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Egr-1 deficiency protects from renal inflammation and fibrosis

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Abstract

NF-κB and TGFβ play critical roles in renal inflammation and fibrosis, and their regulation in the kidney is thus of great interest. Early growth response-1 (Egr-1), a transcription factor belonging to the immediate early gene family, has been found to regulate inflammation and fibrosis in non-kidney tissues, but its role in renal failure has not been clear. In this study, wild-type and $Egr1^{-/-}$ mice were fed with an adenine-enriched diet to induce tubulointerstitial nephritis (TIN), and primary tubular epithelial cells (PTECs) were treated with pro-inflammatory and pro-fibrotic cytokines. Kidney tissues from patients with or without renal failure were stained for Egr-1. Our results showed that Egr-1 expression was upregulated in the kidney with TIN, and the tubular epithelial cell is the primary site for Egr-1 upregulation and nuclear translocation. $Egr1^{-/-}$ mice were protected from renal failure, reflected by low levels of serum urea and creatinine. The protective effect was related to an attenuation of tubular injury, immune cell infiltration, NF-κB activity, and cytokine/chemokine expressions in the kidney. Renal fibrotic area and TGFβ signaling were also reduced in $Egr1^{-/-}$ mice. In vitro study showed that Egr-1 deficiency attenuated the ordinary responses of PTECs to TNFα and TGFβ. Importantly, Egr-1 is of clinical significance since the activity of Egr-1 in renal tubular cells was upregulated in renal failure patients. Our study highlights the integrative role of Egr-1 in renal inflammation and fibrosis. Thus, Egr-1 may serve as a therapeutic target for human kidney diseases.

Key messages

- Renal failure activates Egr-1 in human and mouse tubular cells.
- Egr-1 deficiency attenuates NF-κB and TGFβ-mediated renal inflammation/fibrosis.
- $Egr1^{-/-}$ PTECs respond weakly to pro-inflammatory or pro-fibrotic stimulation.

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Introduction

Chronic kidney disease is a pathological process of renal inflammation and fibrosis. Kidney injury activates NF-κB pathway, which in turn leads to renal inflammatory responses including macrophage infiltration and release of pro-inflammatory cytokines and chemokines [1]. The nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is also a potential regulator for renal inflammation [2]. Knocking down the components of NLRP3 inflammasome or its downstream inflammation-related genes attenuates tubulointerstitial nephritis (TIN) induced by an adenine-enriched diet [3]. Chronic renal inflammation leads to fibrosis, and transforming growth factor β (TGFβ) is central to the whole fibrogenic processes, mediating epithelial-mesenchymal transition (EMT) of tubular epithelial cells, and excess deposition of extracellular matrix (e.g., collagen) [4, 5]. Given the critical roles of NF-κB and TGFβ in inflammation and fibrosis, identifying how they are regulated is of great importance for the treatment of human renal diseases.

Early growth response-1 (Egr-1), an 80-kDa zinc-finger transcription factor of the immediate early gene family, has been found to regulate inflammation and fibrosis in various tissues. Egr-1 promotes inflammation in the animal models of cholestatic liver injury [6], ischemic and reperfusion lung injury [7], and atherogenesis [8]. Egr-1 upregulation is also essential for TGFβ-induced fibrosis in skin and lung [9, 10]. Several potential mechanisms have been proposed for Egr-1 in regulation of inflammation and fibrosis. For example, Egr-1 may enhance the expression of tumor necrosis factor-α (TNFα), NF-κB, and TGFβ, since the promoters of these genes all contain Egr-1 binding site [11–13]. Egr-1 also modulates NF-κB activity post-transcriptionally by direct interaction with NF-κB subunit [14], or indirectly via inducing activator of NF-κB, such as TNFα [15]. On the other hand, some studies found Egr-1 is able to antagonize NF-κB and TGFβ-mediated responses. For example, Egr-1 counteracts lipopolysaccharide (LPS)-induced NF-κB activation in human enterocytes [16], and hepatic fibrosis induced by carbon tetrachloride is enhanced in $Egr1^{-/-}$ mice [17]. Thus, the consequences of Egr-1 activation in NF-κB and TGFβ pathways vary according to the insults and organs.

The role of Egr-1 in kidney injury has only been addressed in a few studies. During renal ischemia and reperfusion injury, Egr-1 protein is rapidly expressed and accumulated in the nuclei of the thick ascending limbs and collecting ducts [18]. Transfecting DNA enzyme to suppress Egr-1 activity in renal fibroblasts inhibits interstitial fibrosis in rats via

downregulation of TGFβ, α-smooth muscle actin (αSMA) and type I collagen [19]. Despite these studies, the role of Egr-1 in renal inflammation and fibrosis has not been firmly established and not been tested in the animal model with systemic renal failure. In addition, it would be interesting to identify the major cell type responsible for Egr-1 upregulation in the kidney.

The aim of this study is to comprehensively explore the role of Egr-1 in kidney injury. The effects of Egr-1 deficiency on NF-κB-mediated renal inflammation and TGFβ-mediated renal fibrosis were verified, for the first time, in the animal model of TIN. The responses of Egr-1-deficient primary renal tubuloepithelial cells (PTECs) to pathological insults were also examined. Finally, Egr-1 expression in human kidney samples was assessed to correlate the bench work findings with clinical conditions.

