Decoy receptor 2 mediation of the senescent phenotype of tubular cells by interacting with peroxiredoxin 1 presents a novel mechanism of renal fibrosis in diabetic nephropathy

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Premature senescence of renal tubular epithelial cell (RTEC), which is involved in kidney fibrosis, is a key event in the progression of diabetic nephropathy. However, the underlying mechanism remains unclear. Here we investigated the role and mechanism of decoy receptor 2 (DcR2) in kidney fibrosis and the senescent phenotype of RTEC. DcR2 was specifically expressed in senescent RTEC and associated with kidney fibrosis in patients with diabetic nephropathy and mice with streptozotocin-induced diabetic nephropathy. Knockdown of DcR2 decreased the expression of α-smooth muscle actin, collagen I, fibronectin and serum creatinine levels in streptozotocin-induced mice. DcR2 knockdown also inhibited the expression of senescent markers p16, p21, senescence-associated beta-galactosidase and senescence-associated heterochromatic senescent markers p16, p21, senescence-associated beta-DcR2 knockdown also inhibited the expression of metalloproteinase 2 in vitro and in vivo. However, DcR2 overexpression showed the opposite effects. Quantitative proteomics and validation studies revealed that DcR2 interacted with peroxiredoxin 1 (PRDX1), which regulated the cell cycle and senescence. Knockdown of PRDX1 upregulated p16 and cyclin D1 while downregulating cyclin-dependent kinase 6 expression in vitro, resulting in RTEC senescence. Furthermore, PRDX1 knockdown promoted DcR2-induced p16, cyclin D1, IL-6, and TGF-β1 expression, whereas PRDX1 overexpression led to the opposite results. Subsequently, DcR2 regulated PRDX1 phosphorylation, which could be inhibited by the specific tyrosine kinase inhibitor genistein. Thus, DcR2 mediated the senescent phenotype of RTEC and kidney fibrosis by interacting with PRDX1. Hence, DcR2 may act as a potential therapeutic target for the amelioration of diabetic nephropathy progression.

Translational Statement

Premature senescence is a key event in the progression of diabetic nephropathy (DN). This study for the first time demonstrates that decoy receptor 2 (DcR2) mediates the senescent phenotype of tubular cells by interacting with peroxiredoxin 1 (PRDX1), which ultimately contributes to renal fibrosis. These results suggest that DcR2 may serve as a specific marker of tubular senescence and can be used to sort senescent cells. DcR2 may be a potential biomarker for the evaluation of DN progression from the perspective of cell senescence. Furthermore, DcR2 is a potential target for the elimination of senescent cells to prevent DN progression.

D N occurs in 20% to 40% of patients with diabetes and is considered to be the leading cause of end-stage renal disease.1,2 End-stage renal disease arising from diabetes accounts for approximately 16.4% of all cases, according to Chinese Renal Data System statistics.2 Numerous studies have proven that renal tubulointerstitial fibrosis (TIF) plays a crucial role in the progression of DN. The severity of TIF is associated with renal malfunction, which further predicts the prognosis of DN.3–6 However, the mechanism is complex and has not yet been elucidated.

Recent findings have revealed that stress-induced premature senescence is among the key mechanisms in the occurrence of renal fibrosis.7,8 Stress-induced premature senescence is an accelerated senescence that can be induced by various cellular stress signals, independent of replicative senescence and age. Previous published studies have shown that cell senescence mainly relies on the p16-retinoblastoma and alternative reading frame–p53–p21 signaling pathways.8,9 Cell senescence plays an important role not only in embryonic development, but also in the progression of major diseases such as diabetes mellitus, cancer, and cardiovascular disease,
through the senescence-associated secretory phenotype (SASP), which consists of cytokines, chemokines, growth factors, and proteases.10–12

The renal tubular epithelial cell (RTEC) is the main component of renal parenchyma and contains numerous mitochondria. In cases of hyperglycemia, ischemia, and hypoxia, these mitochondria are more susceptible to damage resulting in cell senescence.7,13 A recent study found that tubular cell senescence occurred on day 10 of streptozotocin (STZ) induction in diabetic rats.14 Furthermore, the number of senescent RTECs gradually increased with the progression of DN in human renal tissue specimens.15,16 The above-mentioned evidence indicates that RTEC senescence is the early cellular event of diabetes, playing an important role in the progression of DN. Therefore, it is necessary to clarify the role and mechanism of RTEC senescence in the progression of DN.

