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GSDMD Mediates Ang II-Induced Hypertensive Nephropathy by Regulating the GATA2/AQP4 Signaling Pathway

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Aim: Hypertensive nephropathy is a common complication of hypertension. However, no effective measures are currently available to prevent the progression of renal insufficiency. Gasdermin D (GSDMD) is a crucial mediator of pyroptosis that induces an excessive inflammatory response. In the present study, we aimed to determine the effect of GSDMD on the pathogenesis of hypertensive nephropathy, which may provide new insights into the treatment of hypertensive nephropathy.

Methods: C57BL/6 (wild-type, WT) and Gsdmd knockout ($Gsdmd^{-/-}$) mice were subcutaneously infused with angiotensin II (Ang II) via osmotic mini-pumps to establish a hypertensive renal injury model. Recombinant adeno-associated virus serotype 9 (AAV9) carrying GSDMD cDNA was used to overexpress GSDMD. Renal function biomarkers, histopathological changes, and inflammation and fibrosis indices were assessed. Transcriptome sequencing (RNA-seq) and cleavage under targets and mentation (CUT & Tag) experiments were performed to identify the downstream pathogenic mechanisms of GSDMD in hypertensive nephropathy.

Results: GSDMD was activated in the kidneys of mice induced by Ang II ($P < 0.001$). This activation was primarily observed in the renal tubular epithelial cells ($P < 0.0001$). GSDMD deficiency attenuated renal injury and fibrosis induced by Ang II ($P < 0.0001$), whereas Gsdmd overexpression promoted renal injury and fibrosis ($P < 0.01$). Mechanistically, GSDMD increased Ang II–induced GATA binding protein 2 (GATA2) transcription factor expression ($P < 0.01$). GATA2 also bound to the aquaporin 4 (*Aqp4*) promoter sequence and facilitated *Aqp4* transcription ($P < 0.001$), leading to renal injury and fibrosis. Moreover, treatment with GI-Y1, an inhibitor of GSDMD, alleviated Ang II–induced renal injury and fibrosis ($P < 0.01$).

Conclusion: GSDMD plays an important role in the development of hypertensive nephropathy. Targeting GSDMD may be a therapeutic strategy for the treatment of hypertensive nephropathy.

Keywords: gasdermin D, hypertensive nephropathy, pyroptosis, angiotensin II, aquaporin 4, GATA binding protein 2

Introduction

Hypertension is a chronic illness worldwide.[1] Hypertensive nephropathy is the main consequence of hypertension and it affects patient prognosis. Hypertensive nephropathy is characterized by progressive fibrotic and inflammatory changes in the kidneys that are associated with hypertension.[2] Pathological manifestations of hypertensive nephropathy include inflammatory cell infiltration, renal interstitial fibrosis, tubular atrophy, and glomerular sclerosis.[3] Although patients with hypertensive nephropathy can be diagnosed before the development of end-stage renal disease (ESRD), no effective

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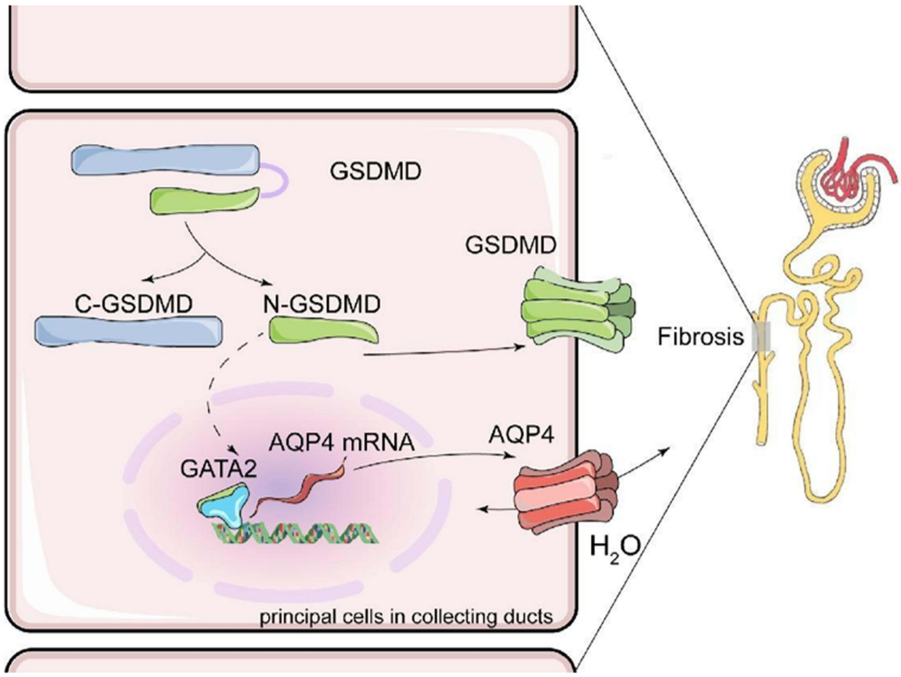
Graphical Abstract



measures are available to prevent progression of renal insufficiency. Hence, clarifying the pathogenesis of hypertensive nephropathy and identifying potential therapeutic targets has important scientific and clinical significance.

The pathogenesis of hypertension involves various mechanisms. Among these, the renin-angiotensin-aldosterone system (RAAS) is recognized as a crucial component.[4] Angiotensin II (Ang II) is a major RAAS effector. It can increase blood pressure and cause abnormal renal hemodynamics, thereby impairing renal function.[5] Recently, the non-hemodynamic effects of Ang II in hypertensive nephropathy have received increased attention. Ang II is reportedly involved in regulating inflammatory responses in the renal tubular epithelial and endothelial cells.[6,7] Ang II-mediated infiltration of inflammatory cells is closely associated with renal fibrosis.[8]

Pyroptosis is a form of cell death associated with inflammation that results in an excessive inflammatory response.[9] Gasdermin D (GSDMD) is a crucial mediator of pyroptosis.[10,11] GSDMD consists of a C-terminal fragment (GSDMD-C) and N-terminal fragment (GSDMD-N) with a stem-loop structure. Under stable conditions, the GSDMD was maintained in an inactive state. When relevant danger signals appear, GSDMD is cleaved by the activated inflammatory caspases to produce GSDMD-N.[12] GSDMD-N is transferred to the cell membrane and oligomerizes to form pores, resulting in cellular injury and release of inflammatory cytokines.[13] GSDMD-dependent neutrophil extracellular traps reportedly promote renal fibrosis in obstructive nephropathy by facilitating the macrophage-to-myofibroblast transition and inflammation.[14] Moreover, GSDMD-mediated pyroptosis contributes to the occurrence and progression of diabetic nephropathy.[15,16] However, whether GSDMD is implicated in the development of Ang II–induced hypertensive nephropathy has not been determined.

In this study, we observed a significant increase in the expression of GSDMD-N in renal tubular epithelial cells stimulated by Ang II. The absence of GSDMD alleviated Ang II–induced kidney injury and fibrosis in mice, indicating that GSDMD plays a critical role in the pathogenesis of Ang II-mediated kidney damage. Consequently, we further investigated the molecular mechanisms underlying the involvement of GSDMD in hypertensive nephropathy. Additionally, this research employed a GSDMD inhibitor GI-Y1 to assess its effects on hypertensive nephropathy. This study aims to provide a potential target and a therapeutic agent for the clinical management of hypertensive nephropathy.

Materials and Methods

Animal Experiments

All experimental procedures received the approval from the Ethics Approval Committee of the Animal Experimental Center at Second Affiliated Hospital of Jiaxing University (no. JEXY2021JX044) as well as those outlined in the NIH Guide for the Care and Use of Laboratory Animals. C57BL/6 mice with a Gsdmd knockout ($Gsdmd^{-/-}$) were obtained from GemPharmatech Co., Ltd. (strain no. T010437). Hypertensive renal injury in mice was induced by subcutaneous administration of Ang II via osmotic mini-pumps for four weeks (1000 ng/kg/min, Alzet Model 1004, CA, USA). Normal saline was used as the vehicle for Ang II injection. A blood pressure telemetry system (BP-2010A, Softron Biotechnology, Tokyo, Japan) was used to monitor the blood pressure weekly from 1:00 pm to 5:00 pm.[1] Age- and weight-matched (8-week-old) male wild-type C57BL/6 mice (WT) and male $Gsdmd^{-/-}$ mice were randomly divided into four groups (n=6): WT-saline, $Gsdmd^{-/-}$-saline, WT mice subjected to Ang II injection (WT-Ang II group), and $Gsdmd^{-/-}$ mice subjected to Ang II injection ($Gsdmd^{-/-}$-Ang II group).[2] Overexpression of Gsdmd: The recombinant adeno-associated virus serotype 9 (AAV9) virus carrying Gsdmd cDNA (CMV-betaGlobin-GSDMD -3Flag-SV40 PolyA) was used for Gsdmd overexpression ($Gsdmd^{oe}$). Male $Gsdmd^{-/-}$ mice (8 weeks old) were randomized into two groups (n = 6): the experimental group received a tail vein injection of AAV9 twice at a total dose of 2×10^{11} vg, followed by Ang II injection (Ang II-$Gsdmd^{oe}$), and the negative control group received an equivalent volume of vehicle via the tail vein, followed by Ang II injection (Ang II–Vehicle).[3] For GI-Y1 treatment, male C57BL/6 mice (8 weeks old) were randomly divided into three groups (n=6): saline vehicle, Ang II–vehicle, and Ang II-GI-Y1 groups. GI-Y1 (50 mg/kg, i.g., every other day) was administered to the Ang II + GI-Y1 group for 4 weeks, whereas the other two groups received an equivalent volume of vehicle. At the end of the experiment, the mice were euthanized using 1% pentobarbital sodium (50 mg/kg, intraperitoneal injection). Kidney tissue, serum, and urine samples were collected. The levels of renal function biomarkers, including serum creatinine, urine nitrogen, urinary albumin, and urinary creatinine, were measured using a Beckman Coulter AU480 Chemistry Analyzer (PBMA).

