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Targeting connexin 43 protects against the progression of experimental chronic kidney disease in mice

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Excessive recruitment of monocytes and progression of fibrosis are hallmarks of chronic kidney disease (CKD). Recently we reported that the expression of connexin 43 (Cx43) was upregulated in the kidney during experimental nephropathy. To investigate the role of Cx43 in the progression of CKD, we interbred RenTg mice, a genetic model of hypertension-induced CKD, with Cx43 + / − mice. The renal cortex of 5-month-old RenTgCx43 + / − mice showed a marked decrease of cell adhesion markers leading to reduced monocyte infiltration and interstitial renal fibrosis compared with their littermates. In addition, functional and histological parameters such as albuminuria and glomerulosclerosis were ameliorated in RenTgCx43 + / − mice. Interestingly, treatment with Cx43 antisense produced remarkable improvement of renal function and structure in 1-year-old RenTg mice. Similar results were found in Cx43 + / − or wild-type mice treated with Cx43 antisense after obstructive nephropathy. Furthermore, in these mice, Cx43 antisense attenuated E-cadherin downregulation and phosphorylation of the transcription factor Sp1 by the ERK pathway resulting in decreased transcription of type I collagen gene. Interestingly, Cx43-specific blocking peptide inhibited monocyte adhesion in activated endothelium and profibrotic pathways in tubular cells. Cx43 was highly increased in biopsies of patients with CKD. Thus, Cx43 may represent a new therapeutic target against the progression of CKD.

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The number of patients suffering from CKD is growing worldwide because of aging of the population, improved survival from cardiovascular diseases, and spreading of type-2 diabetes. CKD is characterized by chronic inflammation leading to abnormal accumulation of extracellular matrix components (mainly collagens type I and III) and structural alterations within all renal compartments.[1,2] Thus, arresting or reversing the progression of renal disease represents one of the major challenges in public health and there is an urgent need to identify new targets of therapy.

Recruitment of leukocytes from the blood to the injured site is an important step in the inflammatory cascade, and gap junctions (GJs) are highly implicated in this process.[3,4] GJ channels are formed by the multimeric assembly of connexins (Cxs), being specialized in the control of direct exchange of small metabolites between adjacent cells. To date, over 20 Cx isoforms have been characterized in mammalian cells.[5,6] One GJ channel results from the docking of two hemichannels or connexons. Each connexon is assembled from six Cx proteins and can open under both physiological and pathological conditions. Connexons and GJ channels may be formed by different Cxs.[7,8] Each type of Cx-made channel has unique inherent gating properties or permeabilities to various molecules and ions. Thus, the Cx composition of GJ channels appeared to determine selectivity for different second messengers.[9]

We have previously reported that Cxs are able to regulate monocyte adhesion in chronic vascular inflammatory diseases.[10,11] We have also recently demonstrated that Cx43 expression was increased in the early stages of hypertension-induced and obstructive nephropathy in the renal cortex of the diseased mice. The Cx43 upregulation was paralleled closely by that of cell adhesion molecules, indicating that this Cx may be considered an early signal of renal inflammation during the progression of CKD.[12] In the present study we investigated whether targeting Cx43 expression by genetic and pharmacogenetic manipulation could alter the progression of renal disease. First, we generated a double transgenic strain by crossbreeding RenTg mice, a genetic model of

hypertension-induced CKD, with mice in which Cx43 expression was genetically reduced by half (Cx43 + / −). In addition, we used Cx43 + / − to perform unilateral ureteral obstruction (UUO), another model of CKD in which the initiating cause is tubulointerstitial inflammation. Finally, we inhibited the Cx43 upregulation in wild-type (WT) mice by delivering an oligodeoxynucleotide antisense (AS) in the two above-mentioned models of experimental nephropathy. Our results demonstrate for the first time that reduction of Cx43 expression limited inflammatory cell infiltration as well as renal fibrosis and markedly improved renal structure and function, indicating that this protein may represent a new therapeutic target against the progression of CKD.

RESULTS

Decreased Cx43 expression ameliorates renal structure and prevents the decline of renal function in hypertension-induced CKD

We have previously demonstrated that Cx43 expression is increased in RenTg mice since the early stages of the disease.[12] In addition, immunofluorescence showed that Cx43 was strongly increased within injured kidneys during the progression of disease (Supplementary Figure S1 online). To obtain further insight into the implication of Cx43 in hypertension-induced CKD, we first attempted to inhibit the Cx43 upregulation in these mice. Given that the Cx43 knockout mouse dies shortly after birth,[13] we interbred RenTg with Cx43 + / − to generate RenTgCx43 + / − mice. As the role of Cx43 in the modulation of blood pressure is controversial,[14–16] we first compared arterial pressure between RenTgCx43 + / −, RenTgCx43 + / +, and WT mice aged 5 months (mo). Systolic blood pressure was significantly increased in hypertensive mice compared with WT controls (Figure 1a). However, decreasing expression of Cx43 did not modulate systolic blood pressure (150 ± 6.5 mm Hg in RenTgCx43 + / + and 152 ± 6.1 mm Hg in RenTgCx43 + / −). Therefore, phenotypic observations due to the genetic deletion of Cx43 are independent from blood pressure. In addition, we confirmed the overexpression of Cx43 messenger RNA (mRNA) in RenTgCx43 + / + mice compared with WT healthy animals. This upregulation was blunted in 5-mo-old RenTgCx43 + / − mice (Figure 1b). Next, we checked whether decreased expression of Cx43 could affect the renal structure. Masson's trichrome staining showed that 5-mo-old RenTgCx43 + / + mice presented established lesions typical of hypertension-induced CKD, such as perivascular and periglomerular inflammation, glomerular ischemia, glomerulosclerosis, and tubular dilation, compared with WT healthy animals (Figure 1c). In RenTgCx43 + / − mice the renal structure was preserved, as glomerulosclerosis and tubular dilation were significantly decreased (Figure 1d and e, respectively) and consequently microalbuminuria was highly reduced (Figure 1f). Thus, decreased expression of Cx43 was associated with improved renal structure and function during the progression of hypertension-induced CKD.

Next, we investigated at the same time point the relation between decreased Cx43 expression and inflammation during the progression of the disease. Quantitative PCR showed that mRNA expression for C-C chemokine receptor type 2 and vascular adhesion molecule-1 (VCAM-1) was highly upregulated in RenTgCx43 + / + mice compared with WT healthy animals (Figure 2a and b, respectively). In RenTgCx43 + / − this increase was significantly restricted. In addition, F4-80 immunostaining showed a pronounced inflammatory cell infiltration in cortical slices of RenTgCx43 + / + mice (Figure 2c). In contrast, monocyte recruitment was highly reduced in RenTgCx43 + / − animals (Figure 2d). To explore whether Cx43 could control the leukocyte recruitment, we compared adhesion of the RenTgCx43 + / + and RenTgCx43 + / − monocytes collected after peritoneal lavage with an activated mouse endothelial cell monolayer. As shown in Figure 2e, the number of adherent Cx43-deficient monocytes was considerably reduced. Thus, reduced Cx43 expression leads to restricted interstitial inflammation during the progression of hypertension-induced CKD.

