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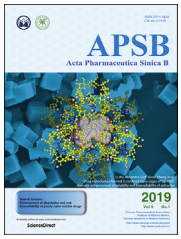

ORIGINAL ARTICLE

Gene deficiency or pharmacological inhibition of PDCD4-mediated FGR signaling protects against acute kidney injury

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KEYWORDS

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Cell death;
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Inflammation

Abstract Recent studies have shown that programmed cell death 4 (PDCD4) modulates distinct signal transduction pathways in different pathological conditions. Despite acute and chronic immune responses elicited by ischemia contributing to the functional deterioration of the kidney, the contributions and mechanisms of PDCD4 in acute kidney injury (AKI) have remained unclear. Using two murine AKI models including renal ischemia/reperfusion injury (IRI) and cisplatin-induced AKI, we found that PDCD4 deficiency markedly ameliorated renal dysfunction and inflammatory responses in AKI mice. Consistently, upregulation of PDCD4 was also confirmed in the kidneys from patients with biopsy confirmed acute tubular necrosis from a retrospective cohort study. Moreover, we found that overexpression of *Fgr*, a member of the tyrosine kinase family, dramatically aggravated renal injury and counteracted the protective effects of PDCD4 deficiency in AKI mice. We discovered that FGR upregulated NOTCH1 expression through activating STAT3. Most importantly, we further found that systemic administration of ponatinib, a tyrosine kinase inhibitor, significantly ameliorated AKI in mice. In summary, we identified that PDCD4 served as an important regulator, at least in part, of FGR/NOTCH1-mediated tubular apoptosis and inflammation in AKI mice. Furthermore, our findings suggest that ponatinib-

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mediated pharmacologic targeting of this pathway had therapeutic potential for mitigating AKI.

1. Introduction

Acute kidney injury (AKI) is a common complication with high morbidity and mortality worldwide, which is often induced by ischemia/reperfusion injury (IRI), sepsis or toxins[1]. During the process of AKI, the tubular epithelial cells (TEC) is not merely a passive victim of injury as evidenced by mitochondrial dysfunction, ATP depletion, impaired solute/ion transport, loss of cellular polarity, cytoskeletal disruption, and cell death[2], but also an active participant in inflammatory responses in AKI. Therefore, a better understanding of the molecular and cellular aspects underlying tubular epithelium injury has important therapeutic implications for treating AKI patients.

Programmed cell death 4 (PDCD4) is expressed ubiquitously in different tissues, and is a multifunctional tumor suppressor that inhibits cellular growth, tumor invasion, metastasis and induction of apoptosis. The expression of PDCD4 is often downregulated in tumors but is maintained at high levels in a large number of diseases. PDCD4 has been considered as a potential target for anticancer therapies in recent years[3,4]. Recently, emerging evidence has also shown that PDCD4 can affect gene transcription and can also inhibit protein translation *via* two distinct mechanisms[5]. One of these mechanisms acts in a translation-initiation-factor-4A (eIF4A)-dependent manner. By binding competitively to eIF4A, PDCD4 inhibits the combination of the RNA helicase, eIF4A, and the scaffold protein, eIF4G, which initiates the translation of mRNA[6−8]. In addition to this eIF4A-dependent mechanism, PDCD4 may directly bind to mRNAs such as those encoding antiapoptotic proteins, BCL-XL and X-chromosome-linked inhibitor of apoptosis (XIAP), to suppress their translation[9]. Several studies have reported that PDCD4 is involved in the induction of inflammation. PDCD4 has been found to be involved in macrophage- and T-cell-mediated immune responses[10,11]. Other studies have indicated that PDCD4 can inhibit the production of IL-10 through the Twist2/c-MAF pathway[12]. Furthermore, *Pdcd4*-deficient mice are resistant to the induction of inflammatory diseases such as autoimmune encephalomyelitis and diabetes[13], indicating that PDCD4 regulates both carcinogenesis and inflammation[5]. In atherosclerosis, PDCD4 increases both the formation of foam cells and the release of inflammatory mediators[10]. Despite acute and chronic immune responses elicited by ischemia or toxicity contributing to the functional deterioration of the kidney, the contributions and mechanisms of PDCD4 to AKI have remained unclear.

Overactivation of NOTCH1 signaling results in a series of pathological consequences, including the formation of different types of tumors[14]. In addition to these effects, activated NOTCH1 signaling is also reported to contribute to kidney fibrosis and is involved in the podocytic injury, indicating its important role in the kidney[15,16]. In injured cells, NOTCH1 signaling is activated through the binding of the ligand, Jagged, to its membrane receptor. The NOTCH1 intracellular domain is an active intracellular domain of NOTCH1 and translocates to the nucleus to stimulate signaling cascades[17]. In the present study, we found that PDCD4 deficiency protected against the model of AKI in mice, at least in part, by regulating FGR/NOTCH1-mediated apoptosis and immune responses. Importantly, we further found that systemic administration of ponatinib, a third-generation tyrosine kinase inhibitor, significantly ameliorated AKI in mice. Taken together, our findings suggest that a better understanding of the biological activities of PDCD4 may provide unexpected opportunities for developing novel therapies for ameliorating AKI.

2. Materials and methods

Procedures for detailed mouse models of AKI, RNA extraction, real-time reverse-transcription polymerase chain reaction (RT-PCR), Western blot analysis, assessment of renal function, immunohistochemistry, morphological examinations, caspase-3 activity assays and luciferase reporter assays were presented online in the Supporting Information.

2.1. Animal studies and drug treatments

Twelve-week-old male *Pdcd4* deficient mice (20−25 g) in C57BL/6 background were obtained from ViewSolid Biotech (Beijing, China). Experiment mice were randomly divided into different groups using a random-block grouping. When assessing the outcome and analysis of the results, a double-blind method employed. AKI was induced by renal IRI or cisplatin as described previously[18], and the detailed procedures can be found in our online supplemental materials. The experiments were also designed to evaluate the preventive and potential therapeutic effects of ponatinib on AKI. Mice received ponatinib (15 mg/kg, i.p., Selleckchem, Houston, TX, USA) at 6 h before the induction of ischemia; the vehicle group received an equal volume of vehicle (DMSO:PEG300:Tween80:NS = at a ratio of 1:6:1:33). All protocols were approved by the Medical Ethics Committee of Medical School Shandong University (201401049) and were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.2. Cell culture and treatments

Immortalized rat renal proximal tubule (NRK-52E) cells, human podocytes (HPC), glomerular endothelial cells (GENC), and glomerular mesangial cells (RMC) were cultured and treated as described previously[19]. These cells were certified using short-tandem-repeat genotyping and were tested for mycoplasma contamination once per month.

