Activation of platelet-activating factor receptor exacerbates renal inflammation and promotes fibrosis

Matheus Correa-Costa[1], Vinicius Andrade-Oliveira[1], Tarcio T Braga[1], Angela Castoldi[1], Cristhiane F Aguiar[1], Clarice ST Origassa[2], Andrea CD Rodas[1], Meire I Hiyane[1], Denise MAC Malheiros[3], Francisco JO Rios[1,4], Sonia Jancar[1] and Niels OS Câmara[1,2]

Platelet-activating factor (PAF) is a lipid mediator with important pro-inflammatory effects, being synthesized by several cell types including kidney cells. Although there is evidence of its involvement in acute renal dysfunction, its role in progressive kidney injury is not completely known. In the present study, we investigated the role of PAF receptor (PAFR) in an experimental model of chronic renal disease. Wild-type (WT) and PAFR knockout (KO) mice underwent unilateral ureter obstruction (UUO), and at kill time, urine and kidney tissue was collected. PAFR KO animals compared with WT mice present: (a) less renal dysfunction, evaluated by urine protein/creatinine ratio; (b) less fibrosis evaluated by collagen deposition, type I collagen, Lysyl Oxidase-1 (LOX-1) and transforming growth factor β (TGF-β) gene expression, and higher expression of bone morphogenetic protein 7 (BMP-7) (3.3-fold lower TGF-β/BMP-7 ratio); (c) downregulation of extracellular matrix (ECM) and adhesion molecule-related machinery genes; and (d) lower levels of pro-inflammatory cytokines. These indicate that PAFR engagement by PAF or PAF-like molecules generated during UUO potentiates renal dysfunction and fibrosis and might promote epithelial-to-mesenchymal transition (EMT). Also, early blockade of PAFR after UUO leads to a protective effect, with less fibrosis deposition. In conclusion, PAFR signaling contributes to a pro-inflammatory environment in the model of obstructive nephropathy, favoring the fibrotic process, which lately will generate renal dysfunction and progressive organ failure.

Laboratory Investigation (2014) **94,** 455–466; doi:10.1038/labinvest.2013.155; published online 3 February 2014

KEYWORDS: chronic kidney disease; platelet activating factor receptor; renal fibrosis; renal inflammation

Renal fibrosis is considered as the hallmark of chronic kidney disease (CKD), and epidemiological studies have shown that the prevalence of patients with progressive renal failure is increasing worldwide. As a consequence, CKD has become not only a global health issue, but also an economic problem, affecting individuals, families and societies.[1–5] Under normal conditions, the renal interstitial space is formed of few cells (fibroblasts and resident immune cells) and peritubular capillaries. These structures are incorporated in the extracellular matrix (ECM), and the production of its components is well controlled to keep tissue homeostasis. In a fibrotic situation, the interstitial space is filled with numerous cells (fibroblasts, myofibroblasts, infiltrating immune cells and atrophic tubular cells), there is rarefaction of peritubular capillaries and concomitant excessive production of ECM proteins.[6]

A suggested mechanism that contributes to the fibrotic process is the epithelial-to-mesenchymal transition (EMT), in which epithelial cells undergo differentiation and start to express mesenchymal markers, changing their phenotype and producing ECM compounds.[7] In this sense, fibroblast-specific protein 1 (FSP-1), a filament-associated molecule, present in fibroblast, but not in epithelium, mesangial cells or embryonic endoderm, can be used as a marker of EMT.[8] Such process has been described in a variety of different tissues, such as kidney, liver, lung, colon and heart, although the molecular mechanisms involved are not yet completely understood.[3,9–13]

[1]Department of Immunology, Institute of Biomedical Sciences IV, University of São Paulo, São Paulo, Brazil; [2]Nephrology Division, Federal University of São Paulo, São Paulo, Brazil; [3]Pathology Department, Faculty of Medicine, University of São Paulo, São Paulo, Brazil and [4]British Heart Foundation, Glasgow Cardiovascular Research Centre, Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow, UK
Correspondence: Dr M Correa-Costa, PhD, Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, Cidade Universitária, Av Professor Lineu Prestes, 173, São Paulo 05508-900, Brazil.
E-mail: matheusccosta@usp.br

Received 2 June 2013; revised 20 November 2013; accepted 19 December 2013

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*glycero-3-phosphocholine) is a lipid mediator of inflammation. During kidney inflammation, PAF is synthesized by inflammatory cells that infiltrate the tissue and/or by mesangial cells.[14] PAF acts on a G-protein coupled receptor (PAFR) expressed in monocytes/macrophages, polymorphonuclear leukocytes, platelets, endothelial cells and many other cell types.[15] PAF or oxidized molecules with PAF-like activity generated during stress situations such as hypoxia can engage the PAFR, which activates intracellular signaling pathways that lead to transcription of pro-inflammatory genes.[16]

Although PAF is involved in acute renal dysfunction[17] its role in renal fibrosis and in EMT is not completely known. One of the most used experimental model of CKD is the unilateral ureter obstruction (UUO) model. Such procedure leads to cellular infiltration (mainly macrophages), disequilibrium between apoptosis and proliferation of tubular cells, as well as fibroblast activation and proliferation, with consequent ECM production and fibrosis deposition. On the basis of this, the aim of this study was to investigate the involvement of PAFR in CKD. To this purpose, we employed the UUO model and compared mice lacking PAF receptor (PAFR knockout (KO)) with wild-type (WT) animals.

MATERIALS AND METHODS

Ethics Statement

The animals used in this work were housed in individual standard cages and were kept on a 12-h light/dark cycle in a temperature-controlled room at 21–23 °C, with free access to water and food. All procedures were approved by the internal ethical committee of the University of São Paulo (Documents 118/2009 and 122/2012).

