Figure 3. GM6001 Treatment attenuated renal tubulointerstitial fibrosis in the UIR mice. (A) Mice underwent UIR with or without GM6001 treatment for 14 days, representative images of Masson's trichrome-stained kidney sections are shown. Scale bar, 100 μm. (B,C) Western blot images and quantitative analysis of MMP9 in UIR mice. (D) Fibrosis-related gene expression after UIR with or without GM6001 treatment. (E) Protein expression of Fibronectin and α-SMA was decreased after GM6001 treatment compared with UIR group, assessed by Western blot. All $n = 4$ to 6 per group unless stated, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

degradation of the ECM, which not only promotes tissue damage but also stimulates the further secretion of SASP factors from senescent cells. This creates a detrimental feedback loop that accelerates senescence and worsens tissue degeneration. Interestingly, recent studies have suggested that inhibiting MMP9 may help break this cycle. For example, hydrogen sulfide has been shown to mitigate cardiac senescence by downregulating MMP9 [30], and MMP9-targeting peptide-modified nanoparticles have been demonstrated to reduce cellular senescence in osteoarthritis by inhibiting pyroptosis [31]. These findings highlight the potential of targeting MMP9 to slow down cellular senescence and its associated diseases. In this study, treatment with GM6001, an MMP9 inhibitor, not only reduced fibrosis but also improved senescence markers, suggesting that the inhibition of MMP9 may alleviate both the fibrotic and senescent aspects of kidney injury.

P53 is a tumor suppressor protein, which plays an important role in cell stress response, DNA repair, cell cycle regulation, apoptosis, and senescence [32]. Upon exposure to stressors such as DNA damage or oxidative stress, P53 is activated to initiate cellular responses that can lead to cell cycle arrest or apoptosis if the damage is severe [33]. In the context of kidney injury, P53 is upregulated and induces cell cycle arrest by activating downstream targets such as P21 and P16, which are markers of cellular senescence. Additionally, P53 modulates the inflammatory environment by activating SASP factors, contributing to the progression of fibrosis. Previous studies have shown that MMP9 upregulates P53 expression through the activation of the transmembrane protein Notch [34]. The activation of P53 then triggers the DNA damage response, which is a key hallmark of cellular senescence [35]. In our experiment, after using an MMP9 inhibitor, we observed a decrease in P53 protein levels. Based on these findings and previous literature, we hypothesize that inhibiting MMP9 can reduce P53 expression, thereby alleviating cellular senescence during the transition from AKI to CKD, and ultimately mitigating fibrosis.

The study demonstrated that GM6001, an MMP9 inhibitor, effectively reduced both fibrosis and senescence in the kidney following ischemic injury. *In vitro* experiments further confirm that GM6001 inhibited MMP9 expression and