In vivo Randomization and Blinding Procedures

For animal experiment, we utilized a random number table for the purpose of randomization. In summary, all animal experiments conducted in this study were performed and analyzed in a blinded manner. Treatment groups were assigned randomly. Each mouse was given a temporary random number within the specified weight range. After mice had been allocated into their respective groups, they received permanent numerical designations within their cages. For each group, one cage was selected at random from the total pool of cages. All data were collected and analyzed by two observers who remained unaware of group assignments or treatment conditions affecting the animals.

Cell Culture and Treatment

Mouse glomerular mesangial cells (SV40 MES-13) and human embryonic kidney 293T cells (293T cells) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Mouse renal tubular epithelial cells (TCMK-1) and a murine podocyte cell line (MPC-5) were obtained from Shanghai Tongpai Biological Technology (Shanghai, China). 293T and MPC-5 cells were incubated in DMEM (Gibco, Grand Island, NY, USA) supplemented with 1% penicillin/streptomycin (Sbjbio, Nanjing, BC-CE-007) and 10% fet bovine serum (FBS; Thermo Fisher Scientific). SV40 MES-13 cells were maintained in a medium supplemented with 1/4 DMEM/F12, 1% penicillin/streptomycin, and 10% FBS. TCMK-1 cells were maintained in 1640 medium (Gibco, C11875500BT) supplemented with 1% penicillin/streptomycin and 10% FBS. Upon reaching 80–90% confluence, the cells were treated with Ang II (1 μM) for 24 h to induce renal injury. In the control group, the cells were treated with an equal volume of the corresponding solvent. To confirm the role of AQP4, the cells were treated with the AQP4 inhibitor TGN020 for 2 h before Ang II stimulation to mimic renal injury.

GSDMD was silenced in TCMK-1 cells using a small interfering RNA (siRNA; 5′-GGAAATGGAGTTGAGACAA-3′). Sequences were constructed using RiboBio (Guangdong, China). All the transfections were performed using Lipofectamine 2000 (Invitrogen). GSDMD was overexpressed using an encoding plasmid (GeneChem) in combination with Lipofectamine 3000 (Invitrogen).

Cleavage Under Targets and Tagmentation

CUT and Tag experiments were conducted using a Novo NGS CUT and Tag 3.0 high-Sensitivity Kit (Novaprotein, N259-YH01), and all steps were performed according to the manufacturer's instructions. For targeted proteins, 0.1 million cells were used. The primary antibodies were used at a 1:50 dilution. Protein concentration was increased by overexpressing the GSDMD plasmid in 293T cells. The obtained DNA was subjected to agarose gel electrophoresis to determine the optimal number of cycles followed by PCR amplification. Subsequently, the DNA levels of different samples were quantified using qPCR. The predicted binding sites of the GATA2 and AQP4 promoters were retrieved from the JASPAR database (https://jaspar.genereg.net/analysis). The three sites and their corresponding primers are listed in Supplementary Tables S1 and S2, respectively.

RNA-Sequencing (RNA-Seq)

RNA was extracted from kidney samples using TRIzol (Invitrogen, CA, USA). Sample separation and purification, along with library construction for transcriptome sequencing, were conducted by LC-BIO Technologies (Hangzhou) Co., Ltd. The R package edgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html) was utilized to analyze differentially expressed genes across sample, defining significant differential expression as fold change > 2 or fold change < 0.5 and with p value < 0.05 (using a parametric F-test comparing nested linear models). Finally, Gene Ontology (GO) enrichment analysis for the identified genes was conducted using DAVID software (https://david.ncifcrf.gov/).

Correlation Analysis

Based on the promoter sequence of 2000 bp upstream from the transcription start site of AQP4, which was obtained from NCBI, we predicted the transcription factors for mouse AQP4 using three different online resources: TFDB (https://guolab.wchscu.cn/AnimalTFDB4//#/), GTRD (https://gtrd20-06.biouml.org), and PROMO (https://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). The intersection of these predicted transcription factors was identified, and a pearson correlation analysis between their expression levels and that of GSDMD in our transcriptome sequencing data was performed using OmicStudio tools (https://www.omicstudio.cn/tool) to generate network plots.

Statistical Analysis

The experiments were conducted in a randomized blinded manner. Data are expressed as mean ± SEM (Standard Error of the Mean) from three independent experiments. For in-vitro experiments, as each experimental data set is an average of a large number of cultured cells, we assumed the data was normally distributed based on the central limit theorem. For the analysis of in-vivo experiments, our sample sizes were above or equal to 6, the normal distribution of each data was evaluated for normal distribution using the Shapiro–Wilk test. $P > 0.05$ on test indicated that the data were approximately normally distributed for each group. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA). One-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons or Student's *t*-test for two groups was performed. Statistical significance was set at $P < 0.05$.

An expanded Materials and Methods section is available in the *Supplementary Information*.

Results

GSDMD is Activated in Renal Tubular Epithelial Cells Induced by Ang II

Our first objective was to determine the GSDMD levels in the kidneys of Ang II-treated mice. After reviewing the data that were made accessible to the public on the GEO website, we discovered that mice with Ang II–induced hypertensive nephropathy exhibited higher levels of GSDMD gene expression in the kidney (NCBI GEO; GSE207102[17] and GSE182517[18]) than in the respective controls (Figure 1A). Moreover, the number of GSDMD-positive cells was increased in hypertensive nephropathy patients according to single-cell sequencing data (NCBI GEO; GSE174219[19], Figure 1B). Based on these data, the GSDMD-N protein levels in the kidneys of Ang II–induced hypertensive mice were assessed. Western blotting and immunohistochemical staining revealed that GSDMD expression was dramatically upregulated in the kidney tissues of mice following Ang II induction (Figure 1C and D). These findings provide evidence