Materials and methods

Mice

$Egr1^{-/-}$ mice, kindly provided by J. Milbrandt (Washington University, St. Louis, MO, USA) [20], were maintained on the C57BL/6 genetic background and housed in a specific pathogen-free barrier facility. For the control group, wild-type littermates were generated by intercrossing $Egr1^{+/-}$ mice. At 8 weeks of age, the mice were fed ad libitum with a 0.25 % adenine-enriched diet (LabDiet, St. Louis, MO) to induce TIN or a regular chow diet (Purina laboratory Rodent Diet 5001; PMI Nutrition International, Richmond, IN) as controls. After feeding for 10–13 days, the mice were sacrificed to harvest the kidneys. All procedures for handling animals were done in accordance to ethical statements approved by the Institutional Animal Care and Use Committee of National Cheng Kung University.

Primary cell culture and treatment

Mouse PTECs were isolated for culture according to the method of Tang and Tannen [21]. Briefly, mouse kidneys were removed, decapsulated, and the cortical tissues were minced and digested with 0.5 mg/ml collagenase type II (Worthington, Lakewood, NJ). The proximal tubules were isolated after density gradient centrifugation using 42 % Percoll solution as suspension and cultured in DMEM-F12 medium (Sigma-Aldrich) supplemented with 1× insulin-transferrin-selenium, 1× MEM non-essential amino acids, 0.1 μM hydrocortisone (Invitrogen, Carlsbad, CA), 100 IU/ml penicillin, 100 μg/ml streptomycin (Sigma-Aldrich), and 1 % FBS (Invitrogen). To prove that authentic proximal tubuloepithelial cells were obtained, the expression of proximal tubule differentiation markers, including sodium-glucose cotransporter

type 2 (*Sglt2*) and sodium-hydrogen exchanger isoform 3 (*Nhe3*), was analyzed [22]. For the treatments, PTECs were incubated in serum-free medium for 24 h and then treated with TNFα (PeproTech, Rocky Hill, NJ) at 10 ng/ml or 100 ng/ml for 1 h or 24 h, or with TGFβ (PeproTech) at 10 ng/ml for 4 h to 72 h.

Human renal tissues

Human renal tissues were obtained from the Human Biobank, Research Center of Clinical Medicine, National Cheng Kung University Hospital. Ten tissues were kidney biopsy samples with histological diagnosis of diabetic nephropathy, confirmed by light microscopic, immunofluorescent, and electron microscopic examinations. These samples were classified as the "renal failure" group since the creatinine levels of these patients were above the normal limit (Supplementary Table 1). The other 12 renal tissues were derived from non-diabetic patients with serum creatinine level within normal limits and underwent nephrectomy for urothelial malignancy. The renal tissues of these patients, which were adjacent to the tumor, were classified as the "normal" group because no histological abnormality could be identified under microscopy. The study was conducted according to the study protocol approved by the Institutional Review Board of National Cheng Kung University Hospital (permit number: B-ER-101-008).

RNA analysis

Tissues and cells were stored in RNA*later* solution (Ambion Inc., Austin, TX) and total RNA was extracted using REzol RNA extraction reagent (Protech Technology, Taipei, Taiwan). Samples of messenger RNA (mRNA) were analyzed by SYBR green-based real-time quantitative real-time polymerase chain reaction (RT-PCR) (Applied Biosystem, Foster City, CA), with β-actin (*Actb*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), or cyclophilin A (*Ppia*) as the reference gene in each reaction. The sequences of the primers are given in Supplementary Table 2.

Western blotting

Total proteins of kidneys were subjected to electrophoresis; transferred to PVDF membranes and probed with antibodies against Egr-1, apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1 (Santa Cruz Biotechnology, CA); IκB, phosphorylated IκB kinase-α/β (p-IKKα/β), Smad3, phosphorylated-Smad3 (Cell Signaling Technology, Beverly, MA); TGFβ (PeproTech), NLRP3 (Abcam, Cambridge, MA); Interleukin-1β (IL-1β) (R&D

systems, Minneapolis, MN); and β-actin (Sigma-Aldrich). Immunoreactive proteins were detected using an enhanced chemiluminescence detection system (GE Healthcare, Pittsburgh, PA).

Renal morphology

The kidneys were fixed in 4 % para-formaldehyde and embedded in paraffin. Sections with a thickness of 5 μm were cut and stained with hematoxylin and eosin (H&E) or Masson's trichrome. The H&E stained sections were used to determine the tubular injury score, which estimates the percentage of injured tubules (tubule flattening, necrosis, apoptosis, or presence of cast) in the cortex and outer medulla. The score is as follows: 0, none; 1+, <10 %; 2+, 10–25 %; 3+, 26–45 %; 4+, 46–75 %; and 5+, > 75 %. The average score was determined by 10 random, non-overlapping ×400 magnification fields per kidney section. To quantify interstitial fibrosis, kidney sections with Masson's trichrome staining were evaluated using a computer-aided evaluation program (AxioVision LE, 40 × 64v, Zeiss, Germany). The percentage of fibrotic area (stained in blue) relative to the entire field without glomeruli, and large vessels was calculated. Ten randomly selected fields of renal cortex per section were examined at ×400 magnification.