Given that Cx43 downregulation limited monocyte infiltration, we hypothesized that this protein may influence the development of renal fibrosis. As expected, quantitative PCR showed a marked upregulation of the mRNAs of transforming growth factor-β1 (TGF-β1) and type I collagen (Col1) in RenTgCx43 + / + mice. This upregulation was blunted in the RenTgCx43 + / − animals (Figure 2f and g, respectively). In accordance, Sirius Red coloration demonstrated a decreased interstitial collagen deposition in the cortex of RenTgCx43 + / − mice (Figure 2h). We can thus conclude that Cx43 contributes to interstitial renal fibrosis in addition to monocyte infiltration during the progression of hypertension-induced CKD.

Targeting Cx43 protects against the progression of hypertension-induced CKD

To investigate whether Cx43 could be a potential therapeutic target against the progression of CKD, we blocked its overexpression by using Cx43AS, known to specifically decrease Cx43 expression.[17–20] This AS was administered to 11-mo-old RenTg mice, suffering thus from advanced hypertension-induced CKD, for 1 mo via minipump infusion. Scrambled (SCR) sequence was used as control. As illustrated in Figure 3a, Masson's trichrome showed that the renal structure of 1-year-old RenTg mice treated with SCR was considerably damaged. In contrast, RenTg mice treated with Cx43AS showed a substantial improvement in renal structure as glomerulosclerosis and tubular dilation were significantly reduced (Figure 3b and c, respectively). Consequently, CKD progression was hindered as renal function was highly ameliorated (Figure 3d). In addition, decreased expression of Cx43 (Figure 3e) led to a lesser upregulation of VCAM-1 at both mRNA (Figure 3f) and protein levels (Supplementary Figure S2 online), and consequently limited monocyte infiltration (Figure 3g). Furthermore, upregulation of col1 and TGF-β1 mRNA levels was blunted in RenTg animals

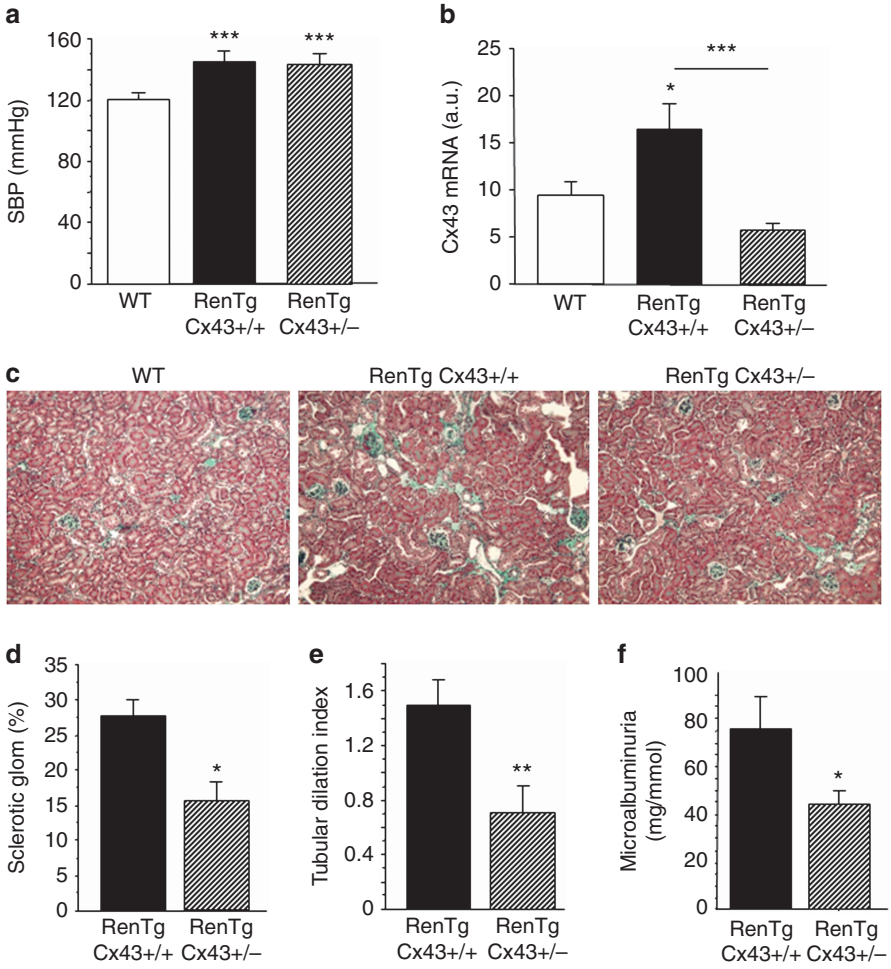

Figure 1 | Decreased expression of connexin 43 (Cx43) improves renal structure and function during the progression of hypertension-induced chronic kidney disease (CKD). Measurements of systolic blood pressure (SBP) in RenTgCx43+/+ and RenTgCx43+/− mice indicate that decreased Cx43 expression did not modulate blood pressure values (**a**). Quantitative PCR (qPCR) analysis in the renal cortex showed a marked increase in Cx43 messenger RNA (mRNA) expression in RenTg mice aged 5 months (mo) compared with normotensive wild-type (WT) mice. Cx43 upregulation was blunted in RenTgCx43+/− mice (**b**). Representative examples of renal cortical histology revealed by Masson's trichrome in 5-mo-old WT, RenTgCx43+/+, and RenTgCx43+/− mice. Note the substantial improvement in the renal histology in RenTgCx43+/− mice (**c**). Renal parameters such as glomerulosclerosis (**d**), tubular dilation (**e**), and proteinuria (**f**) were significantly ameliorated in these mice ($n=9$ mice from each group; *$P<0.05$, **$P<0.01$, ***$P<0.001$; original magnification of microphotographs ×200).

following AS infusion (Figure 3h and i, respectively) and consequently interstitial fibrosis was also reduced (Figure 3j). These data suggest that Cx43 may be used as a therapeutic target against the progression of hypertension-induced CKD.