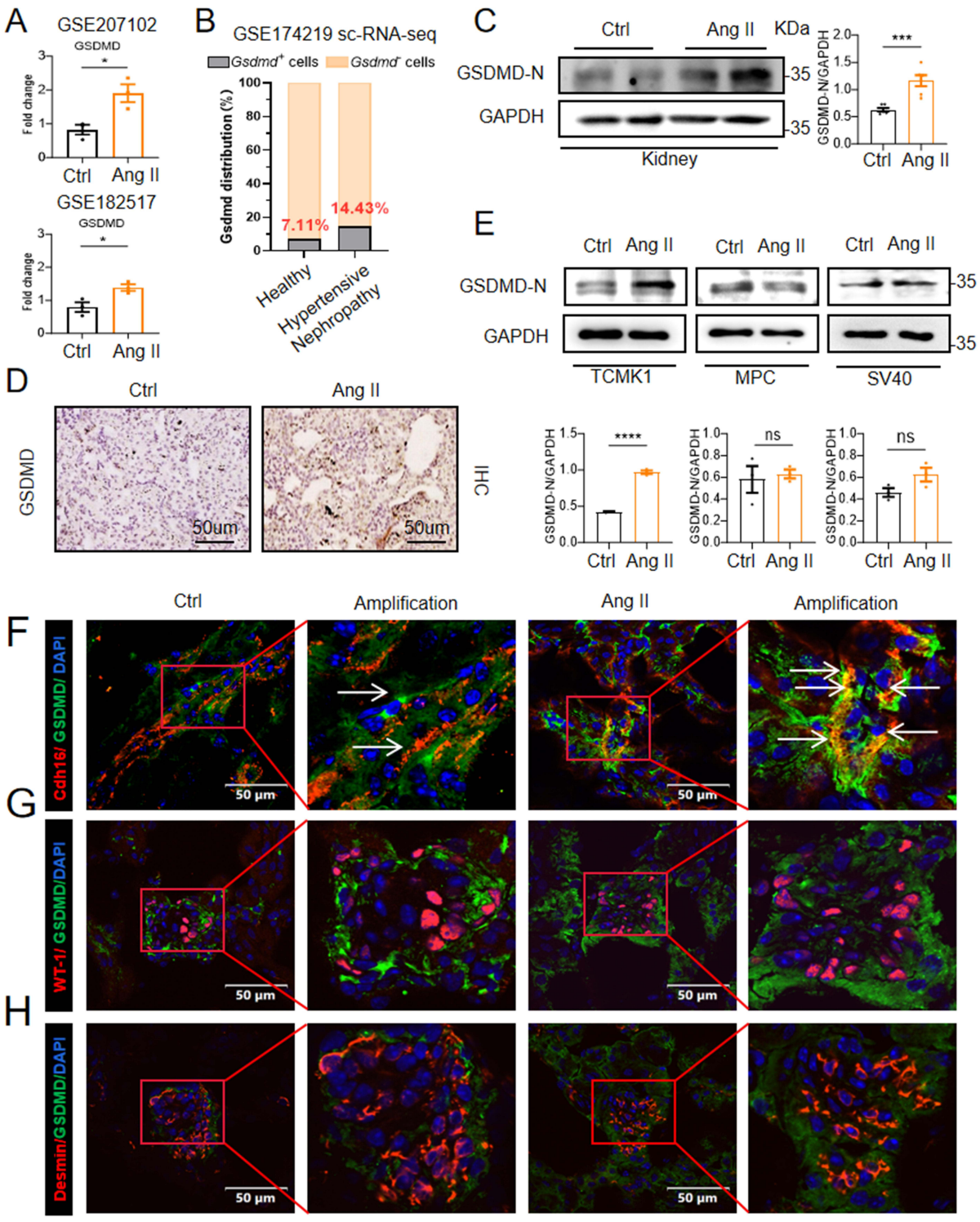


Figure 1 GSDMD is activated in renal tubular epithelial cells induced by Ang II. (**A**) RNA-Seq analysis of relative *Gsdmd* mRNA levels in the kidney tissues of mice induced by Ang II. The data were obtained from the GSE207102 and GSE182517 datasets. (**B**) Single-cell sequencing analysis of *Gsdmd* expression in kidney tissues from healthy individuals and hypertensive nephropathy patients. The data were retrieved from the RNA sequencing dataset GSE174219. (**C**) Expression of the GSDMD-N protein in kidney tissue from control and Ang II–induced mice was tested by Western blotting (WB) and the corresponding statistical results (n = 6). (**D**) GSDMD immunohistochemical staining of kidney tissues from control and Ang II–induced mice (scale bar = 50 μm). (**E**) Expression of GSDMD-N in control and Ang II–induced mouse glomerular mesangial cells (SV40-MES-13), mouse renal tubular epithelial cells (TCMK-1) and a murine podocyte cell line (MPC-5) and corresponding statistical results (n = 3). (**F**) Representative immunofluorescence images showing the colocalization of GSDMD and Cadh16 (a renal tubular epithelial cell marker) in kidney tissues from control and Ang II–induced mice (scale bar = 50 μm). (**G**) Representative immunofluorescence images showing the colocalization of GSDMD and WT-1 (a podocyte cell marker) in kidney tissues from control and Ang II–induced mice (scale bar = 50 μm). (**H**) Representative immunofluorescence images showing the colocalization of GSDMD and Desmin (a glomerular cell marker) in kidney tissues from control and Ang II–induced mice (scale bar = 50 μm). The data are expressed as the mean ± SEM (Standard error of mean). ****$P < 0.0001$; ***$P < 0.001$; *$P < 0.05$; ns, $P > 0.05$.

that GSDMD is involved in the development of Ang II–induced hypertensive nephropathy. To investigate the distribution of GSDMD induced by Ang II in the kidneys of mice, the protein levels of GSDMD in mouse renal tubular epithelial (TCMK-1) cells, mouse glomerular mesangial (SV40 MES-13) cells, and mouse podocytes (MPC-5) were analyzed. As depicted in Figure 1E, GSDMD expression was significantly increased in TCMK-1 cells compared to that in the other two cell types. Furthermore, the distribution of GSDMD in the kidney was determined by an immunofluorescence assay using different cell markers, including Cadh16, which encodes renal tubular epithelial cells[20], WT1, which encodes podocytes[21], and desmin, which encodes glomerular cells.[22] Immunofluorescence double staining showed that GSDMD colocalized with Cadh16 but not WT1 or Desmin (Figure 1F–H), indicating that the increase in GSDMD induced by Ang II was primarily derived from tubular epithelial cells. Taken together, these findings suggest that GSDMD is activated in renal tubular epithelial cells after Ang II stimulation.

Gsdmd Knockout Ameliorates Ang II-Induced Renal Injury and Fibrosis in Mice

To determine the effect of GSDMD on the pathogenesis of hypertensive renal injury, Gsdmd$^{-/-}$ mice were used. Subcutaneous implantation of Ang II via osmotic mini-pumps was performed in mice to establish a hypertensive nephropathy model. Serum creatinine, urea nitrogen, and urine albumin-to-creatinine ratios, which are crucial indicators of renal function, were measured. The results showed that, compared with WT mice, Gsdmd$^{-/-}$ mice treated with Ang II exhibited lower serum creatinine and urea nitrogen levels and urine albumin-to-creatinine ratio (Figure 2A–C), indicating that Gsdmd knockout mitigated Ang II–induced renal injury. Moreover, H&E staining revealed a reduction in renal structural abnormalities in Ang II–infused mice with a GSDMD deficiency (Figure 2D). The effect of GSDMD on Ang II–induced renal fibrosis was examined. Masson's and Sirius Red staining revealed that GSDMD deficiency significantly reduced Ang II–induced renal interstitial collagen deposition (Figure 2E–H). Fibronectin (FN) is an extracellular matrix protein that can bind to various components on the cell surface and in the extracellular matrix. During the process of renal fibrosis, the expression of FN increases.[23] collagens type III (COL-III) and collagens type I (COL-I) is principal components of the extracellular matrix,[24]. The over expression and accumulation of COL-III and COL-I are significant indicators of fibrosis.[25] Transforming growth factor-beta (TGF-β) is a pleiotropic cytokine, TGF-β facilitates the progression of fibrosis through multiple mechanisms.[26,27] Myofibroblasts are the primary source of increased deposition of extracellular matrix (ECM) in renal fibrosis. α-SMA is a marker protein for myofibroblasts, whose increased expression is often an important indicator of abnormal transformation of myofibroblasts.[28] Immunohistochemical staining for COL-I and TGF-β in kidney tissue was carried out and the results showed that the positive staining area of COL-I and TGF-β was decreased in Gsdmd$^{-/-}$-Ang II mice (Figure 2I–L), indicating that GSDMD deficiency markedly improved Ang II–induced renal fibrosis. Furthermore, the protein levels of FN, COL-III, COL-I, TGF-β and α-SMA in renal tissue were detected. The expression of these indicators about fibrosis all decreased in the kidneys of Ang II-treated Gsdmd$^{-/-}$ mice (Figure 2M–N). To exclude the gender differences in hypertension and kidney disease, we also modeled and sampled female mice, and we found that the protein levels of FN, COL-III, COL-I, TGF-β and α-SMA were also decreased in the kidneys of Ang II-treated Gsdmd$^{-/-}$ female mice (Supplementary Figure S1A and B). In addition, the mRNA levels of *IL-1β* and *IL-18* were detected in the kidneys. The results revealed a significant reduction in the mRNA levels of *IL-1β* and *IL-18* induced by Ang II in the kidneys of Gsdmd$^{-/-}$- mice (Figure 2O). These indicate that Gsdmd knockout alleviates the inflammatory response caused by Ang II-stimulated hypertensive nephropathy. Therefore, we measured the mRNA levels of inflammatory cytokines and chemokines such as IL-6, TNF-α, Cxcl1, Ccl2, Vcam1, and Icam2, and found that the knockout of GSDMD led to their reduction (Supplementary Figure S1C). Given that inflammation is more prominent in the acute model, We further validated this viewpoint in an acute kidney injury model and discovered that the deletion of GSDMD significantly mitigated structural abnormalities in renal tissue induced by ischemia-reperfusion (Supplementary Figure S2A), and reduced the expression levels of FN, COL-III, COL-I, TGF-β and α-SMA in the renal tissue (Supplementary Figure S2B and C). Taken together, these findings demonstrate that GSDMD deletion ameliorates Ang II–induced renal injury and fibrosis by alleviating inflammation.