DcR2 is a transmembrane receptor of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and belongs to the tumor necrosis factor receptor superfamily.17 DcR2 has been considered a hallmark of cell senescence.18,19 A previous study found that DcR2 was highly expressed in oncogene-induced premalignant tumors, similar to other classical senescent markers such as p16 and senescence-associated β-galactosidase (SA-β-gal).18 However, the expression of DcR2 was significantly decreased in the tumor cells, which indicated the relationship of DcR2 to the degree of tumor differentiation.18,19 DcR2 antagonizes the apoptosis effects induced by chemotherapy drugs, resulting in a poor prognosis.20–23 A recent study20 revealed that DcR2 is also highly expressed in activated senescent hepatic stellate cells, which are associated with secretion of the extracellular matrix and liver fibrosis. In addition, DcR2 is expressed in embryonic kidney tissue, which could be related to remodeling, development, and morphogenesis of the embryonic kidney.11 However, the expression and role of DcR2 in the normal kidney tissue and DN are still unclear. Our previous research found that DcR2 was scarcely expressed in adult normal kidney tissue. However, the expression of DcR2 was significantly increased in patients with DN and was positively associated with renal fibrosis scores.24 We also found that DcR2 was specifically expressed in renal tubules and coexpressed with senescence markers such as p16, p21, and SA-β-gal.25 The surrounding areas of DcR2-positive senescent cells deposit a large amount of collagen, which further indicates through the senescence-associated secretory phenotype (SASP), which consists of cytokines, chemokines, growth factors, and proteases.10–12

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Figure 1 | The expression of decoy receptor 2 (DcR2) in streptozotocin (STZ)-induced diabetic nephropathy in mice and the association of DcR2 with renal fibrosis. (a) Fasting glucose, (b) serum creatinine, (c) blood urea nitrogen (BUN), and (d) urinary albumin-to-creatinine ratio (ACR) in each group. *P < 0.05 versus the control group. (e) Quantitative real-time polymerase chain reaction analyzed the expression of DcR2 mRNA levels in each group (n = 6–7 for each group). (f) Representative immunostaining micrographs showed tubular DcR2 expression, and (g) the percentage of positive renal tubular epithelial cells (RTECs) was quantified (n = 6 for each group). Bars = 80 μm. (h) Western blotting detected the renal expression of DcR2, and (i) the relative levels to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified in different groups (n = 6 for each group). *P < 0.05 versus control; **P < 0.05 versus STZ at 4 weeks. (j) The percentage of positive RTECs was quantified according to tubulointerstitial fibrosis (TIF) scores (n = 6 for each group). Data are expressed as the means ± SD for each group. *P < 0.05 versus TIF1; **P < 0.05 versus TIF2. OD, optical density. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
the important role of DcR2-positive senescent cells in the development of renal fibrosis in DN.

In this study, we investigated the role and underlying molecular mechanism of DcR2 in renal fibrosis and the senescent phenotype of RTECs in vitro and in vivo. To the best of our knowledge, this is the first study to screen the interacting proteins of DcR2 in the renal tissues of DN patients and primary RTECs by immunoprecipitation (IP) combined with quantitative proteomics and to clarify the mechanism of DcR2 in the senescent phenotype of RTECs.

RESULTS
DcR2 is increased in STZ-induced DN mice and is also associated with renal fibrosis
An STZ-induced DN mice model was established to study the expression of DcR2 and association with TIF. Fasting blood