Decreased Cx43 expression reduces monocyte infiltration and interstitial fibrosis following obstructive nephropathy

Increased expression of Cx43 was also observed during the progression of obstructive nephropathy within injured kidneys in WT mice (Supplementary Figure S3 online). To investigate whether the potential protective effect of Cx43 blockade is not model dependent, Cx43+/− and their littermates underwent UUO and were euthanized after 7 and 15 days (d). Cx43 mRNA upregulation was confirmed 7d after UUO (Figure 4a), and as expected an increased expression of the mRNA of VCAM-1 (b), P-selectin (c), and chemokine receptor type 2 (d) was also observed. In contrast, at the same time point this upregulation was significantly reduced in Cx43+/− mice. As monocytes have a major role in the inflammatory process in this model of renal nephropathy,[21] we next performed F4-80 immunostaining. An important macrophage infiltration was detected in the interstitium of the obstructed kidneys 7d after UUO, whereas in Cx43+/− mice the infiltrate was significantly reduced (Figure 4e). Next, we studied the impact of the Cx43 deletion on the progression of interstitial renal fibrosis 15d after UUO. We observed that Col1 and Col3 mRNA levels increased significantly after UUO compared with the levels in the

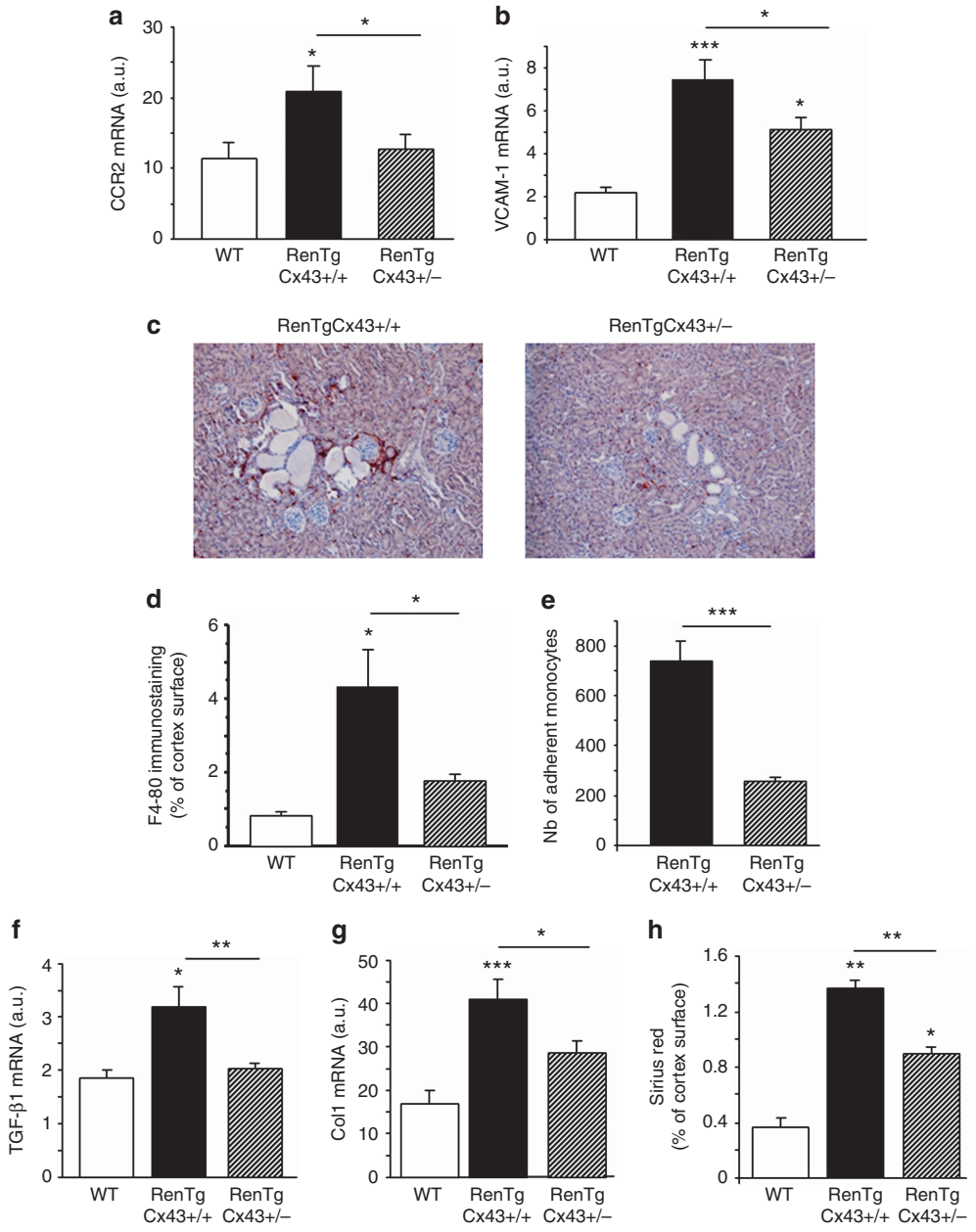

Figure 2 | Reduced connexin 43 (Cx43) expression limits inflammation and interstitial renal fibrosis during the progression of hypertension-induced chronic kidney disease (CKD). Quantitative PCR (qPCR) in the renal cortex showed that in 5-month (mo)-old RenTgCx43 +/− mice messenger RNA (mRNA) expression of chemokine receptor type 2 (CCR-2) (**a**) and vascular adhesion molecule-1 (VCAM-1) (**b**) was significantly inhibited, compared with RenTgCx43 +/+ littermates. F4-80 immunostaining showed that macrophage infiltration was considerably restricted in RenTgCx43 +/− mice (**c**). Histogram shows quantification of the F4-80 macrophage-specific signal (**d**). Adhesion of RenTgCx43 +/− monocytes to an activated endothelial layer was highly decreased compared with RenTgCx43 +/+ control ones (**e**). Upregulation of transforming growth factor-β1 (TGF-β1) (**f**) and col1 (**g**) mRNA expressions was decreased in RenTgCx43 +/− mice. In accordance, Sirius red coloration showed restricted renal interstitial fibrosis in RenTgCx43 +/− mice (**h**) ($n = 9$ mice from each group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; original magnification of microphotographs ×200).

contralateral nonobstructive kidneys (Figure 4g and h, respectively). However, upregulation of mRNA expression of both collagens was blunted in Cx43 +/− and the accumulation of interstitial collagen formation was also reduced (Figure 4i). Furthermore, immunostaining for fibroblast-specific protein-1, a well-established fibrotic marker, showed limited fibroblast-specific protein-1-positive cells after UUO in Cx43 +/− mice (Supplementary Figure S4 online). In conclusion, similar to hypertension-induced nephropathy, decreased expression of Cx43 limited the inflammatory reaction and decreased excessive extracellular matrix accumulation, leading to improved renal structure.