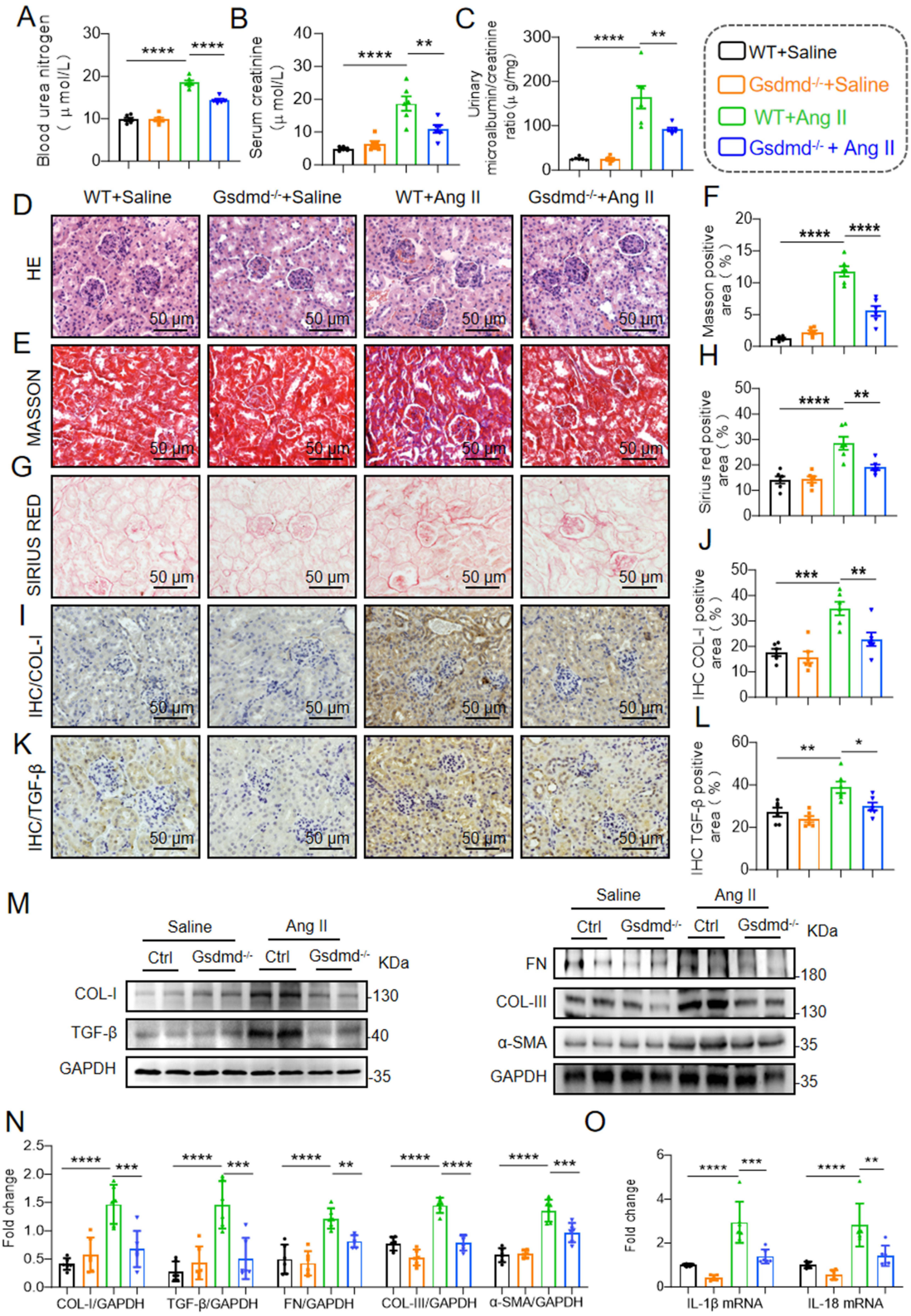


Figure 2 Gsdmd knockout ameliorates Ang II–induced renal injury and fibrosis in mice. (**A–C**) The levels of serum urea nitrogen (**A**), creatinine (**B**), and urinary microalbumin/creatinine (**C**) in WT and Gsdmd$^{-/-}$ mice induced by Ang II or Saline (n = 6). (**D**) Representative H&E images of kidney tissues from WT and Gsdmd$^{-/-}$ mice induced by Ang II or Saline (scale bar = 50 μm). (**E–H**) Representative Masson and Sirius red staining images of kidney tissues from WT and Gsdmd$^{-/-}$ mice induced by Ang II or Saline (scale bar = 50 μm) and statistical analysis (n = 6). (**I–L**) COL-I and TGF-β immunohistochemical staining of kidney tissues from WT and Gsdmd$^{-/-}$ mice induced by Ang II or Saline (scale bar = 50 μm) and the corresponding statistical results (n = 6). (**M** and **N**) Expression levels of the FN, COL-III, COL-I, TGF-β and α-SMA proteins in kidney tissue from WT and Gsdmd$^{-/-}$ mice induced by Ang II or Saline were measured via WB, and the corresponding statistical results (n = 6). (**O**) The mRNA levels of IL-1β and IL-18 in the kidney tissue from WT and Gsdmd$^{-/-}$ mice induced by Ang II or Saline. (n = 6). The data are expressed as the mean ± SEM. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05.

Gsdmd Overexpression Exacerbates Renal Injury and Fibrosis Induced by Ang II in Mice

To further demonstrate the connection between hypertensive renal injury and GSDMD-mediated pyroptosis, an AAV9 virus harboring GSDMD cDNA (CMV-betaGlobin-GSDMD-3Flag-SV40 PolyA) was used to overexpress Gsdmd in Gsdmd$^{-/-}$-Ang II–treated mice. Changes in histological pathology showed that overexpression of Gsdmd increased glomerular abnormalities and collagen deposition, as evidenced by H&E, Masson's trichrome, and Sirius Red staining (Figure 3A–E). Moreover, immunohistochemical staining showed that the positive staining area of COL-I and TGF-β increased in Gsdmd^{oe}- mice induced by Ang II (Figure 3F–I). These results were confirmed by Western blot.

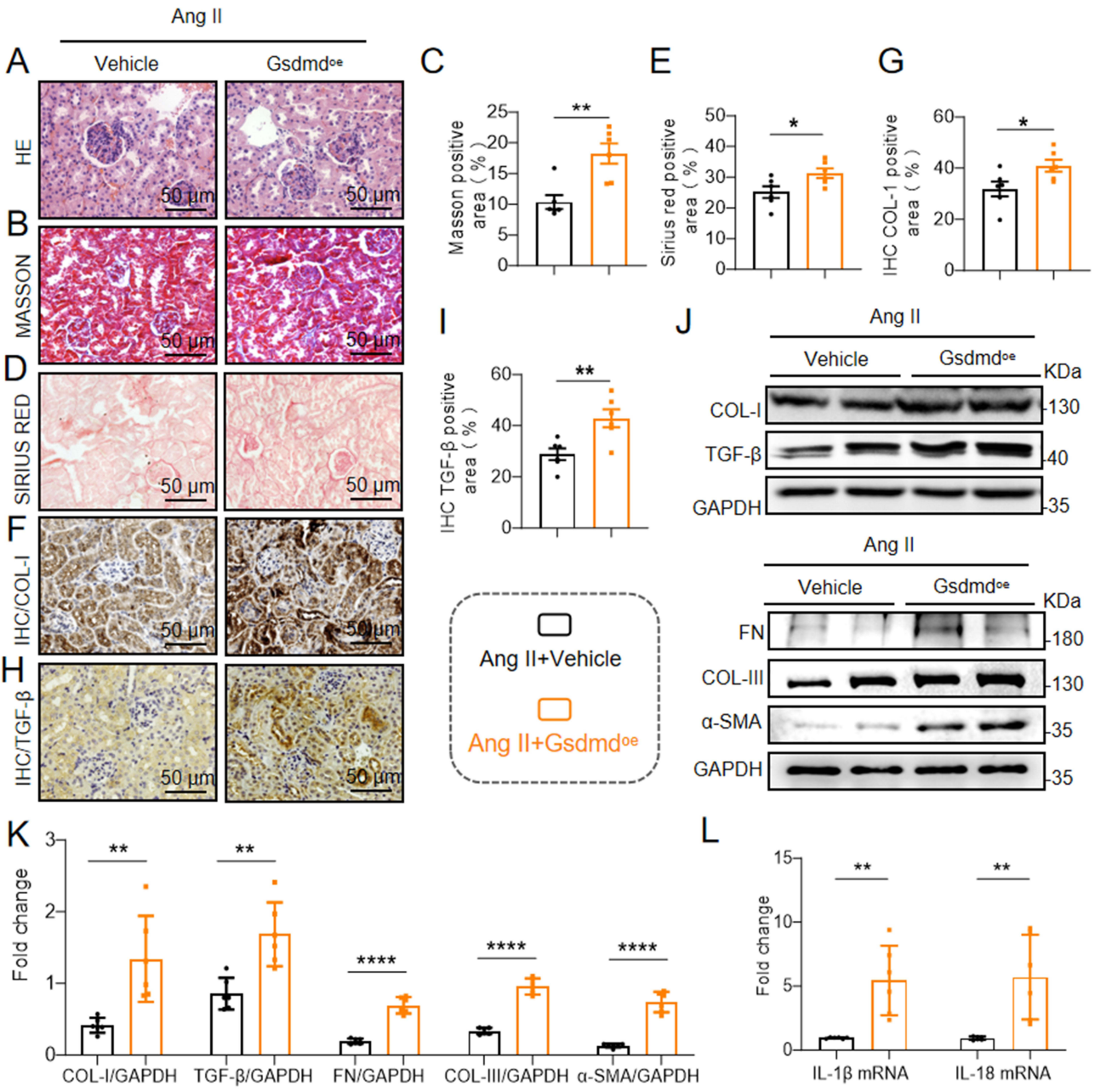


Figure 3 Gsdmd overexpression exacerbates renal injury and fibrosis induced by Ang II in mice. (**A**) Representative H&E images of kidney tissue from Gsdmd$^{-/-}$ and Gsdmd-overexpressing (Gsdmd^{oe}) mice induced by Ang II (scale bar = 50 μm). (**B-E**) Representative Masson and Sirius red staining images of kidney tissue from Gsdmd$^{-/-}$ and Gsdmd^{oe} mice induced by Ang II (scale bar = 50 μm) and statistical analysis (n = 6). (**F-I**) COL-I and TGF-β immunohistochemical staining of kidney tissues from Ang II–induced Gsdmd$^{-/-}$ and Gsdmd^{oe} mice (scale bar = 50 μm) and statistical analysis (n = 6). (**J** and **K**) Expression levels of the FN, COL-III, COL-I, TGF-β and α-SMA proteins in kidney tissue from Gsdmd$^{-/-}$ and Gsdmd^{oe} mice induced by Ang II were measured via WB and the corresponding statistical results (n = 6). (**L**) IL-1β and IL-18 mRNA levels in kidney tissue from Gsdmd$^{-/-}$ and Gsdmd^{oe} mice induced by Ang II (n = 6). The data are expressed as the mean ± SEM. ****$P < 0.0001$; **$P < 0.01$; *$P < 0.05$.

Overexpression of Gsdmd led to an increase in FN, COL-III, COL-I, TGF-β and α-SMA protein levels in kidney tissue (Figure 3J and K). In addition, the mRNA levels of *IL-1β* and *IL-18* were assessed. We found that overexpression of Gsdmd enhanced the Ang II–induced increase in the mRNA levels of *IL-1β* and *IL-18* (Figure 3L). These results indicate that Gsdmd overexpression exacerbates Ang II–induced renal injury and fibrosis in mice.