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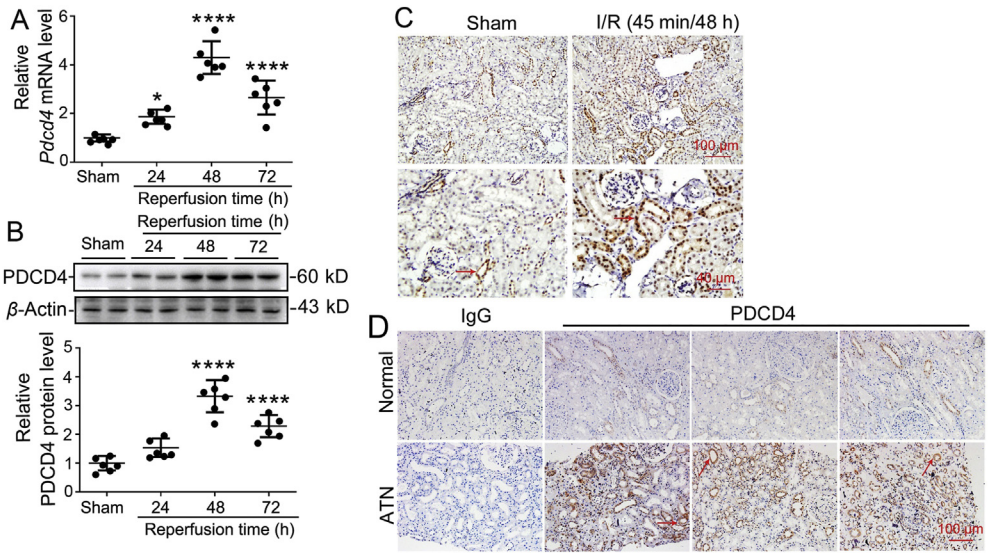

Figure 1 PDCD4 expression is significantly induced in the kidneys from mice with renal IRI. (A) Relative mRNA levels of *Pdcd4* in the kidneys from mice with renal IRI. (B) Representative Western blots and summarized data showing the protein levels of PDCD4 in the kidneys from mice with renal IRI. (C) Representative photomicrographs of IHC staining of PDCD4 in the kidneys from mice with IRI. (D) Representative photomicrographs of IHC staining of PDCD4 in the kidneys from patients with biopsy-confirmed acute tubular necrosis (ATN). Normal kidney tissues were obtained from healthy kidney poles of individuals who underwent tumor nephrectomies without renal disease. All data are displayed as mean ± SD, $n = 6$; *$P < 0.05$, ****$P < 0.0001$ by one-way ANOVA.

2.3. *Flow cytometry*

Cell apoptosis was determined by 7AAD-Annexin V staining as described previously[16].

2.4. *Immunofluorescent staining and confocal microscopy*

Immunofluorescent staining was performed as described previously[20]. We used a LSM780 laser-scanning confocal microscope (ZEISS, Germany) equipped with a Plan-Apochromat 63 × 1.4 objective for all imaging.

2.5. *Intrarenal adeno-associated virus (AAV) delivery*

Overexpression of adeno-associated virus (AAV) constructs of *Fgr* (rAAV9-*Fgr*-GFP) and their negative controls (rAAV9-GFP) was designed and purchased from ViGene Biosciences (Shandong, China). The titers of rAAV9-GFP and rAAV9-Fgr-GFP were approximately 8.0×10^{12} viral genomes/mL and 6×10^{11} viral genomes/L of rAAV9-Fgr-GFP or rAAV9-GFP were delivered to the kidney by means of intraparenchymal injections. The procedure for intraparenchymal delivery was performed as described previously[19,21]. Preliminary studies indicated that AAV-mediated GFP protein expression in the kidney parenchyma was significantly increased after one month, and no toxicity was observed in AAV-treated mice.

2.6. *Statistical analysis*

Data are presented as mean ± standard deviation (SD). Statistically significant differences between the control group and treated groups were determined by one-way analysis of variance (ANOVA) followed by *post hoc* Dunnett's test that were carried out *via* GraphPad Prism 8.0. Differences among multiple groups were examined by Tukey's multiple comparison test *via* GraphPad Prism 8.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1. *PDCD4 expression is significantly induced in the kidneys of renal IRI mice*

First, we found that PDCD4 was expressed in various tissues and in different renal parenchymal cells including rat glomerular mesangial cells (RMC), rat glomerular endothelial cells (GENC), human podocytes (HPC) and rat proximal tubule epithelial cells (NRK-52E cells, Supporting Information Fig. S1). We further observed that the expression of PDCD4 was time-dependently induced in the kidney after 45 min of renal ischemia followed by different time points of reperfusion, as indicated by real-time RT-PCR (Fig. 1A) and Western blot (Fig. 1B) analyses; these findings were also confirmed in paraffin-embedded sections of renal tissues *via* immunohistochemical (IHC) staining (Fig. 1C). To define the tubular segment-specificity increase of PDCD4 expression in the kidneys from mice with renal IRI, we used double immunostaining for PDCD4 (green) and various tubular markers (red) in the kidney. As shown in Supporting Information Fig. S2, PDCD4 was mainly expressed and induced in proximal tubules and collecting ducts. Importantly, we found that compared with renal tissues obtained from patients who underwent tumor nephrectomies without other renal disease, PDCD4 was also unregulated in the kidney from patients with biopsy-confirmed acute tubular necrosis, which presents with AKI and is one of the most common causes of AKI (Fig. 1D).

3.2. *Pdcd4 deficiency ameliorates renal IRI in mice*

Pdcd4$^{-/-}$ mice were phenotypically normal and had no appreciable defects in renal morphology or function. However, *Pdcd4*

deficiency in mice significantly ameliorated renal IRI, as evidenced by a reduction of serum creatinine (Fig. 2A) and blood urea nitrogen (BUN, Fig. 2B), mitigated morphological injury (Fig. 2C and D), and reduced cell death as demonstrated by terminal deoxynucleotidyl transferase-mediated digoxigenindeoxyuridine nick-end labeling (TUNEL) staining (Fig. 2E and F). In addition, *Pdcd4* deficiency also reduced inflammatory responses by decreasing the levels of pro-inflammatory mediators