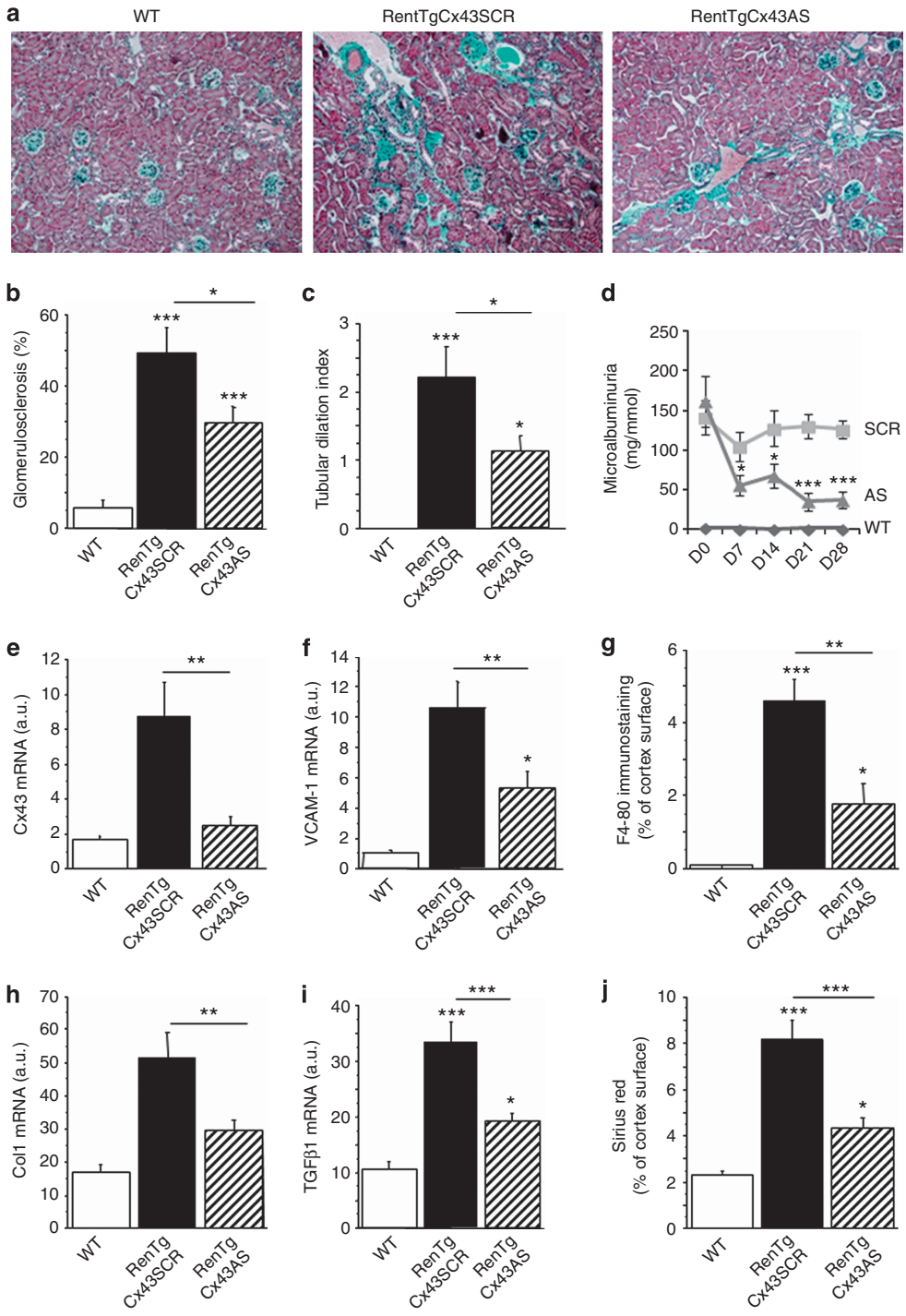

Figure 3 | Targeting connexin 43 (Cx43) upregulation with Cx43-ODN antisense (AS) improves renal structure and function during the progression of hypertension-induced chronic kidney disease (CKD). Representative examples of renal cortical histology revealed by Masson's trichrome in 1-year-old wild-type (WT), RenTg scrambled (SCR), and RenTgCx43AS mice (**a**). Note the substantial improvement in renal histology in RenTg mice that received Cx43AS for 1 month (mo) via minipump infusion. Glomerulosclerosis (**b**), tubular dilation (**c**), and proteinuria (**d**) were also significantly decreased in hypertensive mice that received Cx43-ODN AS (10 μmol/l). Quantitative PCR (qPCR) analysis in the renal cortex showed that upregulation of Cx43 (**e**) and vascular adhesion molecule-1 (VCAM-1) (**f**) messenger RNAs (mRNAs) was blunted in RenTg mice after AS administration. Quantification of the F4-80 macrophage–specific signal (**g**). In addition, mRNA expression of col1 (**h**) and transforming growth factor-β1 (TGF-β1) (**i**) as well as interstitial renal fibrosis (**j**) was also significantly decreased in mice after Cx43-ODN AS infusion ($n = 7$ mice per group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; original magnification of microphotographs ×200).