GSDMD Aggravates Ang II-Mediated Fibrosis in TCMK-1 Cells

Subsequently, the renal tubular epithelial cell line TCMK-1 was used to explore the role of GSDMD in Ang II–induced fibrosis. GSDMD deficiency in TCMK-1 cells was achieved by transfecting siRNA against GSDMD (si-GSDMD) (Figure 4A and B). GSDMD deficiency reversed the Ang II–induced increase in the expression of fibrosis-related proteins including FN, COL-III, COL-I, TGF-β and α-SMA (Figure 4C and D). We also enhanced the expression of GSDMD in TCMK-1 cells by transfecting them with a GSDMD plasmid (pla-GSDMD) (Figure 4E and F). In contrast, Gsdmd overexpression aggravated the expression of FN, COL-III, COL-I, TGF-β and α-SMA (Figure 4G and H). These findings suggest that GSDMD amplifies Ang II–induced fibrosis in TCMK-1 cells.

GSDMD Affects Ang II-Induced Renal Fibrosis by Modulating AQP4

To better characterize the downstream pathogenic mechanism by which GSDMD affects Ang II–induced renal fibrosis, RNA sequencing was performed on kidney tissues from WT and Gsdmd$^{-/-}$ mice induced by Ang II. Gene Ontology (GO) enrichment analysis indicated that the differentially expressed genes were associated with extracellular space and water homeostasis (Figure 5A). It is evident that water homeostasis genes undergo alterations, prompting us to focus on aquaporins. Hence, the number of cells positive for aquaporin genes in the kidneys of hypertensive nephropathy patients was calculated according to the GSE174219 dataset in the GEO database. The expression levels of aquaporin 1(AQP1), aquaporin 2 (AQP2), aquaporin3(AQP3) were high, but the changes were not statistically significant. However, aquaporin 4(AQP4) expression significantly increased (Figure 5B). These findings suggest that AQP4 may be involved in the downstream mechanism by which GSDMD affects Ang II–induced renal fibrosis. Therefore, we assessed the relationship between GSDMD and AQP4 expression. The results showed that AQP4 expression increased in Ang II-stimulated TCMK-1 cells (Figure 5C). Interestingly, GSDMD deficiency reversed Ang II–induced increase in AQP4 expression (Figure 5D). However, Gsdmd overexpression aggravated Ang II–induced increase in AQP4 expression (Figure 5E). To further elucidate the role of AQP4, TCMK-1 cells were pretreated with the AQP4 inhibitor, TGN020, and overexpressing Gsdmd. We found that blocking AQP4 with TGN020 contributed to reduced renal fibrosis induced by Ang II, as evidenced by the decreased expression of FN, COL-III, COL-I, TGF-β and α-SMA proteins (Figure 5F and G). However, after inhibition of AQP4, no significant difference in FN, COL-III, COL-I, TGF-β and α-SMA expression was observed between cells overexpressing Gsdmd and those without Gsdmd overexpression (Figure 5F and G), suggesting that GSDMD affected Ang II–induced renal fibrosis partly through AQP4. Additionally, following Gene Set Enrichment Analysis (GSEA) on the transcriptomic data (Supplementary Figure S3), we uncovered potential relevant downstream pathways linked to mitochondrial functions. Considering previous publications from our team indicating a strong relationship between GSDMD and mitochondrial damage as well as pore formation[29], it seems likely that this protein also plays a critical role in renal fibrosis.

GSDMD Increases Ang II-Induced GATA2 Transcription Factor Expression, Which Further Promotes AQP4 Transcription

Next, we investigated whether GSDMD regulated AQP4 expression via transcription factors. The transcription factors of AQP4 were identified using data predictions from three databases. Several transcription factors of AQP4 been identified, including *Yy 1, Pou5f1, Stat1, Rela, Pou3f1, Gata2, Gata1, and Tal1* (Figure 6A). Correlation analysis between the expression of these transcription factors and the differential gene expression data obtained from RNA sequencing was conducted. The results showed a positive correlation between *Gata2* and differentially expressed genes in the Gsdmd$^{-/-}$-Ang II and WT-Ang II groups (Figure 6B). Subsequently, the mRNA of TCMK-1 was extracted and we found that Gsdmd overexpression enhanced the expression of the *Gata2* transcription factor when stimulated by Ang II (Figure 6C). Moreover, CUT and Tag

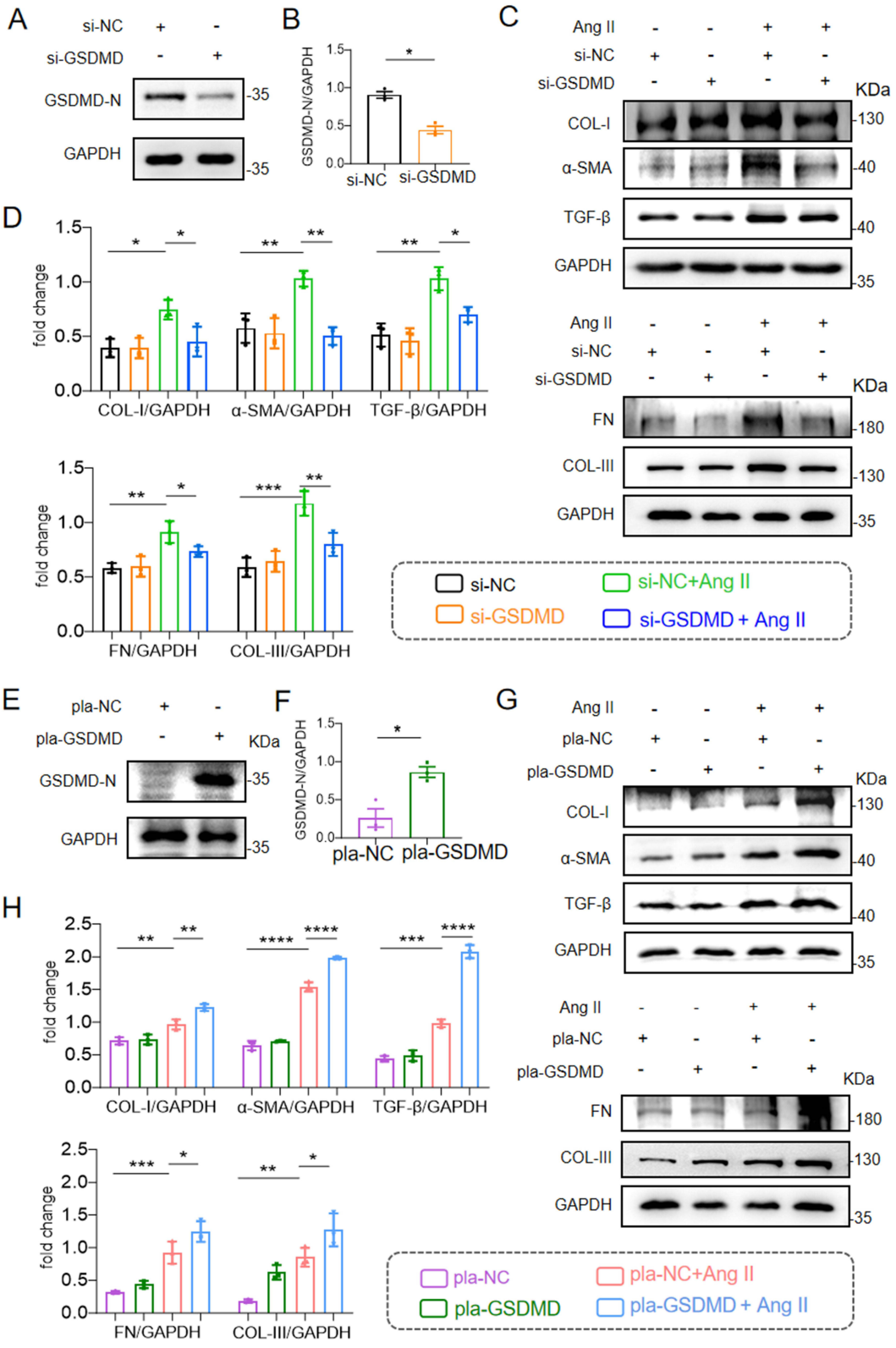


Figure 4 GSDMD aggravates Ang II-mediated fibrosis in TCMK-1 cells. (**A** and **B**)After transfection with GSDMD siRNA (si-GSDMD) or negative control siRNA (si-NC), The expression level of the GSDMD-N protein in TCMK-1 cells was tested by WB and the corresponding statistical results (n = 3). (**C** and **D**) Expression of the FN, COL-III, COL-I, TGF-β and α-SMA proteins in TCMK-1 cells transfected with si-GSDMD or si-NC then induced by Ang II, and the corresponding statistical results (n = 3). (**E** and **F**) After transfection with an encoding GSDMD plasmid (pla-GSDMD) or a negative control vector (pla-NC), The expression level of the GSDMD-N protein in TCMK-1 cells was tested by WB and the corresponding statistical results (n = 3). (**G** and **H**) Expression of the FN, COL-III, COL-I, TGF-β and α-SMA proteins in TCMK-1 cells transfected with pla-GSDMD or pla-NC then induced by Ang II and the corresponding statistical results (n = 3). The data are expressed as the mean ± SEM. ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