Figure 2 | Decoy receptor 2 (DcR2) aggravates renal fibrosis in streptozotocin (STZ)-induced diabetic nephropathy. (a) Fasting glucose, (b) serum creatinine, (c) blood urea nitrogen (BUN), and (d) urinary albumin-to-creatinine ratio (ACR) in each group (n = 9 for each group). (e) Masson trichrome staining showed renal fibrosis with blue color; the percentage of fibrotic area in different groups after quantitative analysis (n = 9 for each group). Bars = 40 μm. (f) Representative immunostaining micrographs show fibrotic markers α-smooth muscle actin (α-SMA), collagen I, and fibronectin expression in different groups (n = 9 for each group). Bars = 40 μm. (Continued)
glucose, serum creatinine, blood urea nitrogen and urinary albumin-to-creatinine ratio were given in Figure 1a to d. DcR2 mRNA and protein levels were gradually increased and peaked at 16 weeks (Figure 1e, h, and i). DcR2 was mainly expressed in the renal tubules, and the percentage of tubular DcR2 gradually increased with higher TIF scores in STZ-DN mice (Figure 1f, g, and j). Moreover, tubular DcR2 was positively associated with TIF scores ($r = 0.763, P < 0.01$). These results are consistent with our previous study, suggesting that DcR2 plays an important role in the renal fibrosis of DN patients and mouse models.

**Role of DcR2 in renal fibrosis in DN**

To investigate the role of DcR2 in renal fibrosis, green fluorescent protein (GFP)-DcR2 small interfering RNA (siRNA) or overexpression plasmids were injected into the kidney via tail vein under ultrasonography guidance as per the established procedures. As shown in Supplementary Figure S1A and B, GFP was expressed in tubules after control vector-- and DcR2-related plasmid transfection, and GFP was coexpressed with DcR2 in mice treated with DcR2-overexpression plasmid. DcR2 mRNA and protein levels were decreased by 74.7% and 69.0% after DcR2-siRNA transfection, respectively, whereas they were increased by 215% and 192% after DcR2-overexpression transfection, respectively (Supplementary Figure S1C–E). Supplementary Figure S1F and G showed that transfection of DcR2-siRNA downregulated DcR2 expression in RTECs, whereas DcR2-overexpression transfection upregulated DcR2 expression. These results suggest that the gene transfection system can effectively regulate the expression of DcR2 in mice. In the STZ-induced DN model, the levels of DcR2 mRNA and protein and expression of tubular DcR2 were suppressed after siRNA plasmid transfection. However, DcR2 overexpression led to the opposite results (Supplementary Figure S2). Moreover, there was no significant difference in fasting blood glucose levels among STZ mice transfected with control vector, DcR2-siRNA, and overexpression plasmids (Figure 2a). However, the levels of serum creatinine, blood urea nitrogen, and urinary albumin-to-creatinine ratio were decreased after DcR2 knockdown, whereas they were increased after DcR2 overexpression (Figure 2b–d).

We further investigated the effects of DcR2 on renal fibrosis. Masson trichrome staining revealed that DcR2 knockdown inhibited renal fibrosis in STZ-DN mice, but DcR2 overexpression aggravated renal fibrosis (Figure 2e). In
addition, DcR2 knockdown downregulated the expression of fibrotic markers such as α-smooth muscle actin (α-SMA), collagen I, and fibronectin (FN), whereas DcR2-overexpression transfection upregulated expression of these markers (Figure 2f). All of the above-mentioned results were further confirmed by Western blot (WB) analysis (Figure 2g–j). These findings suggest that DcR2 is a key driver of renal fibrosis in DN.

**DcR2 mediates the senescent phenotype of RTECs in vivo and in vitro**

The senescent phenotype of RTECs in STZ-DN mice was examined to study the mechanism of DcR2 in promoting renal fibrosis. Figure 3a and b showed that DcR2 knockdown significantly reduced the levels of p16 and p21 mRNA in STZ-DN, however, DcR2 overexpression markedly augmented the effect. These results were also confirmed by WB analysis (Figure 3c–e). Subsequently, DcR2 knockdown significantly decreased tubular SA-β-gal activity in STZ-DN, and DcR2 overexpression led to the opposite results (Figure 3f and g). In addition, several representative markers of SASP in chronic kidney disease such as interleukin 1β (IL-1β), matrix metalloproteinase 2 (MMP-2), and transforming growth factor β1 (TGF-β1) were evaluated. Immunostaining showed that DcR2 knockdown inhibited the expression of IL-1β and MMP-2 in STZ-DN, whereas DcR2 overexpression promoted...