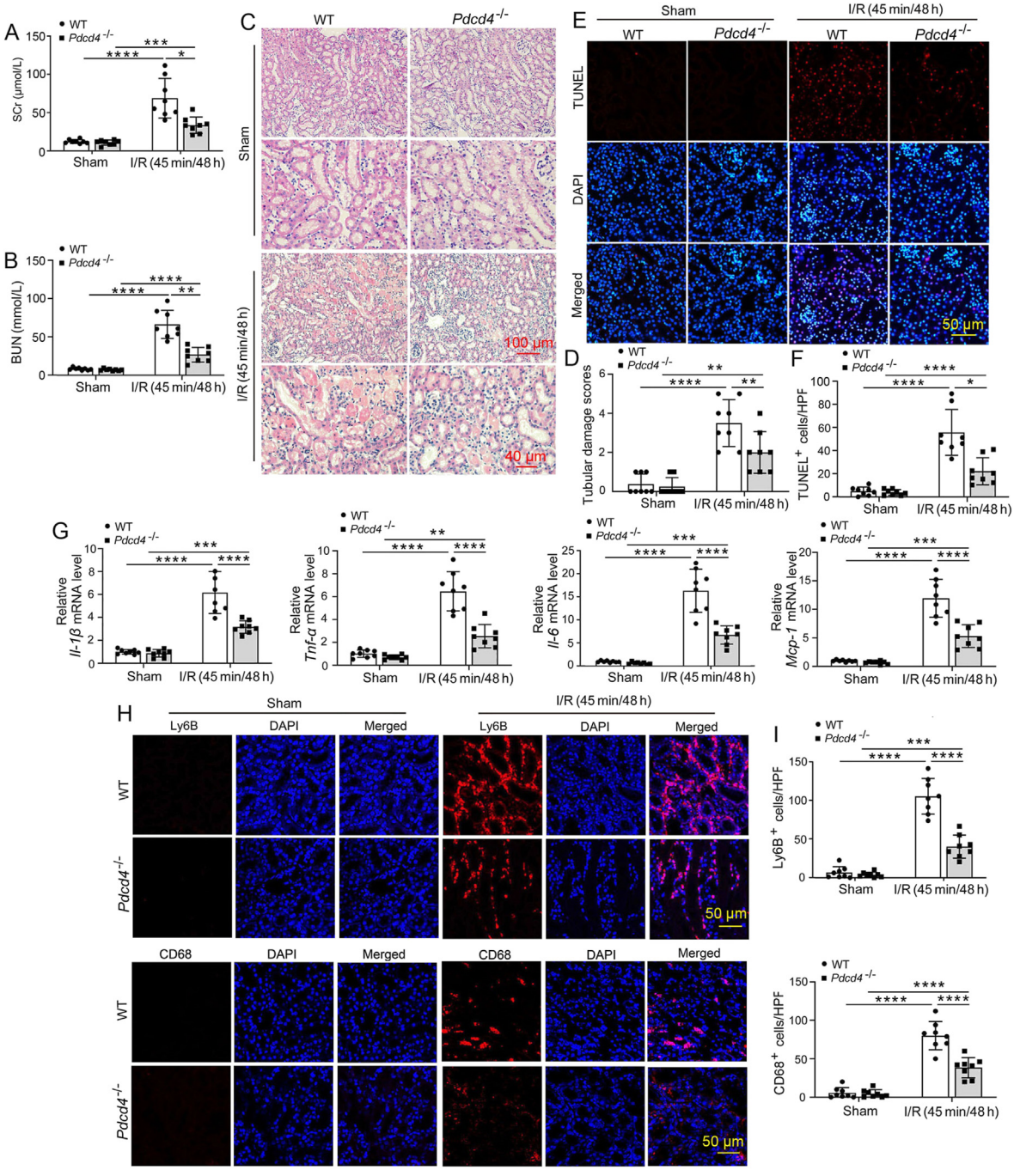

Figure 2 PDCD4 deficiency ameliorates renal IRI in mice. (A) Serum creatinine (SCr) concentrations in different groups of mice. (B) BUN levels in different groups of mice. (C) Representative micrographs showing the morphology of the kidneys from different groups of mice. (D) Quantitative assessment of tubular damage. (E) *In situ* TUNEL assays were performed to assess renal cell death. Nuclei were revealed using DAPI staining. (F) Quantitative assessment of the number of dead cells (numbers per high-power field [HPF]). (G) The mRNA levels of pro-inflammatory mediators including *Il-1β*, *Tnf-α*, *Il-6*, and monocyte chemoattractant protein-1 (*Mcp-1*) in the kidneys from different groups of mice. (H) Representative sections of kidneys stained for macrophages (CD68) and neutrophils (Ly6B) from different groups of mice. (I) Data analysis of macrophage and neutrophil infiltrates in the kidneys (numbers per high-power field [HPF]) from different groups of mice. All data are displayed as mean ± SD, $n = 8$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-way ANOVA.

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(Fig. 2G), as well as macrophage and neutrophil (Fig. 2H and I) infiltration in the kidneys from mice with renal IRI.

3.3. *PDCD4 positively regulates FGR expression*

Using Agilent Whole-Mouse-Genome Oligo Microarrays for global gene expression analysis, we found that IRI-induced the expression of FGR, a member of the SRC family of non-receptor tyrosine kinases, but was significantly abolished by *Pdcd4* deficiency in mice (Fig. 3A), as determined by mRNA (Fig. 3B), Western blot (Fig. 3C), and IHC-staining analyses (Fig. 3D). *In vitro*, we used the following approaches to mimic hypoxic conditions in NRK-52E cells: (1) oxygen−glucose deprivation (OGD, Fig. 3E); and (2) glucose-free medium with antimycin A (AA)/2-deoxyglucose (2-DG) for ATP depletion (90 min, anoxia). Subsequently, we changed the glucose-replete complete growth medium (recovery) and $CoCl_2$ treatment (Supporting Information Fig. S3). All manipulations significantly induced the expression of PDCD4 and FGR. Gene silencing of *Pdcd4* attenuated hypoxia-induced FGR expression (Fig. 3F), indicating that PDCD4 may positively mediate FGR expression.

3.4. *PDCD4 contributes to hypoxia-induced inflammatory responses and cell death in NRK-52E cells via regulating FGR expression*

Overexpression of PDCD4 (Fig. 4A) aggravated hypoxia-enhanced production of pro-inflammatory mediators (Fig. 4C), as well as apoptosis (Fig. 4D) accompanied by increased caspase-3 activity (Fig. 4E), which were counteracted by *Fgr* knockdown (Fig. 4B). Luciferase activity assays further confirmed that PDCD4 enhanced *Fgr* transcription (Fig. 4F). Luciferase activity assays also showed that siRNA-*Pdcd4* decreased *Fgr* transcription, which can be recovered by siRNA-*Daxx* under OGD conditions, indicating that DAXX may regulate transcription of FGR (Supporting Information Fig. S4). We also examined the expression level of DAXX in the kidneys from *Pdcd4*$^{-/-}$ mice and found that *Pdcd4* deficiency dramatically increased the expression of DAXX under AKI conditions (Fig. S4). However, further studies are needed to confirm these results.

3.5. *PDCD4 positively regulates FGR-mediated NOTCH1 signaling through STAT3 activation*

We found that IRI-induced NOTCH1 expression was significantly abolished by *Pdcd4* deficiency in mice, as revealed *via* microarray analysis (Fig. 3A), which was further confirmed by mRNA (Fig. 5A), Western blot (Fig. 5B), and IHC (Supporting Information Fig. S5) analyses. *In vitro*, overexpression of *Pdcd4* increased hypoxia-induced the production of NOTCH1 which was counteracted by *Fgr* knockdown (Fig. 5C) in NRK-52E cells, indicating that NOTCH1 may be a downstream target of the PDCD4/FGR pathway. Considering that STAT3 was identified as a potential transcription factor regulated by SRC kinase[22,23] and that inhibition of STAT3 protects against renal IRI[24], we next investigated whether PDCD4 regulates the FGR/NOTCH1 signaling pathway *via* STAT3. We found that hypoxia-induced STAT3 activation as indicated by increased levels of phospho-STAT3 at