Immunohistochemical and immunofluorescence staining

For the immunohistochemical staining, formalin-fixed paraffin-embedded tissue sections (5-μm thick) were deparaffinized, boiled, blocked, and incubated overnight with the primary antibody against Egr-1 (Santa Cruz Biotechnology). Secondary antibody staining (VECTASTAIN ABC kit; Vector Laboratories) was performed. Slides were developed using 3,3′-diaminobenzidine substrate-chromogen solution (Dako, Glostrup, Denmark) and counterstained with hematoxylin. The percentage of positive nuclear staining of Egr-1 in renal tubule was scored as follows: 0, none; 1+, < 25 %; 2+, 26–50 %; 3+, 51–75 %; and 4+, > 75 %. The intensity of cytoplasmic staining of Egr-1 in renal tubule was scored as follows: 0, absent; 1+, weak; 2+, moderate; and 3+, strong. The average score of each kidney section was determined by 10 randomly selected, non-overlapping ×400 magnification fields.

For the immunofluorescence staining, frozen sections (20-μm thick) or cells were blocked with blocking buffer (Thermo Scientific Pierce) for 30 min and incubated overnight with antibodies against Egr-1 or NF-κB RelA/p65 (Santa Cruz). The sections or cells were then probed by secondary antibodies conjugated with Alexa Fluor 555 (Invitrogen) or 5(6)-TAMRA (Leadgene,

Taiwan), and examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Blood urea nitrogen and serum creatinine

Serum samples of the mice were collected via orbital sinus blood sampling or at the time of sacrifice. Serum levels of blood urea nitrogen (BUN) and creatinine were measured by a colorimetric method using a dry chemistry analyzer (Fujifilm, Tokyo, Japan).

Data analysis

Values are reported as mean±SEM unless otherwise specified. Statistical analyses were conducted using Student's t test. Differences were considered statistically significant at $p \leq 0.05$.

Results

Egr-1 is upregulated in the animal model of TIN

In mammals, adenine is converted by xanthine dehydrogenase (XDH) into 2,8-dihydroxyadenine, an insoluble crystal that precipitates in the tubules of kidney and causes TIN and renal failure. Wild-type mice fed with an adenine-enriched diet for 10 days showed typical features of human chronic kidney disease, including body weight loss, high serum creatinine, and high blood urea nitrogen (BUN) (Supplementary Figure 1A). TIN was successfully induced, revealed by massive immune cell infiltration, interstitial fibrosis (Supplementary Figure 1B), increased renal expression of pro-inflammatory factors (macrophage inflammatory protein-2 [MIP-2], monocyte chemotactic protein-1 [MCP-1], interleukin-6 [IL-6], and TNFα), pro-fibrotic factors (TGFβ and connective tissue growth factor [CTGF]), and markers for macrophage (F4/80), extracellular matrix (type I collagen) and EMT (α-SMA) (Fig. 1a). These TIN pathologies were not associated with changes of XDH expression but associated with a fivefold upregulation of Egr-1 mRNA (Fig. 1a). Interestingly, the increases of Egr-1 expression and its nuclear translocation were prominent in the tubules rather than in the glomeruli (Fig. 1b). Immunoblotting confirmed the nearly twofold upregulation of Egr-1, which is accompanied by the increases of TGFβ protein level, the downstream signaling (Smad3 phosphorylation), and the EMT effector (α-SMA) (Fig. 1c). These results suggest that Egr-1 activation in the renal tubule is likely involved in the pathogenesis of TIN through governing the transcription network for renal inflammation and fibrosis.

TIN associates with increased NLRP3 protein level but not the activation of caspase-1

Since NLRP3 inflammasome has been suggested in TIN development [3], we then determined its expression and activation. mRNA levels of NLRP3 inflammasome components, including NLRP3, ASC, and pro-IL-1β, were all upregulated (Fig. 1a). Immunoblot analyses revealed increases of NLRP3 and pro-caspase-1 but a decrease of ASC (Fig. 1d). This is associated with an indistinguishable change in active caspase-1, the major effector of NLRP3 inflammasome. Since pro-IL-1β and active IL-1β were both upregulated despite no change of caspase-1 (Fig. 1d), the involvement of NLRP3 inflammasome in adenine-induced TIN remains debated.
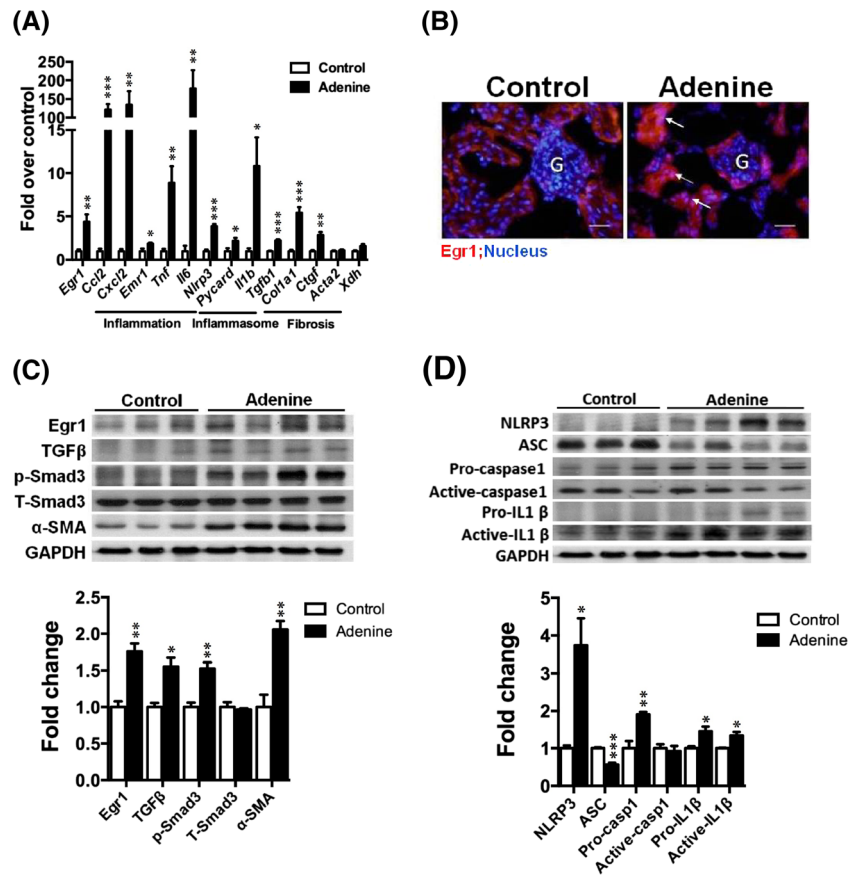
Egr-1 deficiency attenuates renal inflammation