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**Figure 3 | Decoy receptor 2 (DcR2) mediates the senescent phenotype of renal tubular epithelial cell (RTEC) in streptozotocin (STZ) diabetic nephropathy.** (a) Quantitative real-time polymerase chain reaction analyzed the p16 and (b) p21 mRNA levels in each group (n = 6 for each group). (c) Western blotting detected the renal expression of the p16 and p21 protein, and (d,e) the relative levels to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified in different groups (n = 6 for each group). (f) Representative senescence-associated β-galactosidase (SA-β-gal)–staining micrographs indicated senescent tubules with blue color, and (g) the percentage of positive tubules was quantified (n = 6 for each group). Bars = 200 μm. (Continued)
the expression of these markers (Figure 3h–j). Consistently, MMP-2 and TGF-β1 expression presented similar results by WB analysis (Figure 3k–m). These data suggest that DcR2 mediates RTEC senescence and its secretory phenotype in vivo.

In vitro, primary mouse RTECs were used to construct a high glucose (HG)–induced cellular senescent model. First, the time- and dose-effect relationship of DcR2 expression and senescent markers was assessed. As shown in Figure 4a to e, the mRNA levels of DcR2 and p16 were increased at 12 and 24 hours, respectively, and protein levels of DcR2 and p16 were also elevated at 24 and 36 hours, respectively. The results revealed that DcR2 expression occurred earlier than p16, suggesting that DcR2 may be an inducer of RTEC senescence. Then transfection of DcR2 siRNA and overexpression plasmids was performed to determine the effect of DcR2 on RTEC senescence in vitro (Supplementary Figure S3).
Figure 4 † Decoy receptor 2 (DcR2) induces renal tubular epithelial cell (RTEC) senescence leading to fibrosis in high glucose (HG). (a,b) Expression of DcR2 and p16 mRNA was detected at different time points after HG (30 mmol/l) treatment (n = 6 for each group). (c) Representative Western blot shows DcR2 and p16 expression, and (d,e) the relative levels to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified after HG treatment (n = 4 for each group). (f) Representative micrographs of senescence-associated heterochromatin foci (SAHF) in the nuclei using 4',6-diamidino-2-phenylindole staining and (g) the percentage of positive RTECs (n = 3 for each group). Arrows indicate positive staining. Bars = 20 μm. (h) Western blotting showed p16 expression, and (i) the relative levels to GAPDH were quantified after transfection DcR2 plasmids (n = 4 for each group). (j) Representative senescence-associated β-galactosidase (SA-β-gal)–staining micrographs of RTECs, and the percentage of positive RTECs was quantified (n = 3 for each group). Bars = 200 μm. (Continued)

Figure 4f to m showed that DcR2 siRNA significantly inhibited the expression of senescence-associated heterochromatin foci, p16, and SA-β-gal; decreased the percentage of cells in the G0/G1 phase; and reduced the levels of IL-6 and TGF-β1 in the cellular supernatant. However, transfection of DcR2-overexpression plasmid revealed opposed effects. These results indicate that DcR2 mediates RTEC senescence and SASP in vitro. A recent study27 found that cell senescence and SASP accelerate renal fibrosis in acute kidney injury. Therefore, the effects of DcR2-mediated senescence on fibrosis in HG should be evaluated. Figure 4n to p showed that DcR2-siRNA transfection inhibited the expression of α-SMA and FN, and DcR2 overexpression promoted these effects. Figure 4q to s revealed that p16-siRNA transfection presented the similar results, however, DcR2-siRNA distinctly promoted the effect. Accordingly, these results suggest that DcR2 promotes fibrosis by mediating the senescent phenotype of RTEC.
Proteomics identification of PRDX1 is a novel DcR2-interacting protein