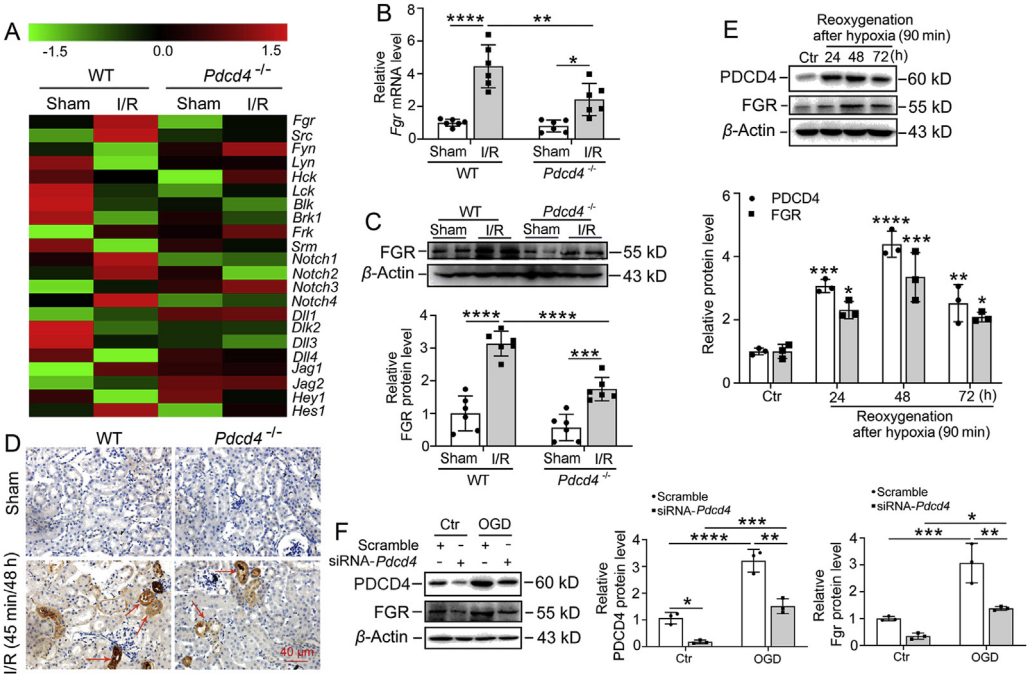

Figure 3 PDCD4 positively regulates FGR expression. (A) Representative heatmap of gene expression levels in the kidneys from mice with IRI, by multiplexed qRT-PCR arrays. (B) Summarized data showing the mRNA levels of *Fgr* in the kidneys from mice with renal IRI ($n = 6$). (C) Representative Western blots and summarized data showing the protein levels of FGR in the kidneys from mice with renal IRI ($n = 6$). (D) Representative photomicrographs of FGR IHC staining in the kidneys from mice with renal IRI. (E) Representative Western blots and summarized data showing the protein levels of PDCD4 and FGR in NRK-52E cells under OGD conditions ($n = 3$). (F) Representative Western blot gel documents and summarized data showing the protein levels of PDCD4 and FGR in NRK-52E cells under OGD conditions by gene silencing of *Pdcd4* ($n = 3$). All data are displayed as mean ± SD; $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by two-way ANOVA.

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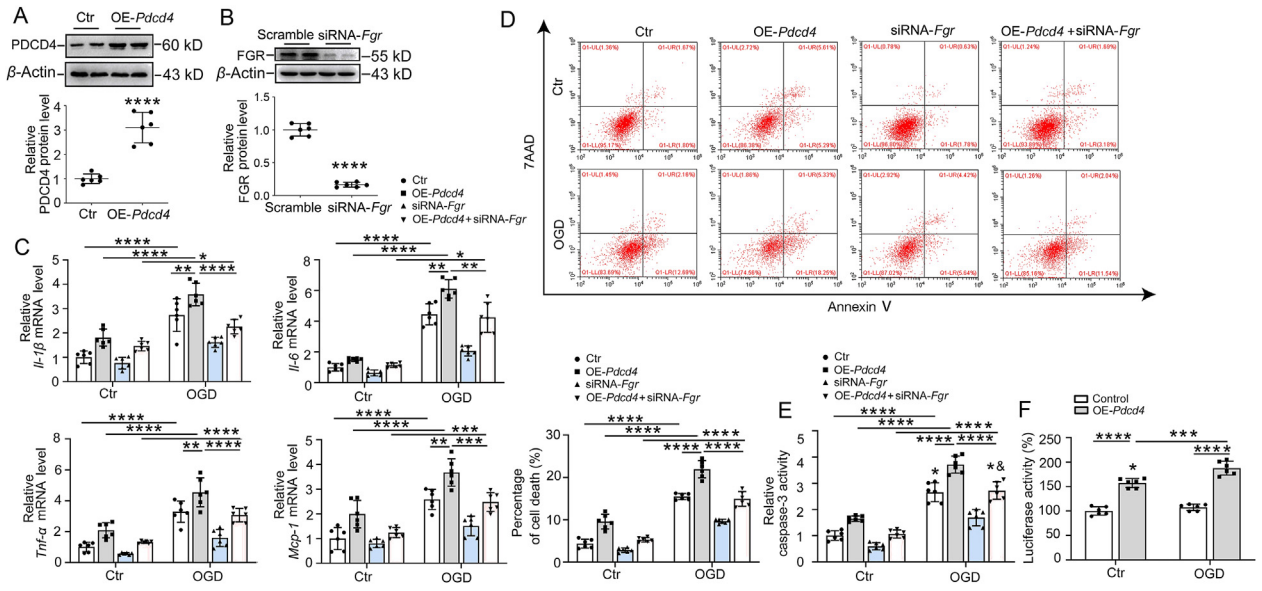

Figure 4 PDCD4 contributes to hypoxia-induced production of pro-inflammatory mediators and cell death in NRK-52E cells *via* FGR. (A) Representative Western blots and summarized data showing the overexpression efficiency of PDCD4 transfection in NRK-52E cells (unpaired two-tailed Student's *t* test). (B) Representative Western blots and summarized data showing the gene silencing efficiency of FGR in NRK-52E cells (unpaired two-tailed Student's *t* test). (C) Summarized data showing the mRNA levels of pro-inflammatory mediators in NRK-52E cells with different treatments. (D) Summarized data showing the overall percentages of cell death, including the amount of apoptotic and necrotic cells determined by flow cytometric analysis, in NRK-52E cells with different treatments. (E) Summarized data showing caspase 3 activity in NRK-52E cells with different treatments. (F) The luciferase activity was quantified in NRE-52E cells transfected with OE-*Pdcd4*. All data are displayed as mean ± SD, $n = 6$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-way ANOVA.

both serine (residue 727) and tyrosine (residue 705) sites (Fig. 5D), as well as by nuclear translocation of STAT3 (Fig. 5E), and increased NOTCH1 levels that were further enhanced by *Fgr* overexpression. Importantly, all of these FGR-mediated effects were counteracted by the STAT3 phosphorylation inhibitor, HO-3867 (Fc tagged, 10 μmol/mL, Selleckchem, Houston, TX, USA) in NRK-52E cells. Similarly, gene silencing of *Fgr* decreased phospho-STAT3 levels and the expression of NOTCH1 (Supporting Information Fig. S6), indicating that FGR mediated NOTCH1 signaling *via* STAT3 phosphorylation.