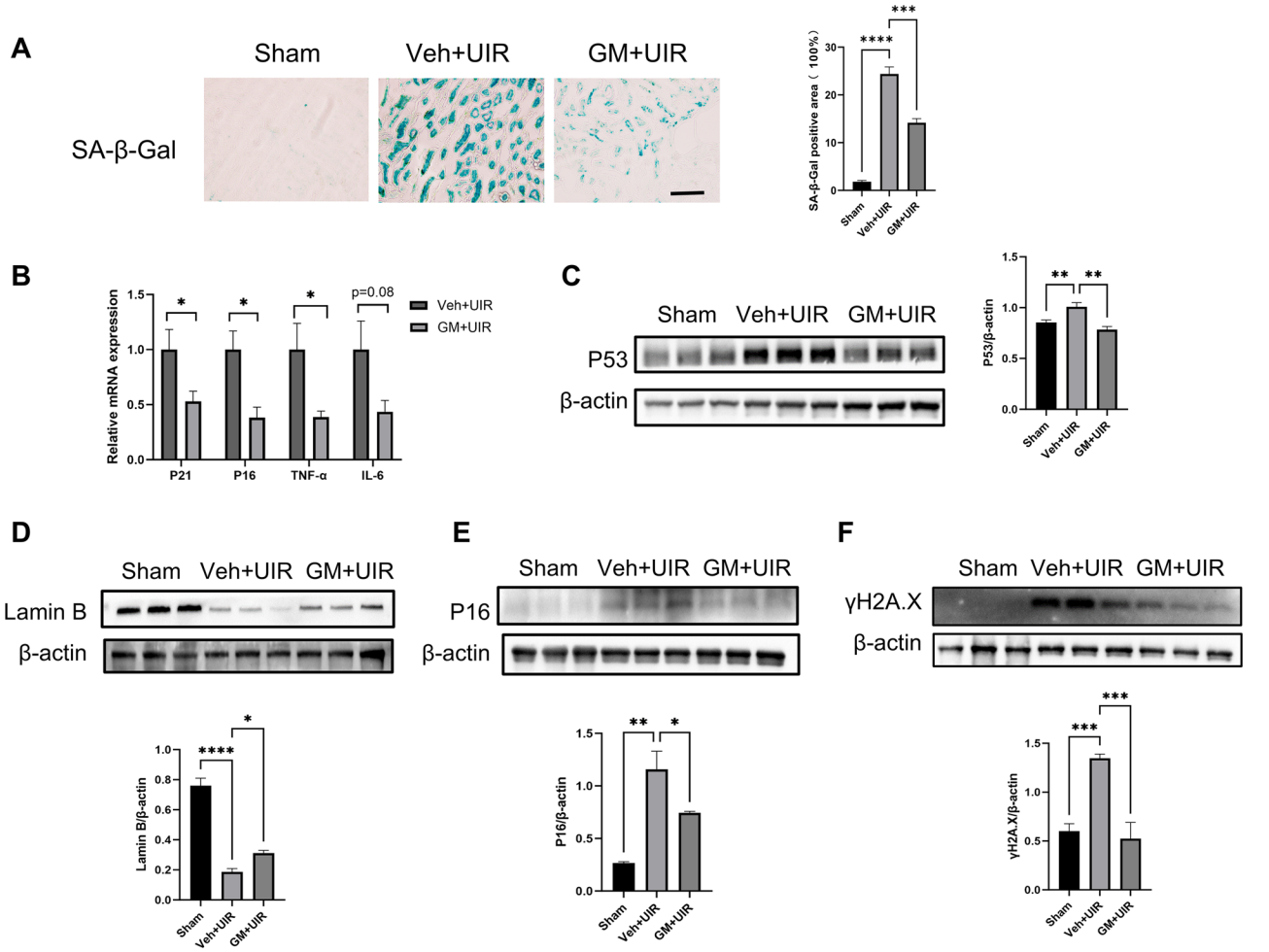

Figure 4. GM6001 treatment attenuated renal senescence in the UIR mice. (A) Representative images and quantitative analysis of SA-β-Gal staining. Scale bar, 100 μm. $n = 3$. (B) PCR showed that renal expression of P21, P16, TNF-α, and IL-6 mRNA expression was decreased after GM6001 treatment. (C–F) Western blot analysis showed that renal expression of P53, P16, and γH2A.X was decreased after GM6001 treatment, while Lamin B expression was increased. All $n = 4$ to 6 per group unless stated, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

suppressed the fibrotic response in TGF-β-stimulated HK-2 cells, a human renal proximal tubular cell line. These findings were consistent with previous research indicating that GM6001 can protect against ischemic AKI by preserving tubular epithelial cell polarity and preventing excessive ECM deposition [17]. The current study expanded on this by showing that GM6001 may also alleviate the transition from AKI to CKD by modulating both MMP9 expression and cellular senescence. While GM6001's antifibrotic effects are well-documented, its impact on cellular senescence in the context of AKI to CKD transition is a novel and significant finding. Cellular senescence is increasingly recognized as a key driver of kidney fibrosis and CKD progression, and targeting senescent cells could provide a therapeutic strategy to mitigate these effects. The ability of GM6001 to reduce cellular senescence markers, alongside its antifibrotic properties, suggests that it may have broad potential in the treatment of renal fibrosis and CKD.

There are some limitations in this study. It primarily focuses on the early progression (up to 14 days) following unilateral ischemic injury, without addressing the long-term effects of GM6001 treatment on CKD progression beyond the acute phase. While the study identifies the inhibition of MMP9 as a key mechanism of GM6001, the specific pathways through which it affects cellular senescence and fibrosis could be further investigated. Additionally, the study uses unilateral ischemic injury as the model for AKI, and it would be valuable to explore whether GM6001's therapeutic effects extend to other types of AKI or renal injuries (e.g., nephrotoxic, septic), which would enhance the generalizability of the findings to a wider range of kidney diseases.

Conclusions

This study provided insights into the mechanisms underlying the progression from AKI to CKD, highlighting the potential roles of MMP9 and cellular senescence in this process. Our findings suggested that suppression of MMP9 may offer therapeutic potential by inhibiting P53 and reducing cellular senescence, which could help in preventing or slowing the development of CKD following AKI. While these results are promising, further research is needed to fully explore GM6001's potential as a therapeutic approach for CKD prevention or treatment.