DcR2-interacting proteins were screened by liquid chromatography (LC)–mass spectrometry (MS)/MS–based quantitative proteomics coupled with IP in the renal tissues of DN patients and HG-induced RTEC senescent model. The clinical characteristics of DN patients and normal controls are presented in Supplementary Table S1. The large-scale identification and functional categorization of differentially expressed proteins (DEPs) were analyzed, and a total of 298 DcR2-interacting proteins were identified. Of these, 135 DEPs displayed fold changes > 2.0 in DN patients versus normal controls (P < 0.05) (Figure 5a and Supplementary Table S2). Considering that the renal tissue contains a variety of cells except RTECs, the screening of DcR2-interacting proteins in vitro was also conducted. Of the 179 DcR2-interacting proteins, 59 DEPs displayed fold changes > 2.0 (P < 0.05) in the HG model versus controls (Figure 5b and Supplementary Table S3). Database for Annotation, Visualization, and Integrated Discovery, or DAVID,28,29 and Protein
Analysis Through Evolutionary Relationships, or PANTHER, tools were used for the enrichment analysis of Gene Ontology (Gene Ontology Consortium, http://geneontology.org/) annotation for DEPs. The data were classified as cellular component, biological processes, and molecular function. As shown in Figure 5c and d, the results of function in both DN and HG were similar. Finally, PRDX1, which is related to cell cycle and senescence was studied.

To verify the interaction of DcR2 and PRDX1, dual immunostaining was conducted and colocalization of DcR2 and PRDX1 in RTEC was observed in DN, STZ-DN, and HG-induced RTEC models (Figure 5e, Supplementary Figure S4A). The interaction was also confirmed by co-IP in both STZ-DN and HG models (Figure 5f and g, Supplementary Figure S4B and C). Moreover, FLAG or Myc was immunoprecipitated with Flag-DcR2 or Myc-PRDX1, followed by immunoblotting with anti-Myc or anti-Flag, respectively (Figure 5h). These results confirm that PRDX1 is a novel DcR2-interacting protein in DN.

The interaction of DcR2 and PRDX1 mediates the RTEC senescent phenotype

The expression of PRDX1 and its relationship with cell senescence in DN were studied. As shown in Figure 6a and b, PRDX1 was mainly expressed in the RTECs, and the percentage of PRDX1 expression was higher in patients with DN than in controls. Similar results were found in STZ mice (Figure 6c and d). Recent studies have shown that PRDX1 regulates the cell cycle by interacting with cyclin-dependent kinase 4 (CDK4) or CDK6 and participates in cellular senescence through the p16 pathway. Figure 6e showed that PRDX1 was coexpressed with CDK6, cyclin D1, and p16.
in patients with DN. Transfection of PRDX1-siRNA inhibited p16 and cyclin D1 expression, but promoted the expression of CDK6 in vitro. However, PRDX1 overexpression led to opposing effects (Figure 6f–i). These results suggest that PRDX1 inhibits RTEC senescence by regulating cell cycle regulatory proteins.

To determine the role of the DcR2-PRDX1 interaction in the RTEC senescent phenotype, PRDX1 and DcR2 plasmids were cotransfected in vitro. PRDX1-siRNA transfection significantly increased DcR2-induced p16 expression in RTECs. However, PRDX1 overexpression led to contrary effects (Figure 6f–i). These results suggest that PRDX1 inhibits RTEC senescence by regulating cell cycle regulatory proteins.

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**Basic Research**

**C Jia et al.: DcR2-PRDX1 mediates senescent phenotype of tubular cells**

**DISCUSSION**

In the present study, we found that DcR2 was specifically expressed in RTECs and associated with renal fibrosis in the
Figure 6 | The role of peroxiredoxin 1 (PRDX1) in the senescent phenotype of the renal tubular epithelial cell (RTEC) in diabetic nephropathy (DN). (a) Representative immunostaining micrographs showed the expression of PRDX1 in patients with DN, and (b) the percentage of positive RTECs was quantified (n = 9 for each group). Bars = 80 μm. (c) Western blotting detected the renal expression of p16 in streptozotocin (STZ)-induced mice, and (d) the relative levels to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified (n = 6 for each group). (e) Representative immunofluorescence staining for decoy receptor 2 (DcR2) (red) and p16, cyclin D1, cyclin-dependent kinase 6 (CDK6) (green) in patients with DN (n = 6 for each group). Bars = 40 μm. (Continued)
STZ-induced DN, which was consistent with results in patients with DN. Our previous study revealed that DcR2 was coexpressed with senescent markers such as p16, p21, and SA-β-gal. This study demonstrated that DcR2 mediated senescent phenotype and renal fibrosis in vitro and in vivo. We also found that DcR2 interacted with PRDX1 and could regulate the level of PRDX1 phosphorylation through PTK. These results clarify a new mechanism by which DcR2 mediates the senescent phenotype of RTEC in DN.