3.6. *In vivo gene overexpression of Fgr counteracts the alleviated renal injury and inflammatory responses in Pdcd4$^{-/-}$ ischemic mice*

To further investigate whether FGR acts as a key regulator linking PDCD4 and inflammatory responses in AKI, a recombinant adeno-associated virus (rAAV) serotype 9 harboring *Fgr* was delivered into mice by means of intraparenchymal injections. Intense green fluorescence was seen in the kidneys at one month after intrarenal delivery of the AAV encoding *Fgr-Gfp*, but not in the kidneys from control mice injected with normal saline (Supporting Information Fig. S7A). Moreover, Western blot analysis also confirmed the FGR overexpression efficiency (Fig. S7B). FGR counteracted the alleviated kidney injury in *Pdcd4*$^{-/-}$ ischemic mice, as evidenced by increased serum creatinine (Fig. 6A) and BUN (Fig. 6B), deteriorated morphologic injury (Fig. 6C and D), enhanced cell death (Fig. 6E and F), and increased inflammatory responses by augmenting levels of pro-inflammatory mediators (Fig. 6G), as well as enhanced macrophage and neutrophil (Supporting Information Fig. S8) infiltration in the kidneys from mice with IRI. Importantly, downregulation of NOTCH1 in *Pdcd4*$^{-/-}$ ischemic mice was partially rescued by *Fgr* overexpression (Fig. 6H).

3.7. *Ponatinib partially rescues renal IRI injury in mice*

Pharmacologically, we tested whether ponatinib, a third-generation of pan tyrosine kinase inhibitor (TKI), could alleviate renal IRI in mice. We found that ponatinib dramatically alleviated renal IRI, as evidenced by reduced serum creatinine (Fig. 7A) and BUN (Fig. 7B), as well as attenuated morphologic injury (Fig. 7C and D), and cell death revealed by TUNEL staining (Fig. 7E and F). However, ponatinib had no significant effect on the renal function of *Pdcd4*$^{-/-}$ ischemic mice, further confirming the involvement of FGR in PDCD4-mediated signaling in AKI.

3.8. *Pdcd4 deficiency protects against cisplatin-induced AKI*

To determine the broad implications of PDCD4 signaling in AKI, we further investigated whether PDCD4 also played a detrimental role in mice with cisplatin-induced AKI. We found that PDCD4, FGR and NOTCH1 expression levels were increased in the kidneys from mice receiving cisplatin treatments (Fig. 8A and B). Furthermore, *Pdcd4* deficiency ameliorated renal dysfunction (Fig. 8C and D), histologic lesions (Fig. 8E and F) and cell death (Fig. 8G and H). Additionally, cisplatin-induced increase in the expression levels of FGR and NOTCH1 (Fig. 8I) were attenuated by *Pdcd4* deficiency. *In vitro*, cisplatin-induced PDCD4 expression and activation of the FGR/NOTCH1 signaling pathway were observed. Finally, gene silencing of *Pdcd4* alleviated cisplatin-mediated inflammation and apoptosis in NRK-52E cells (Supporting Information Fig. S9). Taken together, these results indicate that PDCD4-mediated activation of the FGR/NOTCH1 signaling

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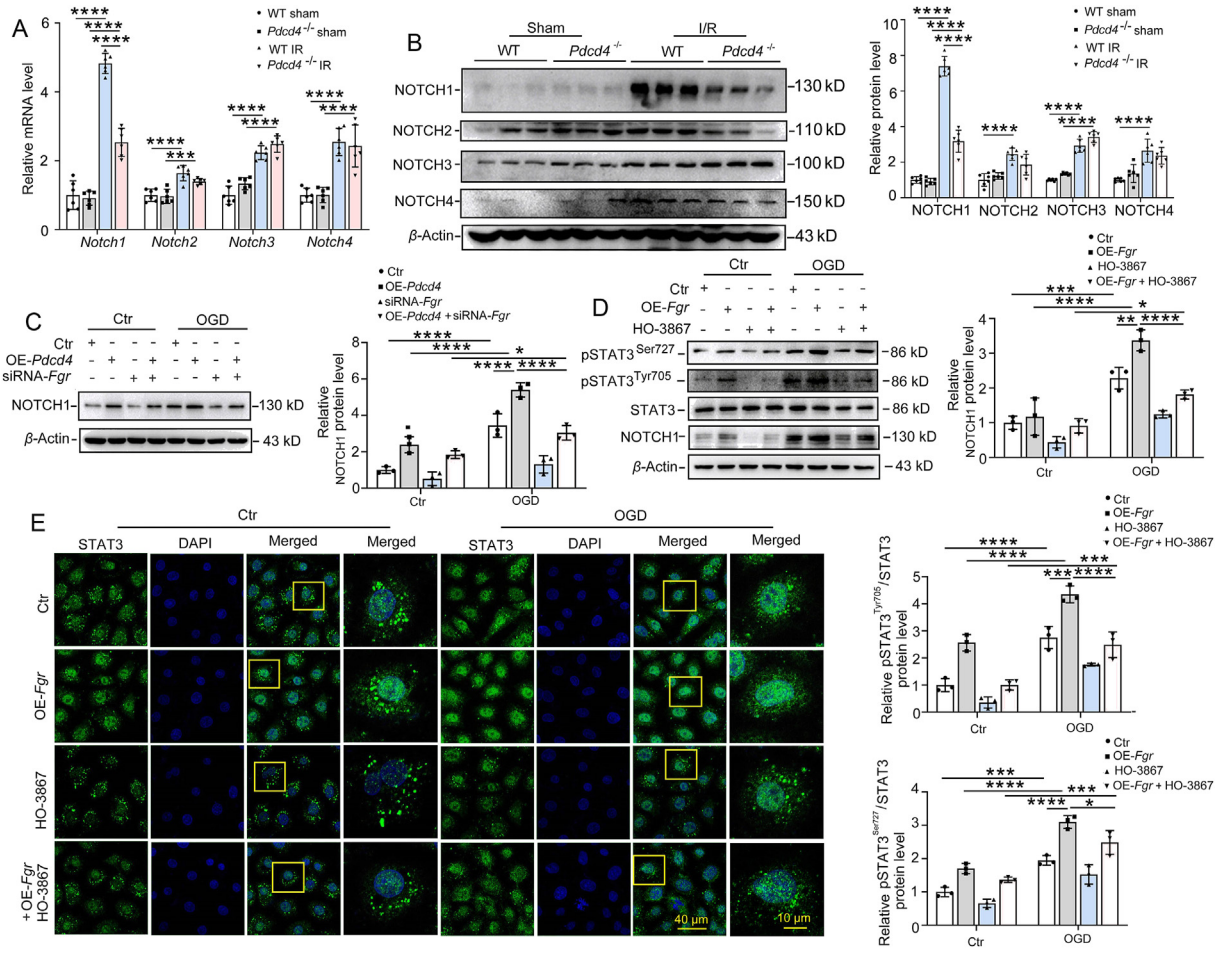

Figure 5 PDCD4 positively regulates FGR-mediated NOTCH1 signaling through STAT3. (A) Summarized data showing the mRNA levels of *Notch1-4* in the kidneys from different groups of mice ($n = 6$). (B) Representative western blots and relevant statistical results showing NOTCH1−4 protein levels in the kidneys from different groups of mice ($n = 6$). (C) Representative Western blots and summarized data showing the protein levels of NOTCH1 in NRK-52E cells under OGD conditions by OE-*Pdcd4* and/or siRNA-*Fgr* transfection ($n = 3$). (D) Representative Western blots and summarized data showing the expression levels of pSTAT3, STAT3 and NOTCH1 in NRK-52E cells with different treatments ($n = 3$). (E) Representative microscopic images of immunofluorescent staining showing the distribution of STAT3 in NRK-52E cells with different treatments. All data are displayed as mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ by two-way ANOVA.

pathway may be a common response in AKI. Using ponatinib or other drugs targeting this pathway may have important therapeutic implications for treating AKI.