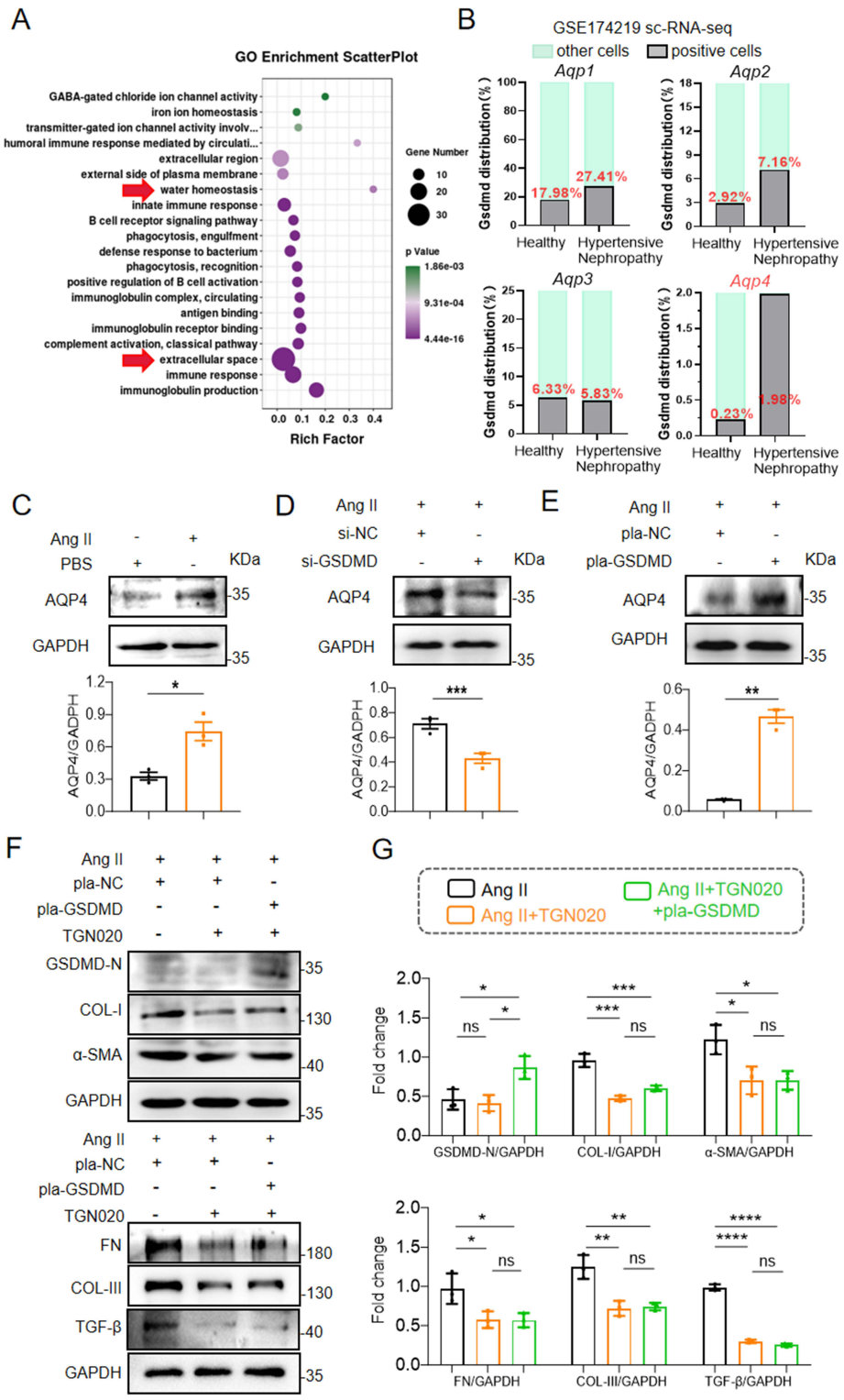


Figure 5 GSDMD affects Ang II–induced renal fibrosis by modulating AQP4. (**A**) Gene Ontology (GO) enrichment analysis of RNA sequencing data from WT and Gsdmd$^{-/-}$ mice stimulated with Ang II. (**B**) Single-cell sequencing analysis of AQP1, AQP2, and AQP3 and AQP4 expression in kidney samples from healthy individuals and patients with hypertensive nephropathy. Data were retrieved from the scRNA-seq data of GSE174219. (**C**) Expression of AQP4 in TCMK-1 cells induced by Ang II was detected by WB and the corresponding statistical results (n = 3). (**D**) Expression of AQP4 in si-NC- or si-GSDMD-transfected TCMK-1 cells induced by Ang II was detected by WB and the corresponding statistical results (n = 3). (**E**) Expression levels of AQP4 in control vector- or pla-GSDMD-transfected TCMK-1 cells induced by Ang II were detected by WB and the corresponding statistical results (n = 3). (**F** and **G**) Cells were transfected with the control vector or Pla-GSDMD and pretreated with TGN020 (an AQP4 inhibitor), followed by Ang II treatment. The expression of GSDMD-N, FN, COL-III, COL-I, TGF-β and α-SMA in TCMK-1 cells from each group was tested by WB and the corresponding statistical results (n = 3). Data are expressed as mean ± SEM. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05; ns, P > 0.05.

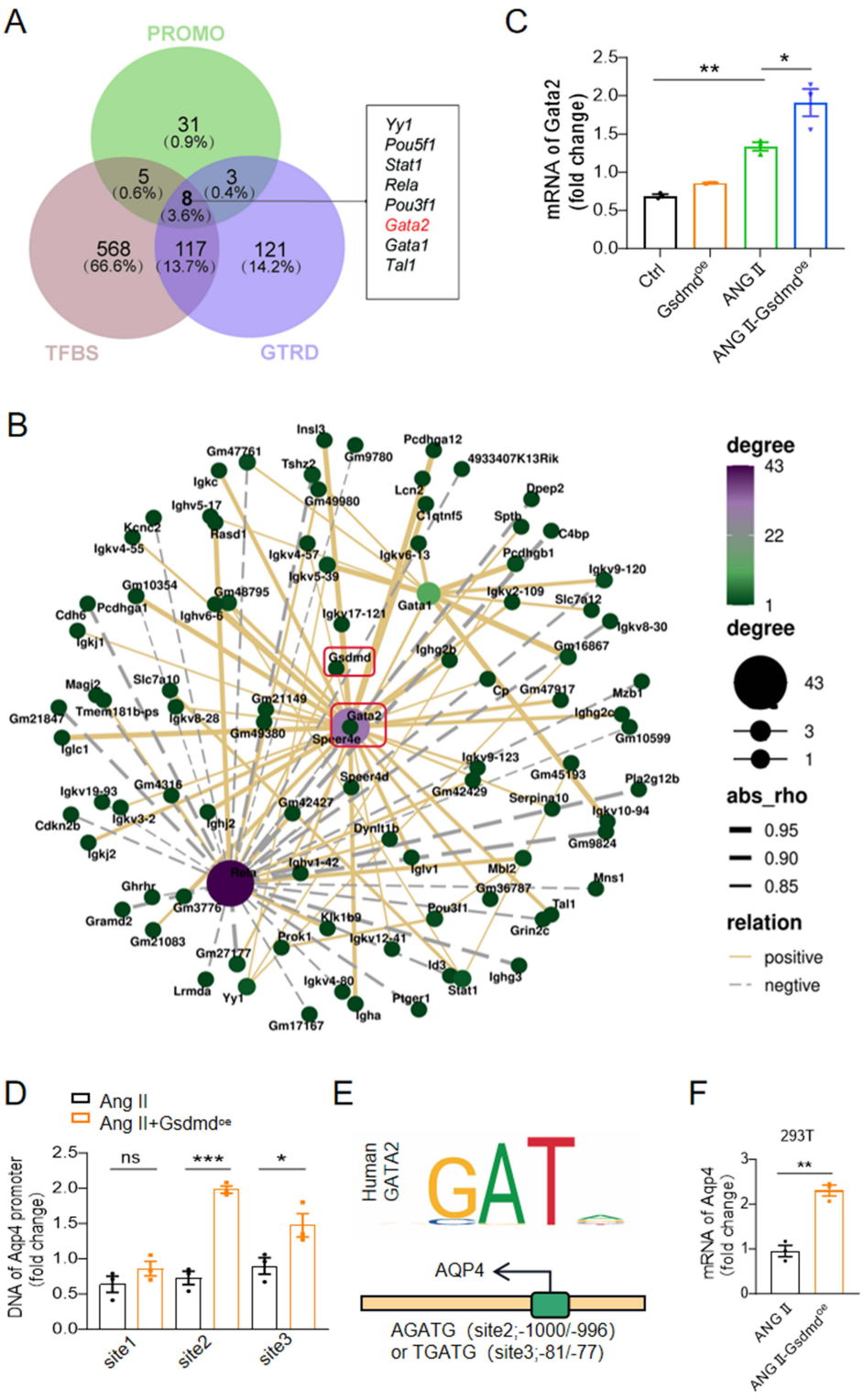


Figure 6 GSDMD increases Ang II–induced GATA2 transcription factor expression, which further promotes AQP4 transcription. (**A**) Venn diagram of the transcription factors of AQP4 based on prediction data from three databases (TFBS, GTRD, and PROMO), which contain *Yy1, Pou5f1, Stat1, Rela, Pou3f1, Gata2, Gata1*, and *Tal1*. (**B**) Correlation analysis of the transcription factor data shown in Figure 6A and significant genes based on RNA sequencing data shown in Figure 5A. (**C**) The mRNA levels of GATA2 in pla-NC- and pla-GSDMD-transfected TCMK-1 cells without Ang II induction (n = 3). (**D**) CUT&Tag analysis showed that the GATA2 antibody could bind to the DNA in the target cells, and among these DNAs, the predicting site2 and site3 of the transcriptional promoter of Aqp4 binding to GATA2 increased after Gsdmd overexpression. (**E**) Depiction of the GATA2 binding sites confirmed in Figure 6C; these sites are located at −1000/-996 (2) and −81/-77 (3) bp upstream of the transcription start of the human AQP4 promoter. (**F**) *Aqp4* mRNA expression in 293T cells transfected with pla-NC or pla-GSDMD, and induced with Ang II (n = 3). Data are expressed as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, $P > 0.05$.

experiments were performed in 293T cells, which showed that *Gata2* could bind to the *Aqp4* promoter sequence and facilitate AQP4 transcription (Figure 6D–F).