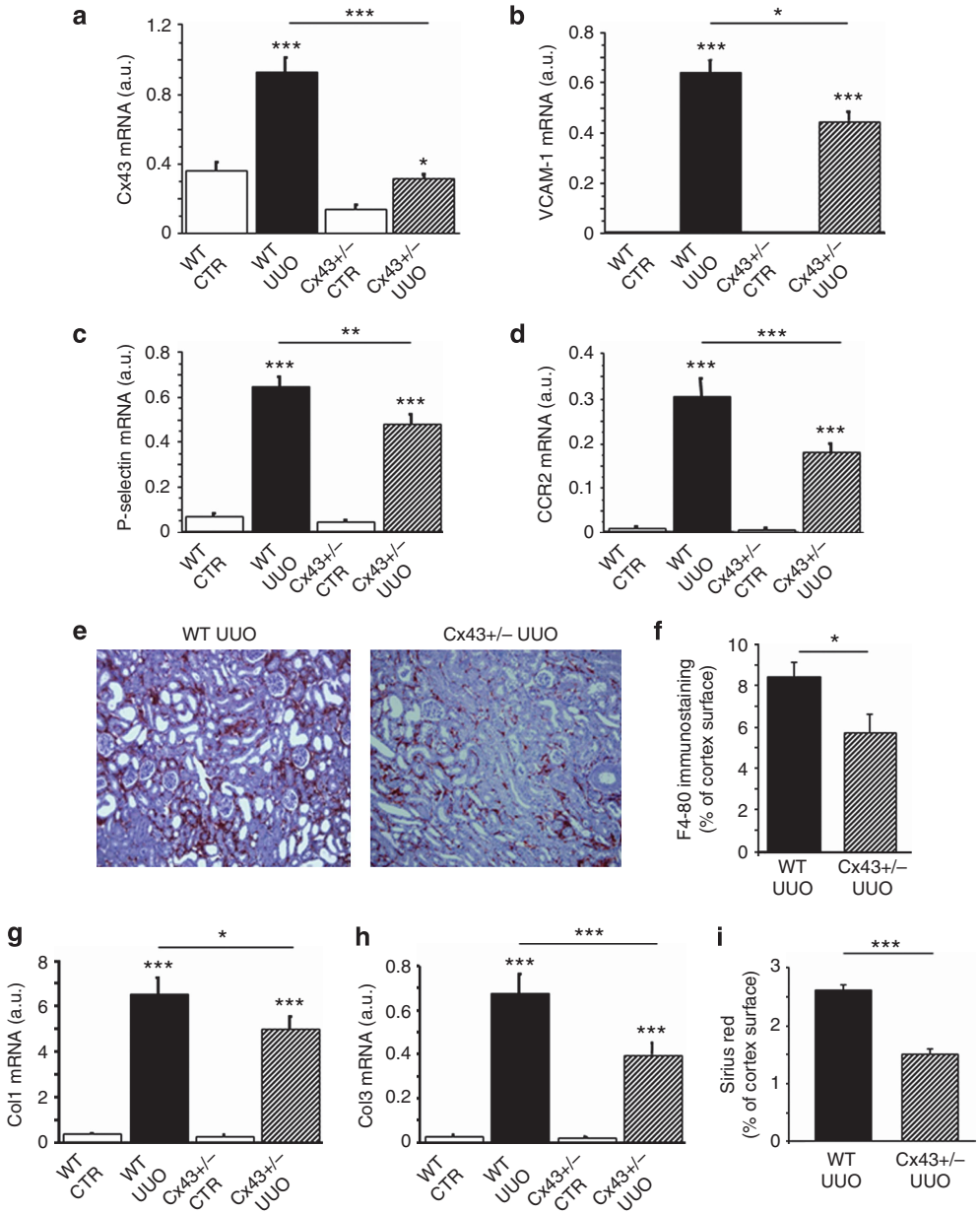

Figure 4 | Connexin 43 (Cx43) heterozygous mice show limited inflammation and fibrosis following obstructive nephropathy. Quantitative PCR (qPCR) analysis in the renal cortex showed that Cx43 messenger RNA (mRNA) upregulation was blunted in Cx43 +/− mice 7 days (d) after unilateral ureteral obstruction (UUO; **a**). Decreased expression of Cx43 significantly inhibited mRNA expression of vascular adhesion molecule-1 (VCAM-1) (**b**), P-selectin (**c**), and chemokine receptor type 2 (CCR-2) (**d**). Macrophage infiltration was considerably restricted in Cx43 +/− mice (**e**). Histogram shows quantification of the F4-80 macrophage staining 7d after UUO (**f**). Fifteen days after UUO, upregulation of col1 (**g**) and col3 (**h**) mRNA expressions was blunted in Cx43 +/− mice. Quantification of Sirius red coloration by morphometric analysis showed restricted renal interstitial fibrosis in Cx43 +/− mice (**i**) ($n = 10$ mice per group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; original magnification of microphotographs ×200).

Targeting Cx43 improves renal structure after UUO

To confirm the protective effect of the Cx43-decreased expression after UUO, we injected a Cx43-ODN AS or a SCR sequence locally in the dilated ureter 24 h after obstruction. Mice were killed 7d after UUO. To quantify the extent of Cx43 knockdown after the AS administration, we assessed Cx43 expression by quantitative PCR. As shown in Figure 5a, the upregulation of the Cx43 mRNA expression was attenuated after the AS injection. In addition, we observed a marked decrease in the VCAM-1 mRNA and protein expressions in the obstructed kidneys that received Cx43AS compared with those that received SCR after UUO (Figure 5b and c, respectively). Interestingly, western blotting for E-cadherin, a marker of renal tubular epithelial phenotype,

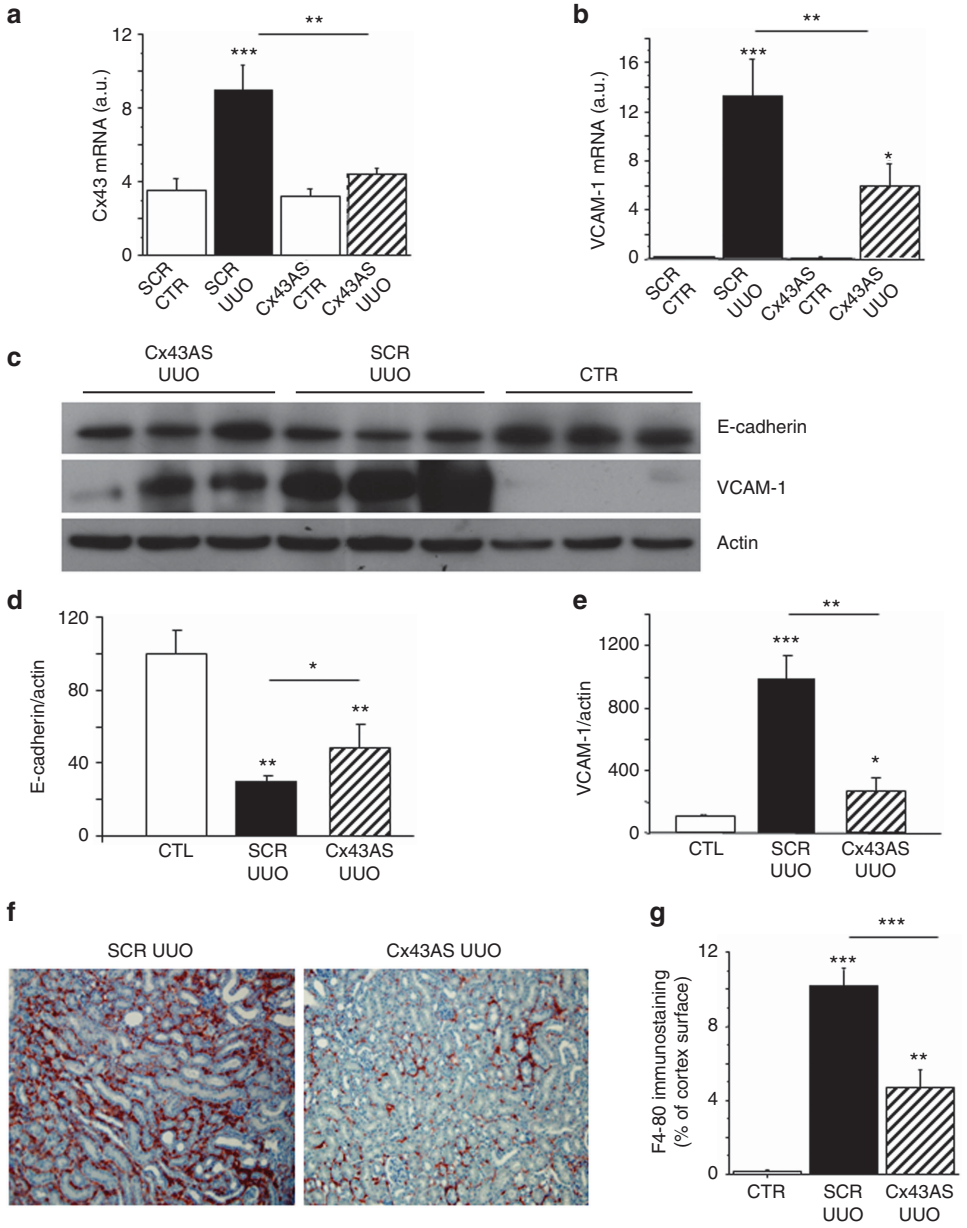

Figure 5 | Local delivery of connexin 43 (Cx43)-ODN antisense (AS) in the dilated ureter decreased monocyte infiltration in obstructed kidneys of wild-type (WT) mice. Quantitative PCR (qPCR) analysis in the renal cortex showed a marked increase in Cx43 messenger RNA (mRNA) expression 7 days (d) after unilateral ureteral obstruction (UUO) compared with nonobstructed contralateral ones. Delivery of Cx43 AS in the dilated ureter blunted Cx43 (**a**) and vascular adhesion molecule-1 (VCAM-1) (**b**) upregulation. Representative western blotting experiments for E-cadherin, VCAM-1, and β-actin were performed by using the renal cortex of contralateral controls and obstructed kidneys treated with scrambled (SCR) or Cx43-ODN AS (**c**). Graphs show quantification of western blots expressed as the ratio of E-cadherin (**d**) and VCAM-1 (**e**) vs. β-actin signal for each sample. F4-80 immunostaining also showed restricted macrophage infiltration after Cx43AS administration (**f**). Quantification of the F4-80 macrophage–specific signal (**g**) ($n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; original magnification of microphotographs ×200).