4. Discussion

Although there has been significant progress in better understanding the structure and function of PDCD4, the contributions and mechanisms of PDCD4 in AKI have remained unknown. In the present study, by using two separate murine AKI models including IRI and cisplatin-induced AKI, we found that PDCD4 deficiency markedly ameliorated renal dysfunction, histologic lesions, and inflammatory responses in AKI mice. We also confirmed the upregulation of PDCD4 in the kidneys from patients with biopsy-confirmed acute tubular necrosis from a retrospective cohort study. We further identified that PDCD4 served as a positive regulator of AKI, at least in part, by mediating FGR/NOTCH1-mediated apoptosis and inflammation. Importantly, the potent FGR activity inhibitor, ponatinib, dramatically prevented AKI in mice, providing promising evidence for its therapeutic potential in ameliorating AKI.

PDCD4 is regulated by a number of mechanisms[25], most notably as a target of miR-21[13]. Some previous studies have demonstrated ischemic-induced and xenon-preconditioning-induced upregulation of miR-21, which mediates renal-protective effects through PDCD4[8,26]. Similarly, the upregulation of miR-21 by ghrelin can ameliorate IR-induced AKI[27]. However, another study has indicated that overexpression of miR-21 alone is not sufficient to prevent TEC death following ischemia[28]. Also, there are many studies that have shown that RNA-binding proteins (*e.g.*, HuR), are upregulated in AKI[29], and can increase the steady-state of PDCD4 mRNA and protein levels[30,31]. Recently, PDCD4 has been found in the lncRNA H19/miR-21/PDCD4 competing endogenous RNA network (ceRNET) and H19 competitively binds with miR-21 to facilitate PDCD4 expression in mouse retinas during I/R, indicating the important role of PDCD4 in I/R injury[32]. Because the post-transcriptional

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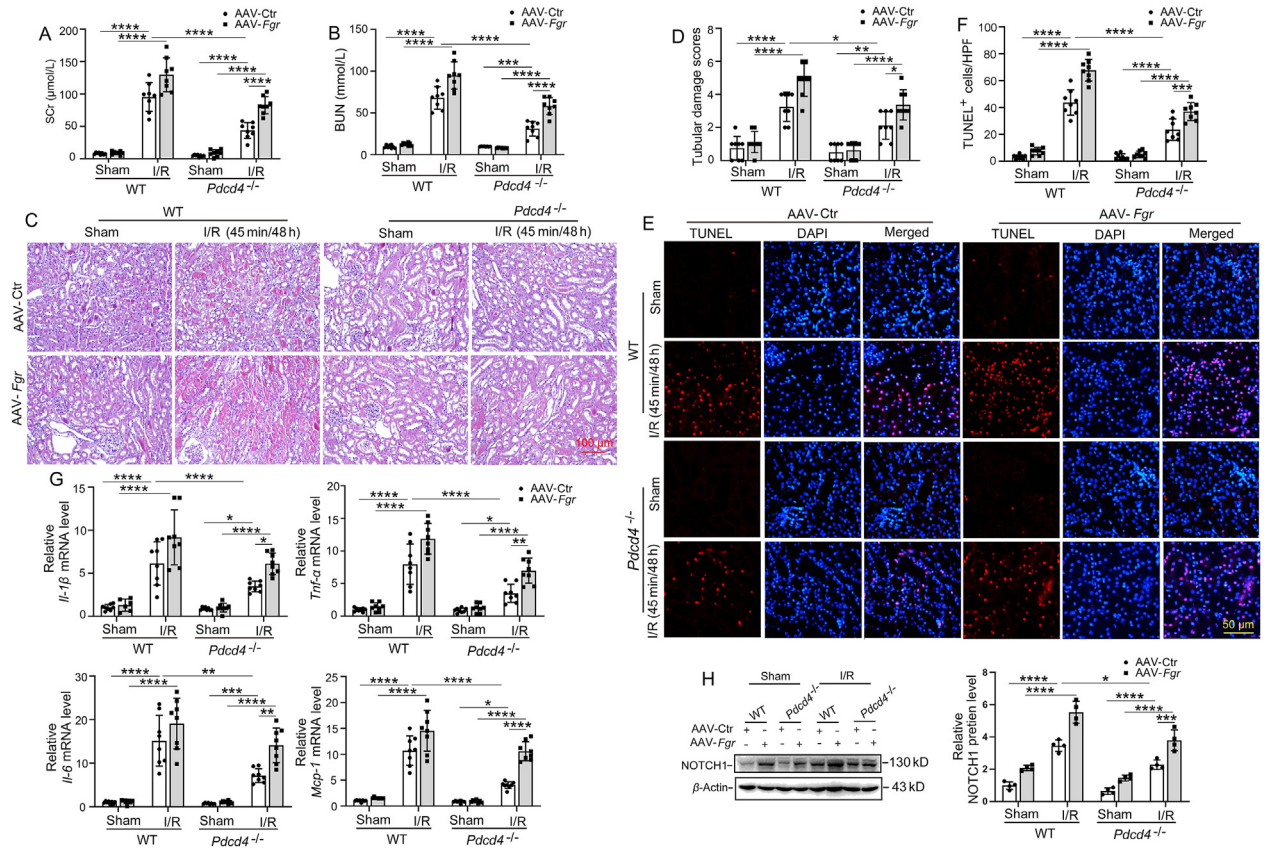

Figure 6 *In vivo* gene overexpression of *Fgr* counteracts the alleviated renal injury and inflammatory responses in *Pdcd4*$^{-/-}$ ischemic mice. (A) Serum creatinine (SCr) concentrations in different groups of mice ($n = 8$). (B) BUN levels in different groups of mice ($n = 8$). (C) Representative micrographs showing the morphologies of the kidneys from different groups of mice. (D) Quantitative assessment of tubular damage ($n = 8$). (E) *In situ* terminal TUNEL assays were performed to assess renal cell death. Nuclei were revealed using DAPI staining. (F) Quantitative assessment of the number of dead cells (numbers per high-power field [HPF]; $n = 8$). (G) The mRNA levels of pro-inflammatory mediators including *Il-1β*, *Il-6*, *Tnf-α*, and *Mcp-1* in the kidneys from different groups of mice ($n = 8$). (H) Representative Western blots and summarized data showing the protein levels of NOTCH1 in the kidneys from different groups of mice ($n = 4$). All data are displayed as mean ± SD; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-way ANOVA.

regulation of PDCD4 and the physiological mechanisms of AKI are complex, investigations of miR-21 or HuR can not fully explain the expression pattern and function of PDCD4 in AKI.

Therefore, in the present study, we first confirmed the upregulation of PDCD4 in AKI mice, as well as in the kidneys from patients with biopsy-confirmed acute tubular necrosis indicating that PDCD4 may play an important role in AKI. Then we used the *Pdcd4*$^{-/-}$ knockout mice to reveal that PDCD4 exacerbated AKI through increasing TEC death and inflammation. Moreover, we explored the detailed mechanisms of PDCD4 in AKI mice and found a positive correlation between PDCD4 and FGR levels in the kidney.