Cell senescence is a state of permanent cell cycle arrest in response to various stressors, leading to cell proliferation and repair disorder, irreversible tissue damage, and organ dysfunction. Senescent cells have metabolic activity and release a large number of SASP, for example, proinflammatory factors. SASP not only causes continuous damage but also accelerates the senescence of adjacent normal cells through the autocrine or paracrine pathway and eventually leads to chronic inflammation and fibrosis. In the premature senescent mouse model, a large number of senescent cells appeared in the kidney, heart, and other organs. Renal fibrosis and myocardial hypertrophy can be improved by the elimination of p16-positive senescent cells. A recent study found that renal histological injury, inflammation, and oxidative stress were severe in old-old parabiosis mice compared with young-old mice. Senescent cells could also be found during the progression of chronic kidney disease such as DN, hypertensive nephropathy, and IgA nephropathy and was associated with renal fibrosis and renal function. These studies determined that senescence markers were closely associated to renal fibrosis in vitro and in vivo, which was consistent with the results in patients with DN. This evidence indicates that cell senescence plays an important role in the progression of DN.

We previously reported that DcR2-positive RTECs possess the senescence phenotype in patients with DN and the expression of DcR2 is associated with the degree of renal fibrosis. These results were further confirmed using the STZ-induced DN mouse model in this study. The renal
fibrosis area, expression of fibrotic markers such as α-SMA, collagen I, and FN; senescent markers such as p16, p21, and SA-β-gal; and SASP markers such as IL-1β, MMP-2, and TGF-β1 were significantly increased after DcR2-overexpression transfection compared with expression of these markers in control mice. The opposite results were observed in DcR2-siRNA transfected STZ mice, suggesting that DcR2 could promote renal fibrosis by mediating the senescent phenotype of RTECs in vivo. These data were further confirmed in vitro using the HG-induced primary RTEC senescent model. Although albumin induced the expression of DcR2 and promoted senescent phenotype, HG distinctly promoted these effects, implying that HG is a key trigger of cell senescence (Supplementary Figure S5).

Interestingly, intervention of p16 inhibited the expression of α-SMA and FN, similar to a previous study. However, concurrent intervention of DcR2 distinctly promoted the effect. These results suggest that DcR2 mediates the senescent phenotype of RTECs and renal fibrosis in DN, and DcR2 may be a potential target for the clearance of senescent cells to alleviate renal fibrosis.

This study further indicated that DcR2 could not interact with its ligand TRAIL and antagonistic receptor death receptor DR5 (Supplementary Figure S6), but it could interact with PRDX1 in patients with DN, STZ-DN mice, and HG-treated RTECs. Recent studies have shown that PRDX1 regulates cell cycle by interacting with CDK4 or CDK6. CDK4/6 and cyclin D1 regulate cell cycle transition from...
the G1 to S phase. Once the expression or activity of CDK4/6 is inhibited by p16, the cell cycle is arrested, leading to cell senescence. In this study, we found that PRDX1 was coexpressed with CDK6, cyclin D1, and p16. Furthermore, PRDX1 regulated the expression of CDK6, cyclin D1, and p16. These data indicate that PRDX1 regulates tubular senescence through the p16/CDK6/cyclin D1 pathway.

The study of DcR2- and PRDX1-plasmids cotransfection showed that DcR2 antagonized the antisenescence effect of PRDX1. The interaction of DcR2-PRDX1 not only regulated the expression of senescent markers such as p16 and cyclin D1, but also affected the secretion of IL-6 and TGF-β1. These results suggest that DcR2-PRDX1 could mediate the senescent phenotype of RTECs. Subsequent data indicated DcR2 expression occurred earlier than PRDX1. DcR2 regulated the level of PRDX1 phosphorylation, but it did not affect PRDX1 mRNA and protein expression. Previous studies have found that the peroxidase activity of PRDX1 can be inhibited