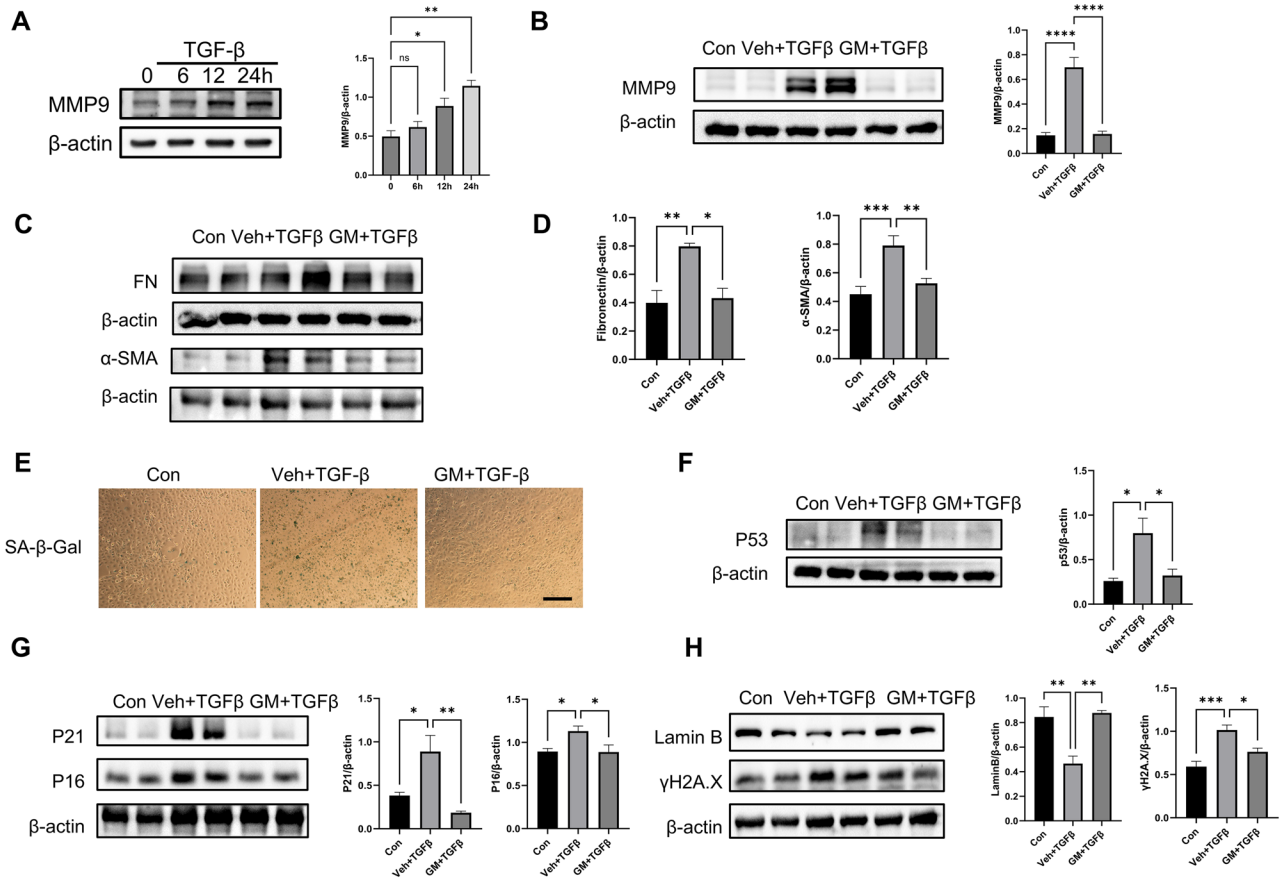

Figure 5. GM6001 alleviated HK-2 cells senescence and fibrosis after TGF-β stimulation. (A) MMP9 expression was gradually increased after TGF-β incubation, shown by western blot. (B) Western blot images and quantitative analysis of MMP9 in HK-2 cells. (C,D) Western blot images and quantitative analysis of Fibronectin and α-SMA in HK-2 cells. (E) Representative images of SA-β-Gal staining in HK-2 cells. Scale bar, 100 μm. (F–H) Western blot images and quantitative analysis of senescent-related proteins, including P53, P21, P16, Lamin B, and γ H2A.X after TGF-β stimulation with or without GM6001. All $n = 3$ per group unless stated, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

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Disclosure statement

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

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Data availability statement

All data reported in this article will be shared by the lead contact upon request.

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