FGR is a member of the SRC family kinases (SFKs), which are the largest family of non-receptor protein tyrosine kinases and include SRC, LCK, FYN, YES, FGR, BLK, LYN and FRK. SFKs can phosphorylate a variety of cellular proteins on tyrosine residues, leading to the activation of protein targets in response to different stimuli, thereby regulating multiple cellular events including cellular movement, differentiation, proliferation, survival, and apoptosis[33–35]. Emerging evidence has shown that SFKs are pivotal mediators in the regulation of renal function[36–38], although the role of some SRKs remains controversial under different renal pathological conditions. In the present study, we observed changes in SFKs by microarray analysis in the kidneys from ischemic mice. Among SFKs, FGR was significantly induced in the kidneys from mice with renal IRI. In addition to FGR contributing to tumor development by preventing programmed cell death and promoting cellular migration[39,40], FGR also mediates inflammatory responses. Dysregulation of FGR is known to result in defective eosinophil recruitment to the lung during allergic airway inflammation and reduced plaque growth and stability by blunting monocyte recruitment[41,42]. In the present study, we found that FGR was significantly induced in TEC in the kidneys from mice with AKI. Gene silencing of *Fgr* decreased hypoxia-induced production of pro-inflammatory mediators and apoptosis in proximal tubule epithelial cells. Surprisingly, we found that FGR induced apoptosis in proximal tubule epithelial cells, rather than inhibiting apoptosis in tumor cells. These opposite effects may depend on different cell types or different disease states. Furthermore, we verified that *Pdcd4* deficiency markedly inhibited AKI-induced FGR expression in the kidney, suggesting that FGR signaling is one of the critical signal transduction pathways that link PDCD4 with the induction of AKI. Although we did not explore the detailed mechanisms by which PDCD4 mediates FGR expression in AKI, PDCD4 has been well documented to act as a regulator of gene expression through

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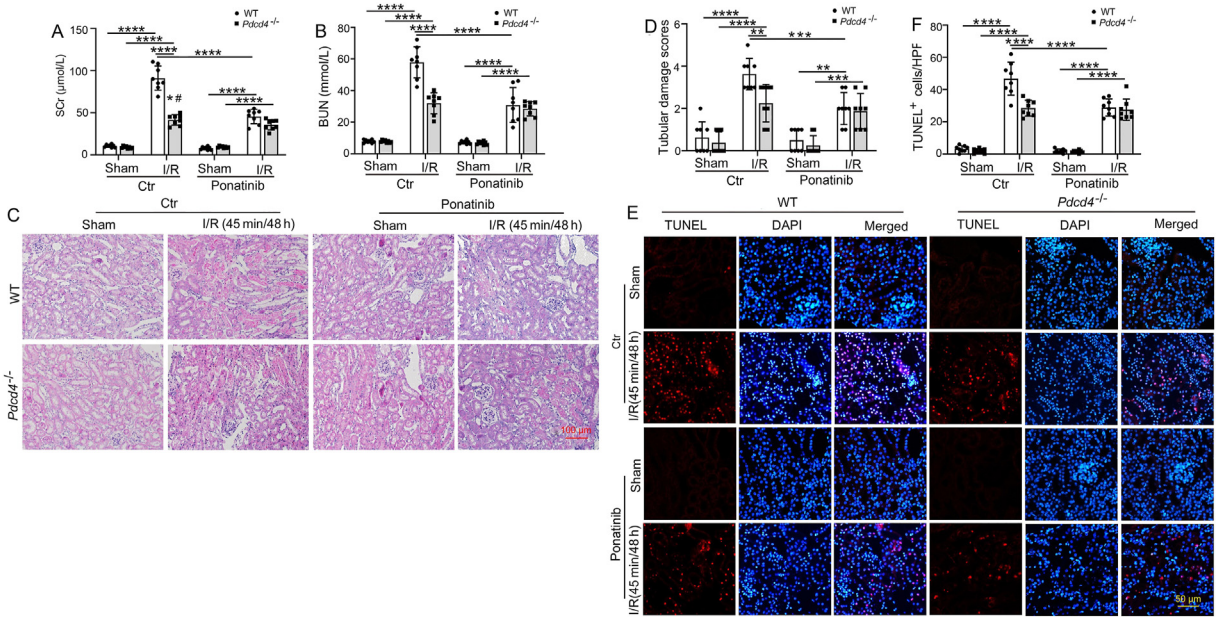

Figure 7 Ponatinib partially rescues renal IRI injury in mice. (A) Serum creatinine (SCr) concentrations in different groups of mice. (B) BUN levels in different groups of mice. (C) Representative micrographs showing the morphologies of the kidneys from different groups of mice. (D) Quantitative assessment of tubular damage. (E) *In situ* TUNEL assays were performed to assess renal cell death. Nuclei were revealed using DAPI staining. (F) Quantitative assessment of the number of dead cells (numbers per high-power field [HPF]). All data are displayed as mean ± SD, $n = 8$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-way ANOVA.

influencing both transcription and translation. Recently, PDCD4 has been identified as a novel interaction partner of death domain associated protein (DAXX) and stimulates the degradation of DAXX[43]. DAXX is a well-established transcriptional repressor that binds to numerous transcription factors, including members of the PAX and P53 families, as well as C/EBPb, ETS1, SMAD4, glucocorticoid receptors and androgen receptors[44]. In the present study, our results indicate that DAXX may be a potential transcription regulator of FGR. However, further studies are needed to confirm these results.

It is well known that the NOTCH pathway is an evolutionarily conserved intercellular signaling pathway responsible for the control of cell fate and tissue morphogenesis during development and disease[45–47]. The activation of tubular epithelial NOTCH1 signaling has been shown to be prolonged in the aging kidney after IRI[48]. In the present study, we observed changes in NOTCH family proteins, especially NOTCH1, in the kidneys from ischemic mice and further confirmed that FGR mediated NOTCH1 expression, at least in part, by activation of signal transducer and activator of transcription 3 (STAT3). STAT3 has long been recognized for its increased transcriptional activity in cancers and autoimmune disorders[49,50]. Recently, STAT3 has received increased attention due to its elucidated role in the progression of kidney disease[51–53]. STAT3 is aberrantly activated in the kidney from autosomal-dominant polycystic kidney disease (ADPKD) patients and in PKD mouse models[54]. Recent studies have also shown that interleukin-6 (IL-6) contributes to the increase in fibroblast growth factor 23 (FGR23) levels through STAT3 phosphorylation in both acute and chronic kidney disease[55]. In the present study, we found that FGR-mediated STAT3 phosphorylation, thereby resulting in increased *Notch1* transcription. Mechanistically, previous studies have shown that phosphorylated STAT3 enters the nucleus, binds to the *Notch1's* promotor, and enhances the *Notch1* transcription[56].