To address the functional significance of Egr-1 upregulation in TIN, $Egr1^{-/-}$ mice were fed with an adenine-enriched diet for 10–13 days. Although the body weight loss was similar between $Egr1^{-/-}$ mice and wild-type littermates, $Egr1^{-/-}$ mice had lower serum creatinine and BUN levels (Fig. 2a), decreased immune cell infiltration and tubular injury score (Fig. 2b, c), and reduced expression of the pro-inflammatory cytokine (TNFα and pro-IL-1β), chemokines (MIP-2 and MCP-1), and the macrophage marker (F4/80) (Fig. 2d). These results suggest that Egr-1 deficiency protects from adenine-induced renal injury. The attenuation of inflammation in $Egr1^{-/-}$ mice was attributed to decreased NF-κB activity, evidenced by a 40 % reduction of phosphorylated IKKα/β level (Fig. 2e). Another evidence supporting the decrease of NF-κB activity in $Egr1^{-/-}$ mice was the reduced protein level of NLRP3 (Fig. 2e), which is known to be regulated by NF-κB activation. Intriguingly, the attenuated expression of NLRP3 in $Egr1^{-/-}$ mice did not lead to a significant decrease in active caspase-1 and IL-1β, the major effector and end product of NLRP3 inflammasome (Fig. 2e). These results suggest that Egr-1 deficiency attenuates NF-κB-related inflammatory responses but has a minor effect on activation of NLRP3 inflammasome.

Egr-1 deficiency attenuates renal fibrosis

After renal failure had been induced for 10–13 days, $Egr1^{-/-}$ mice showed less fibrotic area in the kidney than the wild-type littermates (Fig. 3a). This is associated with lower mRNA levels of TGFβ and type I collagen (Fig. 3b) and a trend toward decrease of TGFβ protein (Fig. 3c). The attenuation of phosphorylated Smad3 and the EMT marker α-SMA in $Egr1^{-/-}$ mice (Fig. 3c) suggests that the suppressed TGFβ signaling is responsible for the decrease of renal fibrosis in Egr-1 deficiency.

Fig. 1 Renal expression of Egr-1, inflammatory, and fibrotic markers in tubulointerstitial nephritis. **a** RT-PCR for the expression of Egr-1, inflammatory markers, NLRP3 inflammasome components, and fibrotic markers of the adenine-fed group relative to the control group ($n = 4$ in each group). **b** Immunofluorescence staining for Egr-1 (*red*) in the kidney. The DAPI nuclear counterstain appears *blue. Scale bars,* 50 μm. G, glomerulus. *Arrows* indicate renal tubular cells with Egr-1 nuclear translocation. **c** Immunoblot analyses for Egr-1 and fibrosis-related proteins. **d** Immunoblot analyses for NLRP3 inflammasome components. The intensities of bands quantified densitometrically relative to the control are represented as the bar graph and expressed as mean ± SEM. $n = 4$ in the adenine-fed and $n = 3$ in the control group. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

Egr-1 deficiency attenuates pro-inflammatory and pro-fibrotic responses in renal tubular cells

Since Egr-1 is predominantly upregulated and activated in the tubular cells, we then used mouse PTECs for the subsequent studies. The highly expressed proximal tubule differentiation markers, *Sglt2* and *Nhe3*, in the cultured PTECs compared to the non-kidney cell lines suggest that authentic epithelial cells were obtained (Supplementary Figure 2). PTECs from $Egr1^{-/-}$ mice and wild-type mice were stimulated with TNFα or TGFβ to mimic an exposure of pro-inflammatory or pro-fibrotic stimulation. The expression of MCP-1, MIP-2, pro-IL-1β, and TNFα upon TNFα stimulation was lower in $Egr1^{-/-}$ PTECs than in wild-type PTECs (Fig. 4a). Egr-1 deficiency also attenuated TGFβ-induced expression of type I collagen, CTGF, TGF-β1, and Snail (Fig. 4b). These results were consistent when being normalized to the expression of different reference genes (Supplementary Figure 3). We further analyzed NF-κB activity in PTECs by immunofluorescent staining of RelA/p65 in response to TNFα. Treatment of TNFα (100 ng/ml) for 1 h induced RelA/p65 nuclear translocation in both genotypes of PTECs (Fig. 4c). However, the number of cells with RelA/p65 nuclear translocation was lower in $Egr1^{-/-}$ PTECs (81/185 = 43.8 %) than in wild-type PTECs (130/254 = 51.2 %). These results suggest

that the attenuated inflammatory responses in $Egr1^{-/-}$ PTECs might be attributed to the downregulation of NF-κB activity. Collectively, these results suggest that Egr-1 is essential for NF-κB-mediated inflammatory responses and the induction of fibrotic responses in renal tubular cells.