showed that its expression was partially restored after AS treatment, indicating an improvement in the renal structure. In accordance, macrophage infiltration was clearly decreased in Cx43AS-treated kidneys (Figure 5f and g). As previously observed, limitation of cell infiltration at the injured site resulted in restricted interstitial fibrosis. Given that GJs are implicated in pathways regulating the transcription of the target genes, we speculated that signaling passing through the Cx43 channels could affect the expression of the *col1* gene after UUO. We focused directly on the extracellular signal-regulated kinase (ERK) signaling pathway that has been shown to regulate the phosphorylation and transcriptional

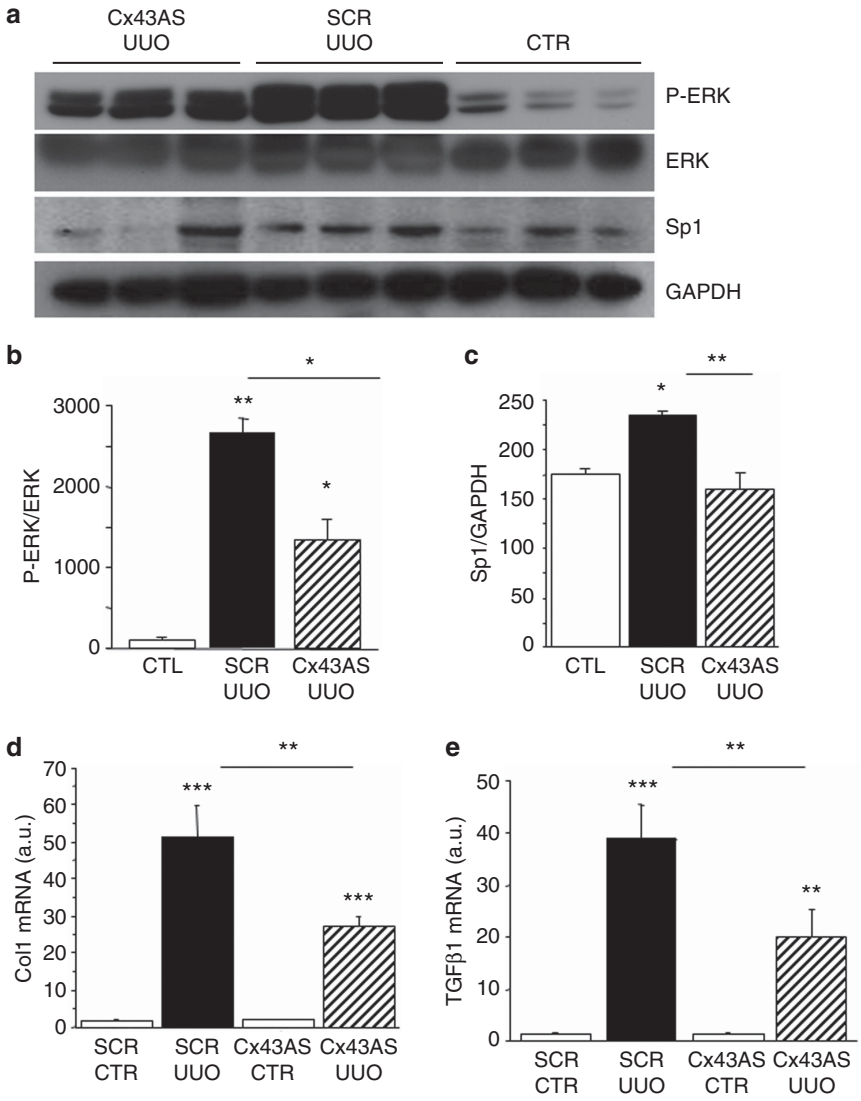

Figure 6 | Connexin 43 (Cx43)-ODN antisense (AS) decreased fibrotic pathways in obstructed kidneys of wild-type (WT) mice. Representative western blotting experiments for P-extracellular signal-regulated kinase (ERK), ERK, Sp1, and GAPDH were performed by using the renal cortex of nonobstructed contralateral controls and obstructed kidneys after administration of Cx43AS or scrambled (SCR) sequence (**a**). Graphs show quantification of western blots expressed as the ratio of P-ERK/ERK (**b**) and Sp1 (**c**) vs. GAPDH for each sample. Quantitative PCR (qPCR) in the renal cortex showed that delivery of Cx43AS in the dilated ureter decreased the Col1 (**d**) and transforming growth factor-β1 (TGF-β1) (**e**) upregulation 7d after unilateral ureteral obstruction (UUO; $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

activity of Sp1, known to be a powerful activator of the transcription of the *col1* gene.[22,23] Mice that received SCR sequence after UUO had a pronounced activation of the ERK activity as the phosphorylation of ERK was highly increased (Figure 6a and b). The phosphorylated form of the Sp1 transcription factor was also markedly increased (Figure 6a and c). At the same time point, the expression of col1 mRNA was increased by almost 50-fold. In contrast, local treatment with Cx43AS reduced the phosphorylation of ERK by half and abolished the phosphorylation of Sp1, leading to a significant reduction in col1 mRNA expression (Figure 6d). Furthermore, Cx43AS significantly reduced mRNA expression of TGF-β1, one of the most important profibrotic cytokines in renal damage (Figure 6e). These data demonstrate that decreasing the Cx43 expression limited the inflammatory process and restricted interstitial renal fibrosis and thus resulted in renal structure preservation.

Cx43-specific blocking peptide inhibits monocyte adhesion as well as profibrotic pathways in renal tubular cells

In the above-mentioned models of CKD, Cx43 seemed to participate in two essential processes during the progression of the disease: monocyte recruitment and tissue fibrosis. To assess the functional importance of Cx43, we used Gap26 peptide, a Cx43-specific GJ blocker that inhibits the docking of the Cx43 hemichannels and consequently the Cx43-mediated GJ intercellular communication (GJIC).[17] As shown in Supplementary Figure S5A online, the number of

monocytes that adhere to an activated mouse endothelial cell monolayer previously incubated with Gap26 was significantly reduced. In addition, freshly extracted monocytes incubated with Gap26 showed considerably less adhesion to the activated bEnd3 monolayer. These data showed that Cx43 expression may have a proadhesive role mainly in monocytes and to a lesser extent in activated endothelial cells.