The GSDMD Inhibitor GI-Y1 Alleviates Ang II-Induced Renal Injury and Fibrosis in Mice

To further confirm the role of GSDMD in hypertensive nephropathy, a specific GSDMD inhibitor (GI-Y1) was used in the subsequent experiments. GI-Y1, a small-molecule GSDMD inhibitor developed by our research team, exerts its function by inhibits GSDMD-N oligomerization in the cell membrane.[30] Firstly, we conducted cellular experiments and observed that GI-Y1 does not affect cell viability (Supplementary Figure S4A) and can effectively reduce Ang II-mediated TCMK-1 cell fibrosis in a dose-dependent manner (Supplementary Figure S4B and C). Compared to the mice induced by Ang II, GI-Y1 treatment reduced the levels of serum creatinine and urea nitrogen, and the urine albumin-to-creatinine ratio (Figure 7A–C). H&E staining showed that the renal structural abnormalities caused by Ang II were reversed by GI-Y1 treatment (Figure 7D). Masson'sand Sirius Red staining showed a notable decrease in interstitial collagen deposition in the kidneys of Ang II-treated mice treated with GI-Y1 (Figure 7E–H). Moreover, the immunohistochemical positive staining areas of COL-I and TGF-β were decreased in Ang II–induced mice treated with GI-Y1 (Figure 7I–L), which was further supported by the results of Western blotting, which showed that the expression of FN, COL-III, COL-I, TGF-β and α-SMA proteins in the kidneys of Ang II–induced mice treated with GI-Y1 was dramatically reduced (Figure 7M and N). The mRNA levels of *IL-1β* and *IL-18* in the kidney tissue were also quantitatively analyzed. GI-Y1 treatment significantly decreased the *IL-1β* and *IL-18* mRNA levels in the kidneys of mice stimulated with Ang II (Figure 7O). Taken together, these findings suggest that mice treated with GSDMD inhibitors are resistant to Ang II–induced renal injury and fibrosis.

Discussion

The aim of this study was to determine the role of GSDMD in the development and progression of hypertensive nephropathy. We found that the expression of GSDMD-N, the active segment of GSDMD, was dramatically elevated in renal tubular epithelial cells stimulated with Ang II. The absence of GSDMD attenuated Ang II–induced renal injury and fibrosis in mice, whereas the overexpression of Gsdmd promoted renal injury and fibrosis. Mechanistically, upon activation, GSDMD mediated inflammation on the one hand, and on the other hand it increased Ang II–induced GATA2 transcription factor expression. GATA2 further enhanced binding to the *Aqp4* promoter sequence and facilitated AQP4 transcription, leading to renal injury and fibrosis. Moreover, treatment with GI-Y1, an inhibitor of GSDMD, alleviated Ang II–induced renal injury and fibrosis, further confirming the effect of GSDMD in hypertensive nephropathy. Our research demonstrated that GSDMD played a pivotal role in the development and progression of hypertensive nephropathy.

Inflammation has been considered to be an important factor contributing to the development of renal fibrosis.[31] Inflammatory cell infiltration and fibroblast activation caused by the overexpression of inflammatory factors contribute to renal interstitial fibrosis and damage.[32] Pyroptosis, a type of death closely related to inflammation, induces the release of excessive inflammatory factors and exacerbates the inflammatory response. When triggered by specific danger signals, GSDMD, the executor of the pyroptosis protein, is cleaved by activated inflammatory caspases to produce an N-terminal fragment that gradually accumulates on the cell membrane and forms membrane pores, leading to cellular damage and inflammatory factor release.[33,34] Inflammatory factors such as IL-1β further mobilize macrophages and infiltrate, contributing to the progression of fibrosis. Studies using various experimental models have indicated that the NLRP3 inflammasome complex partially participates in the development of renal inflammation associated with hypertension.[35,36] Moreover, studies have shown that inhibition of NLRP3 with MCC950 alleviates renal inflammation and fibrosis, thereby improving renal dysfunction in hypertensive mice.[37,38] In our study, the expression of GSDMD-N protein was markedly increased in renal tubular epithelial cells after Ang II stimulation, and this change was accompanied by increased expression of fibrosis-related proteins, including FN, COL-III, COL-I, TGF-β and α-SMA. In addition, we found that the absence of GSDMD attenuated renal injury and fibrosis induced by Ang II, whereas the overexpression of Gsdmd

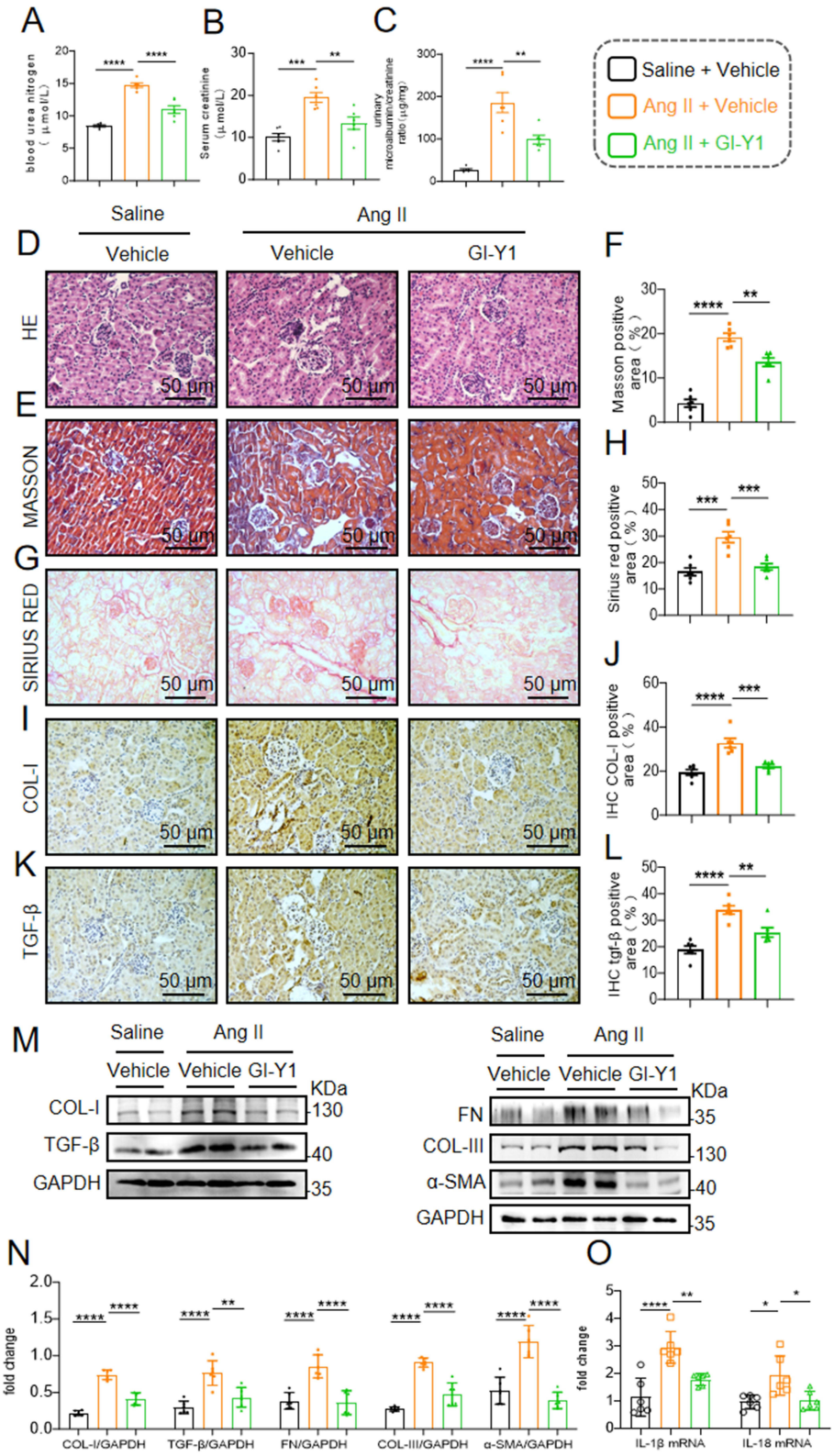


Figure 7 The GSDMD inhibitor GI-Y1 alleviates Ang II–induced renal injury and fibrosis in mice. (**A-C**) The levels of serum urea nitrogen (**A**), creatinine (**B**), and urinary microalbumin/creatinine (**C**) in Ang II–induced mice teated with GI-Y1(n = 6). (**D**) Representative H&E images of kidney tissue from Ang II–induced mice teated with GI-Y1 (scale bar = 50 μm). (**E-H**) Representative Masson and Sirius red staining images of kidney tissue from Ang II–induced mice teated with GI-Y1 (scale bar = 50 μm) and statistical analysis (n = 6). (**I-L**) COL-I and TGF-β immunohistochemical staining of kidney tissue from Ang II–induced mice teated with GI-Y1 (scale bar = 50 μm) and statistical analysis (n = 6). (**M** and **N**) Expression levels of the FN, COL-III, COL-I, TGF-β and α-SMA proteins in kidney tissue from Ang II–induced mice teated with GI-Y1 were detected via WB and the corresponding statistical results (n = 6). (**O**) The IL-1β and IL-18 mRNA levels in the kidney tissue from Ang II–induced mice teated with GI-Y1 were measured (n = 6). The data are expressed as the mean ± SEM. ****P < 0.0001; ***P < 0.001; **P < 0.01; *P < 0.05.

promoted renal injury and fibrosis. These data demonstrated that GSDMD-mediated pyroptosis and inflammation participated in the development of renal fibrosis and dysfunction stimulated by Ang II. Thus, GSDMD may serve as a molecular target for the treatment of hypertensive nephropathy.