For the study of PTECs, it is essential to use equal number of viable cells from wild-type and $Egr1^{-/-}$ mice. For this, several points need to be clarified. First, using the methodology developed by Tang and Tannen [21], we actually obtained segments of renal tubules instead of separated cells from the kidney. The cell-cell contact in these isolated tubules may slow down their transition to mesenchymal cells before the experimental treatment [23]. However, because the cells were clustered together, it is difficult to calculate the cell number directly. Nevertheless, in the analysis of gene expression, we normalized to the expression of several reference genes, including *Actb*, *Gapdh*, and *Ppia*, and found the consistent results (Fig. 4 and Supplementary Figure 3). These reference genes, thought to be expressed in a stable and non-regulated constant level, are involved in processes essential for the survival of cells [24]. Second, we trypsinized the separated cells after growing from primary tubules for 5 days, and seeded equal number (4×10^3 cells) of $Egr1^{-/-}$ or wild-type cells for the assay of LDH activity as an indicator of cell death. After culturing for 24 h, the LDH activity did not differ between

Fig. 2 The effects of Egr-1 deficiency on renal failure and inflammation. a Body weight change, serum creatinine, and BUN levels of adenine-fed $Egr1^{-/-}$ ($n = 10$) and wild-type mice ($n = 12$). b Representative images of H&E stained kidney sections (original magnification ×400). c Tubular injury score of the H&E stained kidney section. d Renal expression of inflammatory markers and NLRP3 components of adenine-fed $Egr1^{-/-}$ mice ($n = 10$) relative to wild-type mice ($n = 12$). e Immunoblot analyses and quantification of phosphorylated IKKα/β, IκB, and NLRP3 inflammasome in the kidney of adenine-fed $Egr1^{-/-}$ and wild-type mice ($n = 4$ in each group). The intensities of bands quantified densitometrically relative to the wild-type are represented as the bar graph and expressed as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$
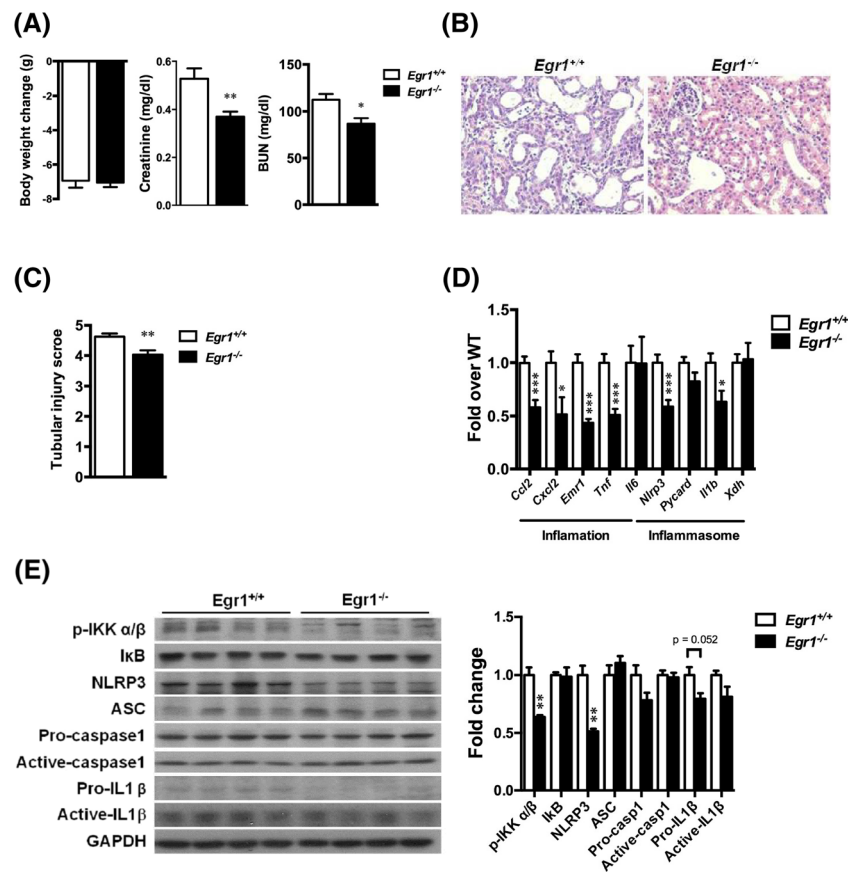
$Egr1^{-/-}$ PTECs (8.5 %) and wild-type PTECs (7.3 %). Since the seeding numbers were equal and cytolysis was not different, it is reasonable to speculate that the numbers of