To study the functional importance of Cx43 in profibrotic pathways we first incubated mouse tubular cells (mCCDs) with TGF-β1. This stimulation led to *de novo* expression of Cx43 mainly between the neighboring cells (Supplementary Figure S5B online). Furthermore, incubation of tubular cells with Gap26 inhibited the TGF-β1-induced ERK pathway (Supplementary Figure S5C and D online), the phosphorylation of the transcription factor Sp1, and the upregulation of col1 (Supplementary Figure S5E and F online, respectively). Thus, Cx43 may participate in the profibrotic effects of TGF-β1 through the exchange of second messengers passing through the Cx43 channels.

DISCUSSION

Impairment of GJIC has been reported to have a large impact on various diseases.[4] In this study we demonstrated for the first time that Cx43 upregulation contributes to structural damages within the renal cortex during the progression of renal disease, and inhibiting its expression improved tissue structure and function.

To investigate whether Cx43 could be a key mediator of renal disease, we took advantage of two different mouse models of CKD. The first, the RenTg model, has been recently described as a powerful tool for studying CKD as it presents all physiopathological characteristics of a slowly progressive hypertension-induced renal disease.[24] The second, the UUO, is a well-established model of tubulointerstitial renal disease leading to renal fibrosis.[25] Although these two experimental models target distinct renal compartments, they are linked by their ability to promote development of chronic inflammation and fibrosis leading to severe damages to the renal structure.

In both models we showed that Cx43 expression was increased during the progression of renal disease (Supplementary Figure S1 and S3 online) and that decreased Cx43 expression was beneficial in chronic inflammation. This process requires cross-talks between leukocytes and the injured tissue and a high level of coordination in which GJs are involved. It has been reported that in various stages of inflammatory diseases Cx43 expression and distribution can markedly change, thus modulating the progression of the injury.[3,26,27] Indeed, in chronic models of inflammation in mice, such as atherosclerosis, an upregulation of Cx43 by the dysfunctional vascular endothelium was detected in the shoulder of the lesions, as well as in intimal smooth muscle cells at the early stages of the disease.[28] Similar temporal expression patterns of Cx43 were also observed in rodents, in the muscular microcirculation following inflammatory stimulation, in obstructed kidneys, and in myocardial endothelial–mesenchymal transition.[29–31] In addition, it has been reported that Cx43 expression was enhanced in damaged tubules and interstitial cells in human kidneys and in rat podocytes in puromycin aminonucleoside nephrosis.[32,33] The Cx43+/− mouse has been used in several diseases to investigate the link between Cx43 and inflammation. Thus, in accordance with our study, in LDLR (low-density lipoprotein receptor)–deficient mice with reduced Cx43 levels, atherogenesis was markedly inhibited and the composition of the atherosclerotic plaques exhibited reduced amount of leukocytes.[34] Additional studies in the same model showed limited local monocyte infiltration leading to faster repair and re-endotheliazation following acute vessel injury.[11] Cx43+/− mice also displayed blunted neutrophil recruitment *in vivo* in an acute lung injury model after instillation of lipopolysaccharide.[26] Interestingly, the authors of this study used mice harboring heterozygous expression of a C-terminal truncation of Cx43, thus creating GJ channels unable to response to chemical stimuli.[35,36] These mice showed enhanced recruitment of neutrophils to the airways and mortality in response to lipopolysaccharide.[26] The role of endothelial Cx43 has also been studied using endothelial-specific Cx43-null mice (Cx43e−/−). Thus, Cx43e−/− mice and blockade of GJIC by chemical inhibitors showed reduced leukocyte adhesion and transmigration upon tumor necrosis factor-α stimulation in the hamster cheek pouch.[29] All these studies point out the crucial role of Cx43 in the inflammatory response in various tissues. The exact molecular mechanisms involving this Cx in the recruitment of inflammatory cells at the site of injury are poorly understood. Recent studies suggested that Cx43 channels may serve as conduits for spreading proinflammatory signals within the endothelial network. Indeed, propagation of $Ca2+$ waves between endothelial lung capillaries through Cx43 channels activated the expression of the leukocyte adherence receptor P-selectin, after intratracheal administration of lipopolysaccharide. Furthermore, peptide inhibitors for Cx43 completely blocked the thrombin-induced microvascular permeability.[37] Accordingly, decreased expression of Cx43 in our experimental models blunted the upregulation of cell adhesion molecules and consequently monocyte adhesion within the injured kidneys. In addition, functional assays using a Cx43-blocking peptide in activated endothelial cells and monocytes showed that Cx43 has a major role in both monocyte adhesion and endothelial cell activation. Interestingly, it seemed that Cx43 GJIC has a proadhesive role mainly in monocytes and to a lesser extent in the endothelial cell activation at least *in vitro*. However, we cannot underestimate the importance of the renal-activated endothelium during the progression of CKD *in vivo*.

Extracellular matrix remodeling is a dynamic process that occurs in response to inflammation. As we observed that decreased Cx43 limited leukocyte adherent receptors and infiltration, we hypothesized that this protein may modulate renal fibrosis during the progression of CKD. We showed that in Cx43+/− mice interstitial collagen deposition was reduced in comparison with Cx43 WT animals. It has been

reported that Cx43 can regulate profibrotic marker expression such as TGF-β and *vice versa*, as well as collagen deposition.[38–40] However, in contrast to the inflammatory process, the role of Cx43 in tissue sclerosis is controversial. Our results are in accordance with those of Zhang *et al.*,[41] who demonstrated that reduced expression of Cx43 inhibited collagen content, thus attenuating ventricular remodeling after myocardial infarction. Moreover, TGF-β was highly upregulated in myocardial infarction hearts but its phosphorylated mediator, pSmad, was markedly decreased in the nuclei of Cx43 +/− hearts after myocardial infarction in mice. Interestingly, it has been shown that Cx43 served as a positive regulator of the TGF-β/Smad signaling pathway in mouse cardiomyocytes.[42] The ability of GJIC to modulate signaling affecting the transcription of profibrotic genes has been well documented in bone biology. Indeed, Cx43 GJ between osteoblasts permits the intercellular propagation of second messengers that activate the ERK signal cascades. These signals regulated the recruitment of the transactivator Sp1 to a Cx-responsive element in the promoter, leading to a robust transcription of the type I collagen gene. When the GJIC was disrupted, the collagen transcription was decreased because of diminished propagated signals among cells.[40] A similar situation may occur during the progression of renal interstitial fibrosis following obstructive nephropathy. Indeed, the blockade of Cx43 upregulation inhibited the phosphorylation of Sp1 via the ERK pathway, resulting in decreased transcription of the type I collagen gene. Similarly, *in vitro* experiments in renal tubular cells confirmed our hypothesis that Cx43 may participate in the profibrotic effects of TGF-β1 via exchange of second messengers passing through the Cx43 channels. Another argument reinforcing our hypothesis that Cx43 participates in the progression of renal fibrosis in CKD is that Cx43 +/− mice showed a restricted number of fibroblast-specific protein-1-positive cells. In contrast, Cx43 +/− LDLR −/− mice presented more collagen deposits within atherosclerotic plaques.[28] Moreover, knocking down Cx43 in a wound-healing model in the skin resulted in increased mRNA for TGF-β1 and collagen content at the wound site.[43] These differences in terms of collagen deposition may reflect different cellular adaptive processes during matrix remodeling and wound healing.