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**Figure 7** (Continued) (e) Western blotting showed p16 and cyclin D1 expression, and (f,g) the relative levels to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified in normal condition (n = 4 for each group). *P < 0.05 versus control; **P < 0.05 versus DcR2 overexpression. (h) Western blotting showed p16 and cyclin D1 expression, and (i-j) the relative levels to GAPDH were quantified in HG (n = 4 for each group). *P < 0.05 versus HG; **P < 0.05 versus HG + DcR2 siRNA. (k) The levels of interleukin 6 (IL-6) and (l) transforming growth factor β1 (TGF-β1) in cellular supernatant under normal or HG condition after transfection (n = 4 for each group). *P < 0.05 versus control; **P < 0.05 versus HG; #P < 0.05 versus DcR2 overexpression; ##P < 0.05 versus HG + DcR2 siRNA. Data are expressed as the means ± SD for each group. DAPI, 4',6-diamidino-2-phenylindole; OD, optical density. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
after phosphorylation, which promotes oxidative stress in cells. In the current study, DcR2 overexpression decreased peroxidase activity and increased levels of reactive oxygen species; however, DcR2-siRNA had the reverse effects (Supplementary Figure S7). The data indicated that DcR2 affected the peroxidase activity of PRDX1 by regulating phosphorylation and eventually participated in cell senescence. Additionally, PRDX1 phosphorylation was inhibited by
specific PTK inhibitor genistein. These results suggest that DcR2 regulates the phosphorylation of PRDX1 through the “bridge” of PTK.

In summary, we showed that DcR2 mediates the senescent phenotype of RTECs and renal fibrosis by interacting with PRDX1 during DN. DcR2 promotes PRDX1 phosphorylation and regulates the expression of cell cycle–related proteins, ultimately leading to cell cycle arrest, cell senescence, and the senescent phenotype (Figure 9). These results suggest that DcR2 could serve as a specific marker of tubular senescence and a potential target for the elimination of senescent cells to prevent DN progression.

METHODS

Animal models

Eight-week-old male C57BL/6J mice were obtained from Army Medical University (Chongqing, China) and housed with free access to food and water under normal light-dark cycle. Mice were injected i.p. with STZ (50 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) for 5 continuous days. After 1 week, mice with blood glucose levels > 250 mg/dl were selected and killed at 24 weeks as previously reported. The experimental protocols were approved by the Ethics Committee of the Army Medical University.

Ultrasonography-mediated gene transfer of DcR2-siRNA or DcR2-overexpression plasmid in vivo

DcR2-siRNA or DcR2-overexpression plasmid (OBiO Technology, Shanghai, China) was transferred into the kidney of mice using the ultrasonography microbubble-mediated gene transfer technique as per our previously published research. Briefly, the DcR2 expression plasmids were mixed with Sonovue (Bracco, Milan, Italy) at a ratio of 1:1 (vol/vol). For the control vector group, the same amount of empty control plasmids was used. The mixed solution of 400 μl was injected after 2 weeks of STZ injection via the tail vein. The ultrasound transducer (Therasonic; Electro-Medical Supplies, Wantage, UK) was immediately applied to the kidney with a continuous wave output of 1 MHz at a power of 2 W/cm² for 5 minutes on each side. Gene transfection was done at the ages of 10, 12, 14, 16, 18, 20, and 22 weeks. All mice were euthanized at 24 weeks.

Histologic examination and immunohistochemical staining

Renal tissue sections (4 μm) were stained with Masson trichrome using the standard protocol and analyzed under an Olympus microscope (Tokyo, Japan). All specimens were incubated with primary anti-DcR2 (ab108412; Abcam, Cambridge, UK), anti-collagen I (ab6308; Abcam), anti-FN (ab6328; Abcam), anti-IL-1β (sc-52012; Santa Cruz Biotechnology, Dallas, TX), anti-α-SMA (BM0002; Boster Biotechnology, Wuhan, China), anti-MMP-2 (BM4075; Boster), and anti-PRDX1 (ab16745; Abcam) antibodies. At least 10 fields were randomly selected to evaluate the percentage of positive RTEC staining.

Immunofluorescence analyses

Tissue sections and cells were incubated with anti-DcR2 antibody followed by Alexa-555 conjugated goat anti-rabbit antibody.
mouse antibody (ab150117; Abcam) at 37°C for 1 hour and cosedimented with 4,6-diamidino-2-phenylindole (C1006; Biyuntian, Shanghai, China). Images were detected by confocal fluorescence microscopy (Leica, Wetzlar, Germany) and analyzed with Image J software (version 1.37; National Institutes of Health, Bethesda, MD).