Fig. 3 The effects of Egr-1 deficiency on renal fibrosis. a Representative images and fibrotic area quantification of Masson's trichrome stained kidney sections (original magnification ×400). b Renal expression of fibrotic markers of adenine-fed $Egr1^{-/-}$ mice ($n = 10$) relative to wild-type mice ($n = 12$). c Immunoblot analyses and quantification of TGFβ, phosphorylated Smad3, total Smad3, and α-SMA in the kidneys of adenine-fed $Egr1^{-/-}$ and wild-type mice ($n = 4$ in each group). The intensities of bands quantified densitometrically relative to the wild-type are represented as the bar graph and expressed as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

viable cell were equal. Third, although, to our knowledge, primary culture for $Egr1^{-/-}$ PTECs has never been performed, Bastianelli et al. showed that the cell proliferation rate of cardiac mesenchymal stem cells (cMSCs) derived from $Egr1^{-/-}$ mice did not differ from that of cMSCs derived from wild-type mice [25]. Therefore, Egr-1 deficiency does not necessarily correspond to the proliferation rate of primary cells ex vivo.
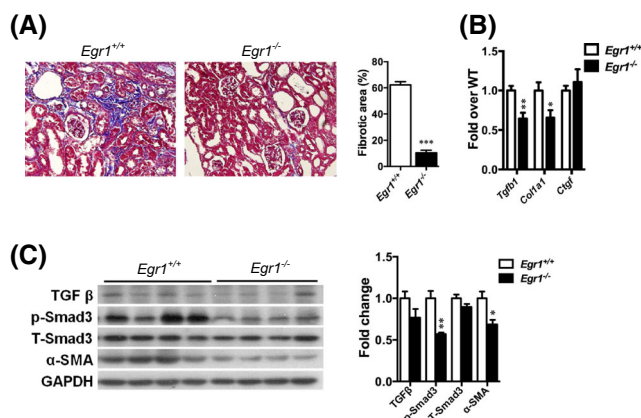
Egr-1 activity increases in patients with renal failure

To study the clinical significance of Egr-1 in human kidney diseases, immunohistochemical staining for Egr-1 was applied to 10 renal biopsy tissues of diabetic patients with serum creatinine concentrations ranged from 1.63 mg/dl to 10.61 mg/dl, as well as to 12 kidney tissues obtained from patients with urothelial malignancy and normal serum creatinine levels. Patients with and without renal failure both expressed Egr-1 in the cytoplasm of renal tubular cells, but only those with renal failure had higher cytoplasmic staining of Egr-1 (Fig. 5a, b). In addition, a higher number of tubular cells with prominent Egr-1 stain within the nuclei was observed in the renal failure patients than in the controls. These results suggest that Egr-1 activity increases with kidney injury.

Fig. 4 The responses of PTECs to cytokine stimulation in Egr-1 deficiency. **a** Expression of inflammatory markers in response to TNFα stimulation, $n = 3$ per experiment condition. **b** Expression of fibrosis markers in response to TGFβ stimulation, $n = 4$ per experiment condition. The reference genes were *Gapdh* for *Col1a1* and *Ctgf*, and *Actb* for the others. mRNA levels are expressed relative to the wild-type PTECs of control treatment. $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ compared with the control treatment; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ compared with the respective wild-type PTECs within the treatment group. **c** Immunofluorescence staining for NF-κB RelA/p65 (*red*) in PTECs. The DAPI nuclear counterstain appears *blue. White arrows* indicate nuclear translocation of RelA/p65 (original magnification ×400)
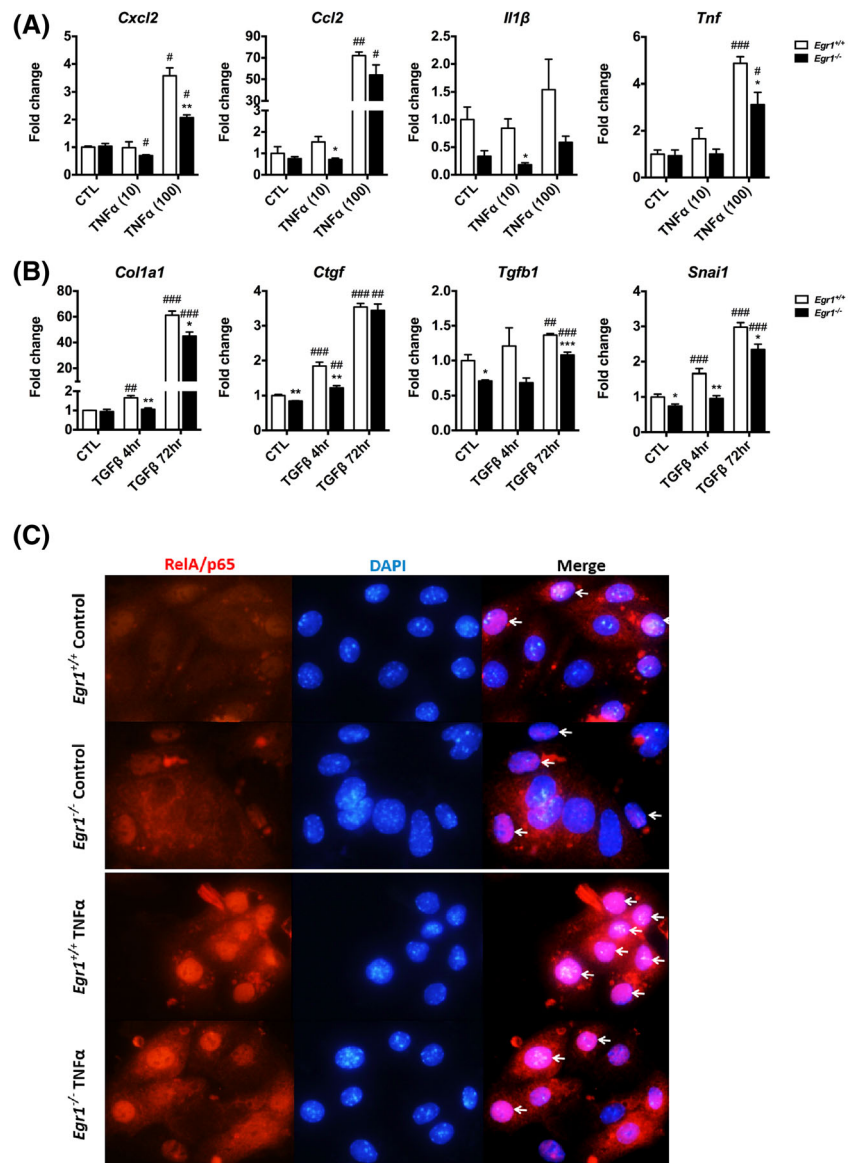
Discussion