Given that FGR plays a vital role in PDCD4-mediated activation of the NOTCH1 signaling pathway in AKI, we further tested whether ponatinib-mediated inhibition of this signaling protects against AKI. Ponatinib (trade name Iclusig), is an orally active multi-tyrosine kinase inhibitor and is currently approved by the U.S. Food and Drug Administration for patients with chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. Due to the multi-targeted characteristics of ponatinib, further studies have demonstrated its ability to target other important tyrosine kinases (*e.g.*, SRC and RET) in other human malignancies[57]. Importantly, we found that ponatinib dramatically alleviated renal IRI in wild-type mice. Our data also indicated that ponatinib had a significant inhibitory effect on the activity of FGR rather than that of SRC or RET (Supporting Information Fig. S10). The reason for this phenomenon may be due to the PDCD4/FGR/NOTCH1 pathway being dramatically activated under AKI conditions. These results further suggest that the therapeutic effect of ponatinib mainly depends on its inhibition of FGR activity. Importantly, in our present study ponatinib had no further effect on the regulation of renal function in *Pdcd4*$^{-/-}$ ischemic mice, indicating the involvement of FGR in PDCD4-mediated renal function in AKI. These studies provide compelling evidence that ponatinib or other drugs targeting FGR may be used as therapeutic agents for the treatment of AKI. It has been reported that the dose of ponatinib is correlated with the incidence of adverse events[58]. Meanwhile, compared with features of anti-tumor treatments, the usage period and dosage of ponatinib to treat AKI are much shorter and lower, which may help to circumvent long-term adverse reactions and further support the use of ponatinib in treating AKI patients. Hence, further studies

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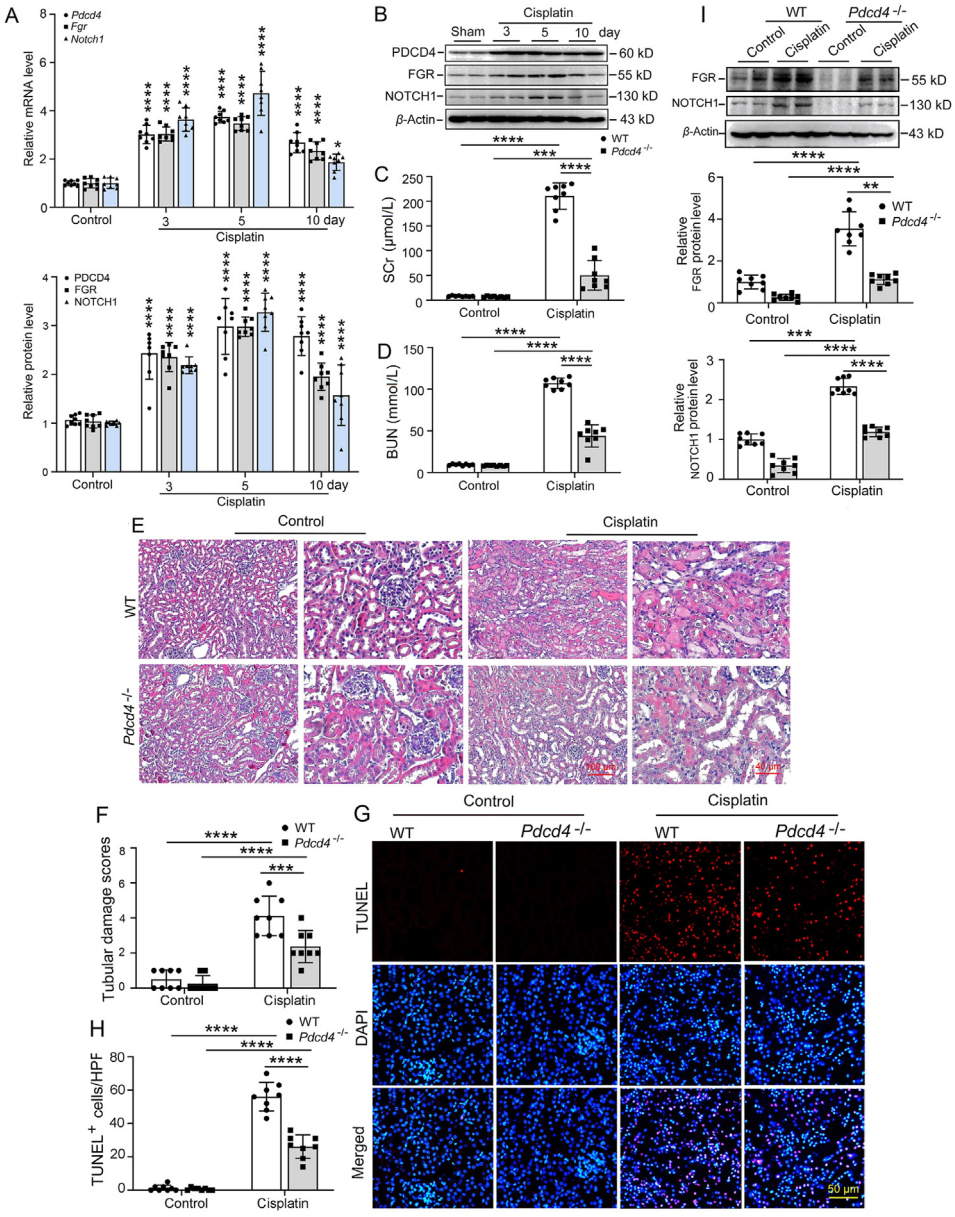

Figure 8 PDCD4 deficiency protects against cisplatin-induced AKI. (A) Summarized data showing the mRNA levels of *Pdcd4*, *Fgr* and *Notch1* mRNA levels in the kidneys from different groups of mice (one-way ANOVA). (B) Representative Western blots and relevant statistical results of PDCD4, FGR and NOTCH1 protein levels in the kidneys from different groups of mice (one-way ANOVA). (C) Serum creatinine (SCr) concentrations in different groups of mice (two-way ANOVA). (D) BUN levels in different groups of mice (two-way ANOVA). (E) Representative micrographs showing the morphologies of kidneys from different groups of mice. (F) Quantitative assessment of tubular damage. (two-way ANOVA). (G) *In situ* TUNEL assays were performed to assess renal cell death. Nuclei were revealed using DAPI staining. (H) Quantitative assessment of the number of dead cells (numbers per high-power field [HPF]; two-way ANOVA). (I) Representative Western blots and summarized data showing the protein levels of NOTCH1 in the kidneys from different groups of mice (two-way ANOVA). All data are displayed as mean ± SD, $n = 8$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

are needed to investigate whether other chemical drugs targeting FGR such as saracatinib (AZD0530), have similar therapeutic effects in AKI.

Collectively, the findings of our present study provide direct evidence showing that FGR/NOTCH1 is one of the critical signal transduction pathways that links PDCD4 to AKI in mice. Therefore pharmacological targeting of PDCD4 signaling pathways at multiple levels, such as *via* inhibition of PDCD4 or FGR activity, may represent novel therapeutic strategies for the treatment of AKI.

5. Conclusions

We found that PDCD4 served as an important regulator of AKI in mice, at least partly *via* FGR/NOTCH1-mediated tubular apoptosis and inflammation. Using ponatinib-mediated

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pharmacologic targeting of this pathway may hold therapeutic potential for mitigating AKI.

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Author contributions

Yan Zhang and Wei Tang designed the study. Xu Jing, Dandan Ren and Fei Gao carried out most of the experiments. Xiao Wu contributed to the preparation of human kidney specimens. Ye Chen interpreted the results. Yue Han and Qingsheng Han contributed to data collection. Liang Li and Xiaojie Wang contributed to the preparation of the figures. All authors contributed to data analysis and the preparation, writing and final approval of the manuscript.

Conflicts of interest

All authors declare no competing interests.

Appendix A. Supporting Information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2020.10.024.

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