To further elucidate the downstream pathogenic mechanisms by which GSDMD affected renal fibrosis and dysfunction stimulated by Ang II, RNA sequencing and GO enrichment analyses were performed. These findings demonstrate that genes with differential expression are linked to extracellular space and even to water homeostasis. Similarly, the classical protein involved in these processes, AQP4, was upregulated in the disease group, according to the GSE174219 dataset from the GEO database. In the present study, the expression of AQP4 varied with GSDMD concentration. GSDMD deficiency leads to a reduction in AQP4 expression, whereas Gsdmd overexpression results in an increase in AQP4 expression, suggesting that AQP4 may be involved in the effect of GSDMD on Ang II–induced renal fibrosis.

Aquaporin (AQP) is a protein that exists in renal tubular epithelial cells and regulates body water homeostasis and urine concentration.[39,40] Among various members of the AQP family, AQP4 is primarily found in the basolateral membrane of the main cells of the collecting duct. The main function of AQP4 is to regulate water reabsorption in the collecting tubule and the fluid volume in the extracellular space. A previous study showed that AQP4 expression is reduced in mice with hydronephrosis.[41] A defect in urinary concentrations has also been observed in *Aqp4* knockout mice.[42] AQP4 is not only involved in regulating body water homeostasis but also acts as a key molecule that initiates intracellular signaling events, regulates the secretion of cell inflammatory factors, and mediates inflammatory responses.[43,44] AQP4 knockout alleviates sepsis-induced neuronal injury and cognitive dysfunction by activating autophagy and inhibiting inflammatory responses in astrocytes by activating the PPAR-γ signaling pathway.[45,46] Moreover, AQP4 inhibition alleviated inflammatory cell infiltration, downregulated the expression of inflammatory cytokines, and suppressed M2 macrophage activation, thus attenuating the severity of lung fibrosis and irradiation-induced injury induced by irradiation.[47] A study demonstrated that blockade of AQP4 attenuated renal fibrosis through the TGF-β signaling pathway in a unilateral ureteral obstruction mouse nephropathy model.[48] In addition, AQP4 was reported to be involved in the development of inflammatory bowel disease through the PPAR-α signaling pathway.[49] In the present study, the expression of AQP4 was elevated in the kidney tissue of mice with Ang II-stimulated hypertensive nephropathy, which was accompanied by an increase in fibrosis markers. To further elucidate the role of AQP4, an AQP4 inhibitor was used in the next experiment. The results showed that blocking AQP4 with TGN-020 contributed to Ang II–induced decrease in FN, COL-III, COL-I, TGF-β and α-SMA protein expression. The increase in fibrosis proteins caused by the overexpression of Gsdmd was also abolished by TGN-020, which further confirmed that GSDMD aggravated Ang II–induced renal fibrosis, partly by modulating AQP4.

Subsequently, correlation analysis between the transcription factor data and differential gene expression data obtained from RNA sequencing was conducted, and the GATA2 transcription factor was shown to be positively correlated with GSDMD. GATA2 participates in a genetic program associated with urinary tract and renal development.[50] GATA2 plays a biological role in regulating AQPs and urinary concentrations.[51] Moreover, GATA2 interacts with Yes-associated protein 1 to promote *Aqp4* transcription.[52] In this study, we found that GSDMD increased the expression of GATA2 stimulated by Ang II. GATA2 further enhanced its binding to the *Aqp4* promoter sequence and facilitated AQP4 transcription. A recent study suggested that the GSDMD cleavage fragment could translocate to the nucleus through nucleoporins, thereby enhancing the transcriptional regulation of CIITA and subsequently increasing MHC II expression.[53] However, whether GSDMD enhances the transcription of GATA2/AQP4 in a similar manner has not yet been determined. These questions require further investigation.

Transforming growth factor-β (TGF-β) is widely recognized for its predominant role in hypertensive renal fibrosis. In this study, we observed that the absence of GSDMD diminished TGF-β expression induced by Ang II, while overexpression of Gsdmd exacerbated TGF-β levels. Previous reports indicate that TGF-β mediates renal fibrosis through regulation of the TGF-β/SMAD3 and NF-kB signaling pathways.[54] We postulate that these pathways may be involved in GSDMD-mediated hypertensive nephropathy, warranting further investigation. Additionally, following Gene Set Enrichment Analysis (GSEA) on the transcriptomic data, we uncovered potential relevant downstream pathways linked to mitochondrial functions. Considering previous publications from our team indicating a strong relationship between GSDMD and mitochondrial damage as well as pore formation[29], it seems likely that this protein also plays a critical role in renal fibrosis.

Inhibition of Gasdermin D (GSDMD) has been demonstrated to play a role in the pathogenesis of hypertensive nephropathy by mitigating inflammation in the present study. Sodium-glucose cotransporter 2 (SGLT-2) inhibitors, a novel class of glucose-lowering agents, primarily target SGLT2 expressed in the proximal tubule of the kidney, and recent studies have illustrated their nephroprotective effects.[55,56] Furthermore, SGLT-2 inhibitors have been reported to suppress renal inflammation and fibrosis.[57,58] However, their underlying mechanisms differ from those associated with GSDMD inhibition. These inhibitors impede the differentiation of monocytes into macrophages while promoting the polarization of macrophages from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype.[59] Additionally, SGLT-2 inhibitors influence tubuloglomerular feedback (TGF), enhancing glomerular pressure and reducing tubular workload by improving renal hemodynamics.[58] Nevertheless, it remains to be determined whether GSDMD inhibition enhances renal function through these aforementioned mechanisms. To verify the effect of GSDMD on hypertensive nephropathy, the GSDMD inhibitor GI-Y1 was used. To date, no GSDMD inhibitors have been approved for clinical use. GI-Y1 is a small-molecule compound selected from a small-molecule library based on its GSDMD-N structure. GI-Y1 has been proven to binds to GSDMD and inhibits GSDMD-N pore formation by targeting Arg7 residue.[30] Compared to other inhibitors of GSDMD, such as disulfiram[60], GI-Y1 has comparable inhibitory effects with fewer side effects, and thus, has potential as a therapeutic agent. The findings of the present study demonstrated that the management of GI-Y1 alleviated renal fibrosis and dysfunction induced by Ang II, which further confirmed the effect of GSDMD in hypertensive nephropathy.

Our previous study examined the effects of the GSDMD inhibitor GI-Y1 on atherosclerosis.[29] Through a relatively long-term experiment (three-month animal experiment), we found that GI-Y1 can ameliorate the progression of atherosclerosis by inhibiting macrophage pyroptosis. This finding indicates that GI-Y1 maintains its efficacy as an anti-pyroptotic agent in the treatment of chronic diseases. In this study, we confirmed that the inhibition of GSDMD by GI-Y1 has a protective effect in hypertensive nephropathy. Importantly, previous studies[61] have shown that neither inhibition nor overexpression of Gsdmd is related to changes in blood pressure (the image is attached below). However, it is worth noting that we currently lack data to confirm the long-term impact of GI-Y1 on hypertensive kidney pathology, which represents a limitation of this study and warrants further investigation. Besides, a comprehensive dose-response analysis for GI-Y1 is lacking. Providing more detailed information on the pharmacokinetic and pharmacodynamic profiles would enhance clinical dose predictions. Such data would be critical in evaluating the potential of GSDMD inhibitor GI-Y1 as a sustainable treatment option. Furthermore, potential side effects or limitations associated with GI-Y1 have not been assessed. To facilitate the translation of therapeutics into clinical practice, safety concerns regarding GI-Y1 must be evaluated more thoroughly in future research. Moreover, utilizing drugs to directly inhibit GSDMD in the treatment of hypertensive nephropathy also come with certain limitations at this stage. Firstly, GSDMD may exhibit opposing physiological effects across different pathological states. For instance, GSDMD-mediated pyroptosis is beneficial for the body's defense against external bacterial and viral infections[62] as well as promoting the apoptosis of tumor cells.[63] Additionally, GSDMD is implicated in maintaining intestinal homeostasis through its involvement in enhancing mucous secretion from goblet cells and contributing to the formation of the protective mucous layer.[64] In conclusion, while GSDMD inhibitors demonstrate significant effects within antihypertensive nephropathy models, they also influence other physiological functions mediated by GSDMD, consequently limiting their clinical applicability. Therefore, targeted delivery of GSDMD inhibitors specifically to the kidneys may represent a promising therapeutic strategy aimed at minimizing potential side effects associated with such treatments.

Conclusion

GSDMD plays a pivotal role in the development of hypertensive nephropathy. Targeting GSDMD may be an effective method to treat hypertensive nephropathy.

Abbreviations

GSDMD, gasdermin D; GSDMD-N, N-terminal fragment of GSDMD; Ang II, angiotensin II; GATA2, GATA binding protein 2; AQP4, aquaporin 4; COL-I, collagen type 1; TGF-β, transforming growth factor β; α-SMA, alpha smooth muscle actin; IL-1β, interleukin-1β; IL-18, interleukin-18; H&E, hematoxylin and eosin.

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Disclosure

The authors report no conflicts of interest related to this work.

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