This study established the crucial role of Egr-1 in chronic kidney disease by showing the expression of Egr-1 in renal tubules during TIN, attenuation of renal inflammation and fibrosis in $Egr1^{-/-}$ mice, and reduced inflammatory and fibrotic responses in PTECs with Egr-1 deficiency. To our knowledge, this is the first study using $Egr1^{-/-}$ mice to verify the renal effects, which provides convincing evidence to support the importance of Egr-1 in the progression of renal failure. Importantly, Egr-1 also plays a role in human kidney disease, as the cytoplasmic expression and nuclear localization of Egr-1 were upregulated in patients with renal failure.

The primary site of Egr-1 activation in our study is the renal tubule, but the expression of Egr-1 has been reported in other cell types of kidney, depending on where the primary site of

injury is in the animal model. For example, a rat model of mesangioproliferative glomerulonephritis induces Egr-1 expression in the mesangial cells [26], while the ischemic-reperfusion model or a model causing selective injury to the watershed area of kidney upregulates Egr-1 in the thick ascending limbs [18, 27]. In the model of tubulointerstitial fibrosis induced by unilateral ureteral ligation, Nakamura et al. showed the transfected DNA enzyme for Egr-1 is predominantly located in the interstitial fibroblasts, but they did not exclude the presence of Egr-1 in renal tubules [19]. In a rat model with 5/6 nephrectomy, Egr-1 is overexpressed in renal tubular epithelial cells [28]. In our study, we induced TIN in animals, and the increases of Egr-1 expression and nuclear translocation were mainly in, if not limited to, tubular epithelial cells. Being a transcription factor, Egr-1 in the nuclei is related to its transcriptional activity. The activity of Egr-1 in
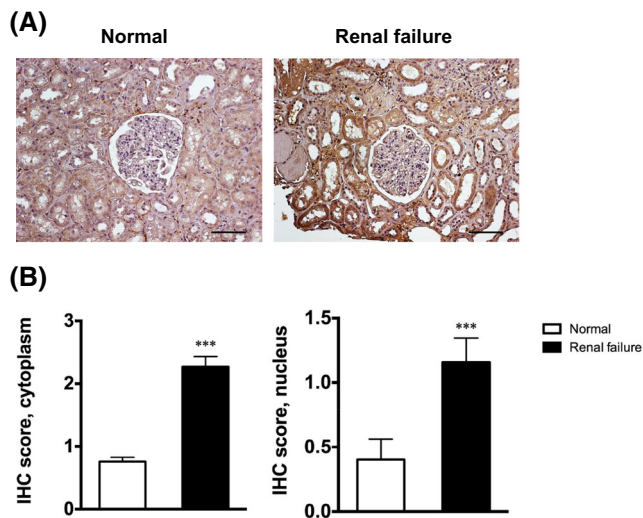
(A)

Normal   Renal failure

(B)

Fig. 5 Immunohistochemical staining for Egr-1 in the kidneys of patients with/without renal failure. **a** Representative images. **b** Scoring for Egr-1 stain intensity in the cytoplasm of tubular cells (IHC score, cytoplasm) and scoring for the proportion of tubular cells with positive Egr-1 stain in the nucleus (IHC score, nucleus). $n = 10$ for renal failure patients, $n = 12$ for patients with normal renal function. *Scale bars*, 100 μm. ***$p \leq 0.001$

renal tubules may be clinically relevant because in our and other studies, immunohistochemistry analysis for patients with kidney diseases showed that increased staining of Egr-1 is mainly in the nuclei of renal tubular cells [28]. Although the concept that tubular cells undergo EMT in vivo has been challenged recently [29], the contribution of tubular cells to the progression of renal failure is still important [30]. We also confirmed Egr-1 deficiency in PTECs attenuated their responses to pro-inflammatory and pro-fibrotic stimuli ex vivo, but how Egr-1 works in tubules in vivo requires further investigations.