Quantitative real-time polymerase chain reaction

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. A total of 1 μg total RNA was synthesized into cDNA in a 20 μl reaction buffer using a reverse transcription polymerase chain reaction kit (Takara, Kyoto, Japan). Thermal cycling conditions included 40 cycles at 95°C for 30 seconds, 95°C for 5 seconds, and 60°C for 40 seconds.

WB analyses

WB analyses were performed on renal lysates or cell extracts with the following primary antibodies: anti-DcR2 (ab108412; Abcam), anti–p16 (ab189034; Abcam), anti–p21 (ab109199; Abcam), anti–cyclin D1 (ab16663; Abcam), anti–CDK6 (ab151247; Abcam), anti–PRDX1 (ab41906; Abcam), anti–collagen I (ab6308; Abcam), anti–FN (ab6328; Abcam), anti–α-SMA (BM0002; Boster), anti–MMP-2 (BM4075; Boster), anti–TGF-β1 (ab179615; Abcam), anti-phospho–PRDX1 (14041S; Cell Signaling Technology, Danvers, MA), anti–β-actin (BM5422; Boster), and anti–GAPDH (BM3876; Boster). The intensity of each band was analyzed using Quantity One software (Bio-Rad Biotechnology, Hercules, CA).

Cell culture and treatment

Primary mouse renal tubular cells were isolated and cultured as per our previously published study.23 Polyplus transfection system was used for the transfection of second-passage RTECs with control vector, DcR2-overexpression, DcR2-siRNA, PRDX1-overexpression, and PRDX1-siRNA plasmids (OBiO Technology). Cells were incubated with genistein (50 μm; Sigma-Aldrich) for 30 minutes, after which the culture medium was changed. Moreover, cultured cells were exposed to bovine serum albumin (10 mg/ml; Sigma-Aldrich) for 12, 24, and 48 hours, and then we collected the cells and supernatant.

Renal tissue and cellular specimens for IP-LC-MS/MS analysis

Eighteen patients with DN diagnosed by renal biopsy and 18 normal controls were enrolled, and the inclusion and exclusion criteria were established according to our previously published study.24 The second-passage RTECs were treated with HG (30 mm) for 72 hours to establish the senescent cell model. The collected lysates was centrifuged at 12,000 g for 15 minutes and incubated overnight with anti-DcR2 (2 μg) at 4°C. A volume of 40 μl protein A–I–G agarose (Bio-Rad Biotechnology) was added to the solution and again incubated at 4°C for 3 hours with continuous shaking. The collected sediments were centrifuged at 2500g for 5 minutes and conjugated with trypsin gel. The trypptic peptides were fractionated by LC, and then each fraction was analyzed by LC/MS (Supplementary Methods).

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AUTHOR CONTRIBUTIONS

CJ performed the experiments and edited the manuscript; CK-H analyzed the data; YJ, XF, DH-Z, and ZJ-G interpreted the results of the experiments; WL-M and WX-Y prepared the figures; HY-N conceived and designed the study.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Figure S1. DcR2 expression in the renal tissue of mice after ultrasound microbubble-mediated gene transfer of DcR2-siRNA or DcR2-overexpression plasmid.

Figure S2. Expression of DcR2 in the renal tissue of STZ-treated mice after DcR2 knockdown or overexpression.

Figure S3. Expression of DcR2 in RTECs after DcR2 overexpression or knockdown in vitro.

Figure S4. Interaction of DcR2 and PRDX1 in the HG-induced RTEC senescent model.
Figure S5. The effect of albumin stimulation on DcR2 expression and senescent phenotype of tubular cell in vitro.

Figure S6. DcR2 does not interact with TRAIL or DRS in DN.

Figure S7. DcR2 regulates the peroxidase activity of PRDX1.

Table S1. Demographic and clinical characteristics of patients with DN and normal control.

Table S2. DEPs in DN compared with the normal control.

Table S3. DEPs in HG compared with the control.

Table S4. The primer sequences used for RT-PCR analysis.

REFERENCES