An additional novel finding of our study is the demonstration that Cx43 is a possible pharmacological target against inflammation and renal fibrosis. The Cx43 blockade considerably improved glomerular injury and renal failure in hypertension-induced CKD and tubular injury after UUO. Moreover, administration of Cx43AS as a therapeutic approach in RenTg mice suffering from an advanced stage of renal disease blunted CKD progression as renal structure and function were close to that of healthy mice. Thus, the protective effects of the Cx43 blockade in different models of experimental nephropathy suggest the involvement of this Cx in common pathophysiological mechanisms associated with the development of renal disease. To our knowledge, curative treatment of mice with AS is the first pharmacological attempt to transiently block Cx43 activity *in vivo* in the context of CKD. Similar to our results, Mori *et al.*[43] showed that recruitment of both neutrophils and macrophages was markedly reduced within Cx43AS-treated wounds. This study suggests that reducing Cx43 expression early in the skin enhanced the wound-healing process because of inflammatory response attenuation. In addition, blocking Cx43 upregulation in different models of spinal cord injury and in corneal scrape injury in rats reduced inflammation and improved functional recovery.[19,20] Finally, in a rat model of collagen-induced arthritis, silencing the expression of Cx43 by short interfering RNA decreased inflammation and joint destruction.[44] All these studies underline the interest for targeting specifically Cx43 to protect against the progression of inflammatory diseases.

The interest to develop Cx43 pharmacological inhibitors in CKD is reinforced by the exaggerated expression of this protein in patients with nephroangiosclerosis or obstructive nephritis in comparison with healthy controls (Figure 7). These results are in accordance with the study by Hillis *et al.*[32] who reported an upregulation and colocalization of Cx43 and

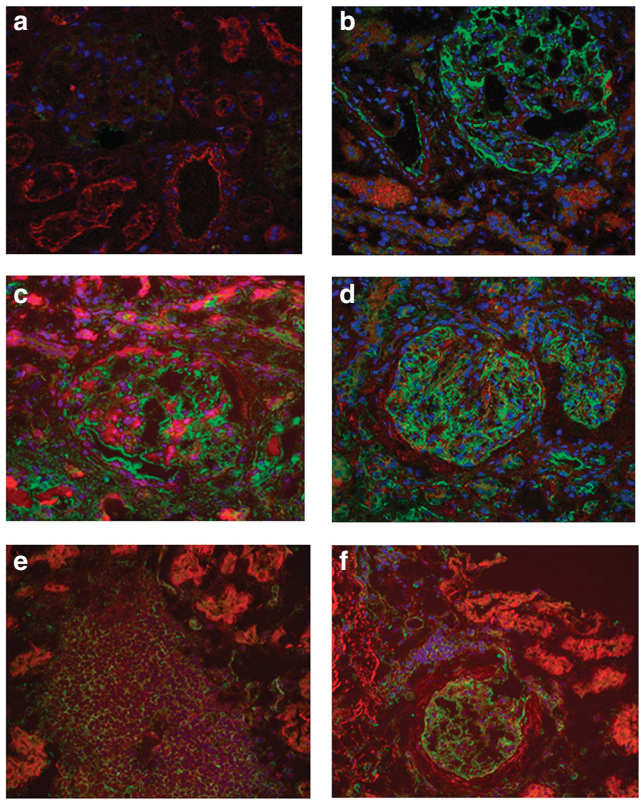

Figure 7 | Connexin 43 (Cx43) immunostaining in human renal biopsies. Cx43 expression is weak in the renal cortex of control kidneys (**a**). Cx43 immunofluorescence (in green) is markedly increased in patients suffering from nephroangiosclerosis (**b**) and obstructive nephritis (**c**). Cx43 expression highly increased in severe nephroangiosclerosis (**d**–**f**). Cryosections were counterstained with Evans blue (red) and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Original magnification of microphotographs ×400.

cell adhesion molecules in human renal biopsies of patients suffering from inflammatory renal diseases. In addition, the expression pattern of Cx43 in humans and in mice suffering from CKD looks rather similar (Supplementary Figure S1 and S3 online). Our data suggest that Cx43 overexpression in the damaged compartment leads to propagated proinflammatory and profibrotic signals within the whole renal tissue. Thus, as the disease progresses throughout the neighboring renal compartments, Cx43 may be expressed by all cell types affected by the disease. In agreement with this assumption, we have observed that in biopsies from renal pathologies, as well as in injured renal tissues in mice, Cx43 is always expressed in proximity to damaged areas.

In summary, we demonstrated that Cx43 has a key role in CKD. Its overexpression in the damaged compartments in response to the injury participates in the progression of renal disease. Thus, Cxs may represent a pertinent therapeutic target and knocking down Cx43 could have widespread applications in renal diseases in improving the structure and function of the injured kidneys.

MATERIALS AND METHODS

Animals

All mice were kept in well-controlled animal housing facilities and had free access to tap water and pellet food. All protocols have been approved by the French Institutional Committee (INSERM and the University 'Paris VI'). Detailed methods of all the *in vivo* experimental procedures can be found in Supplementary Material online.

Renal morphology and immunohistochemistry

Kidneys were fixed in formalin solution (4%) and embedded in paraffin after conventional processing. Sections (3 mm thick) were stained with Masson's trichrome for evaluation of renal damage. Interstitial fibrosis was assessed on Sirius red–stained paraffin sections. All measurements were taken in a blinded manner on coded slides. Measurements of proteinuria were taken as previously described.[24] Immunostaining was performed on paraffin-embedded sections and on cryosections of renal biopsies from patients suffering from renal diseases. Negative controls included omission of first antibodies or preincubation of first antibodies with immunogenic peptides. Detailed methods of all the procedures can be found in Supplementary Material online.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. List of the primers of different genes used for real-time-PCR.
Table S2. Cell numeration in spleens and peripheral blood from Cx43 +/− and wild type mice after 15 days of obstructive nephropathy.
Figure S1. Progressive increase in Cx43 expression in RenTg mice.
Figure S2. VCAM-1 upregulation was blunted in one year old RenTg mice after Cx43AS treatment.
Figure S3. Progressive increase in Cx43 expression in WT mice after obstructive nephropathy.
Figure S4. Cx43 heterozygous mice showed limited FSP-1-positive cells after obstructive nephropathy.
Figure S5. Cx43-specific blocking peptide inhibited monocyte adhesion and profibrotic pathways in tubular cells.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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