Our results suggest that Egr-1 aggravates renal failure via facilitating renal tubular cells in production of pro-inflammatory cytokines and chemokines. Egr-1, induced by TNFα, actively participates in the inflammatory process of non-kidney tissues and cell lines [6–8, 31–33]. Egr-1 usually acts with the transcription factor NF-κB in synergy for the transcription of pro-inflammatory mediators [34–36], but Egr-1 can itself modulate the transcriptional activity of NF-κB [12, 14, 16, 37]. With respect to renal inflammation, the role of Egr-1 has not been well defined in the past. Our study found that $Egr1^{-/-}$ mice with TIN expressed less cytokines (TNFα), chemokines (MIP-2 and MCP-1), and had a lower NF-κB activity (phosphorylated IKKα/β) than wild-type mice. In addition, Egr-1 deficiency attenuated the response of ex vivo PTECs to TNFα stimulation possibly through the downregulation of NF-κB activity. Taken together, our results support the involvement of Egr-1 in NF-κB-mediated renal innate immunity.

In contrast, the present study suggests that the NLRP3 inflammasome pathway may not be involved in Egr-1-

mediated renal inflammation. This is consistent with a recently proposed non-canonical NLRP3 effect, which suggests the influence of NLRP3 on kidney may not be dependent on IL-1β or IL-18 [2]. Andersen et al. showed attenuated kidney injury but intact IL-1β secretion in $Nlrp3^{-/-}$ mice with immune complex glomerulonephritis [38]. Shigeoka et al. reported that NLRP3 deficiency protected mice from post-ischemic acute kidney injury, but IL-1 blockade or IL-1 receptor deficiency did not [39]. Our TIN model in wild-type mice did not find an increase in active caspase-1 despite the significant induction of NLRP3 and pro-IL-1β, suggesting the effect of NLRP3 inflammasome on Egr-1-mediated renal injury is marginal (Fig. 1d). No change of caspase-1 could be related to the downregulation of ASC protein, one key adaptor for NLRP3 inflammasome. We further demonstrated that Egr-1 deficiency downregulated NLRP3 but did not alter the levels of active caspase-1 and active IL-1β (Fig. 2e). These results suggest that Egr-1 may regulate NLRP3 expression in a NF-κB dependent manner, but NLRP3 inflammasome is unlikely to play a major role in renal inflammation during TIN.

Renal fibrosis is a common consequence of chronic kidney disease, and our study showed that Egr-1 participated in this pathological process. The role of Egr-1 in tissue fibrosis has drawn attentions in recent years. Egr-1 is a conserved factor in TGFβ-induced transcription responses across cell-type and species [40]. Given that the promoter of TGFβ1 gene has at least two Egr-1 binding sites, autonomous TGFβ activation may be mediated via Egr-1 [13]. Egr-1 expression driven by TGFβ or other fibrogenic stimuli is identified in animal models of scleroderma, lung fibrosis, and colitis [10, 41, 42], and Egr-1 deficiency ameliorates bleomycin-induced skin and lung fibrosis [43]. However, the profibrotic effect of Egr-1 has been challenged because one study shows carbon tetrachloride-induced hepatic fibrosis is enhanced in $Egr1^{-/-}$ mice [17]. In the kidney, some studies showed that Egr-1 activated in the interstitial fibroblast and tubular epithelial cell is an aggravating factor for fibrosis [19, 28]. Our study also supports the pro-fibrotic role of Egr-1 since Egr-1 deficiency reduced the fibrotic area in the kidney and attenuated TGFβ-induced responses in PTECs. The molecular mechanism for the attenuation of TGFβ signaling in $Egr1^{-/-}$ mice needs further investigations.

It is worth noting a discrepancy between the fivefold upregulation of Egr-1 mRNA (Fig. 1a) and the less than twofold upregulation of Egr-1 protein (Fig. 1c) in TIN. A similar discrepancy was found in our previous publication in pancreatic β cells [44]. The discrepancy may stem from the sensitivity of the detection system (quantitative RT-PCR versus Western blotting). In addition, being a member of the immediate early gene family, Egr-1 protein is both induced and degraded rapidly,

possessing a half-life of less than 2 h [45]. The limited functional period of Egr-1 is also ensured by post-transcriptional regulations. For example, alternative polyadenylation produces two variants of Egr-1 mRNA in the retina with different stability [46]. The binding of HuR protein to the 3'-UTR of Egr-1 mRNA changes the translation rate [47]. Thus, we speculate that a rapid degradation of Egr-1 protein, as well as an altered stability and translation rate of Egr-1 mRNA, might change the Egr-1 mRNA to protein ratio in the injured kidney.

We finally translated our bench findings to the human study and found that Egr-1 was overexpressed and activated in the renal tubular cells of renal failure patients. Our human study has several limitations. First, since normal renal tissues were obtained from patients receiving nephrectomy for urothelial malignancy, some molecular changes may have occurred in these normal appearing tissues. Second, the renal tissues with renal failure were obtained from diabetic patients; thus, we cannot exclude the possibility that Egr-1 is upregulated due to diabetes mellitus rather than renal failure. Third, although Egr-1 upregulation was shown in the renal failure patients, whether Egr-1 plays a protective or detrimental role in these samples remains undefined. Nevertheless, our human data suggest that Egr-1 is involved in regulation of renal failure.

In conclusion, from animal models to human samples, the present study highlights a universal, integrative role of Egr-1 in renal inflammation and fibrosis. This early-responsive transcription factor activated in renal tubule is crucial for amplification of NF-κB and TGFβ-mediated detrimental effects, although its role in NLRP3 inflammasome remains unclear. The strength of our conclusion is derived from the Egr-1 knockout nature of the animal study and the corresponding human data. Thus, we believe our findings provide a solid foundation for future researches considering Egr-1 as a therapeutic target for human kidney diseases.

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