Animals and Treatment

Balb/c PAFR KO mice were kindly provided by Professor Takao Shimizu (University of Tokyo)[18] and were bred and maintained under SPF conditions at Institute of Biomedical Sciences at the University of São Paulo. Balb/c (WT) mice were obtained from our Isogenic Breeding Unit (Immunology Department, Biomedical Science Institute, University of Sao Paulo). Mice were divided into three groups: control ($n=5$)—mice that did not suffer any surgical procedure; WT ($n=5$)—WT Balb/c mice that underwent UUO surgery and PAFR KO ($n=5$)—PAFR gene genetic engineered mice that underwent UUO surgery. In a set of experiments, the animals were treated with the PAFR antagonist WEB2170 (kindly supplied by Boehringer Ingelheim, Pharma KG, Biberach, Germany), at the dose of 5 mg/kg during the first 3 days of surgery (described below).

Experimental Model of UUO

Mice were anesthetized with Ketamine–Xylazine (Agribrands do Brazil, São Paulo, Brazil) unless otherwise stated. On day 0, UUO was performed by complete ligation of the left ureter with 4–0 silk at two points, and an incision made between the points of ligation. Animals were placed in single cages and warmed by indirect light until completely recovered from anesthesia. After recovery, animals were kept under normal housing condition until killing.

Renal Function Evaluation

Urinary protein/creatinine ratio was measured in samples collected from the obstructed pelvis at 7 days post surgery. All samples were analyzed by colorimetric assays using commercially purchased kits for creatinine and protein measurements (Labtest, Lagoa Santa, Brazil). The ratio was normalized by dividing ratio at day 7 by the ratio before surgery in each group of animals. To estimate the urinary albumin concentration, 10 μl of urine was diluted in sample buffer v/v, run on a 10% SDS-polyacrylamide electrophoresis gel and then stained with Commassie blue, as described previously.[19]

Histomorphometric Analysis

Formaldehyde-fixed paraffin sections of the kidneys were stained with Picrosirius for evaluation of fibrosis deposition. Renal histomorphometric analyses were made by two 'blinded' renal histologists. Picrosirius-stained sections were analyzed by an Olympus BX50 microscope with an Olympus camera attached (USA). Manual shots were taken of the cortex, magnified $\times 40$, and observed under polarized light. Photos of at least five different fields in each slide were taken, and structures such as glomeruli, subcapsular cortex, large vessels and medulla were excluded. The pictures were digitalized (HP Scanjet 2400) and then the interstitial volume of collagen in the cortex compared with the overall cortex area was quantified by morphometry. For the morphometric analysis, the Image Processing and Analysis in Java, Image J software was used. The result of the analysis is represented by the percentage, and refers to the proportion of the interstitial volume of collagen in the cortex to the total cortical interstitial volume, and then the arithmetic mean of the analyzed fields was calculated for each slide.

Immunohistochemistry

Localization of FSP-1 (diluted 1:500; DAKO, Ely, UK) and PAFR (diluted 1:200; Cayman Chemical, Denver, USA) was assessed in paraffin-embedded tissue sections. Human slides were used for PAFR expression. The samples were obtained from the healthy portion of the kidney of a nephrectomized patient with renal tumor, and from a biopsy of a patient's kidney with diabetic nephropathy. The slides were deparaffinized, rehydrated and subjected to a Tris-EDTA (pH 9) antigen retrieval solution at 95 °C. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and sections were additionally blocked with Protein Block Solution (DAKO). Slides were incubated with a primary antibody or isotype non-specific IgG as a negative control reagent, followed by incubation with the labeled polymer Envision (DAKO), using two sequential 30-min incubations at room temperature. Staining was completed by a 1- to 3-min

incubation with 3,39-diaminobenzidine (DAB) + substrate chromogen, which stains the specific antigen brown. Hematoxylin counterstaining was also done.

Gene Profiles

Kidney samples were snap-frozen in liquid nitrogen. Total RNA was isolated from kidney tissue using the TRIzol Reagent (Invitrogen, Carlsbad, USA) and protocol according to Invitrogen. RNA concentrations were determined by spectrophotometer readings at absorbance 260 nm. First-strand cDNAs were synthesized using the MML-V reverse transcriptase (Promega, Madison, USA). RT-PCR was performed using the Taqmanor Syber Green real-time PCR assay (Applied Biosystem, USA) for the following molecules: hypoxanthine-guanine phosphoribosyl transferase (HPRT) (Mm00446968_m1), type I collagen (Mm00801666_g1), Lysyl Oxidase-1 (LOX-1) (Mm01145738_m1), TGF-β (Mm01178820_m1), bone morphogenetic protein 7 (BMP-7) (Mm00432102_m1), PAFR (Mm00731507_s1), PAF-acetylhydrolase (PAF-AH) (F-GTCTCTGCTTCAGAGGATGC and R-ACATTGTGATCGTGACCGTG) and Lysophosphatidylcholine acyltransferase (PAF-AT) (F-CCAGGTGGCATTTAAGCTCT and R-TCTTGGCATATTCTGGGTGC). Cycling conditions were as follows: 10 min at 95 °C followed by 45 cycles at 20 s each at 95 °C, 20 s at 58 °C and 20 s at 72 °C. Analysis used Sequence Detection Software 1.9 (SDS). mRNA expression was normalized to HPRT expression. Also, Mouse Extracellular Matrix and Adhesion Molecules PCR array was performed (PAMM013—from Qiagen, The Netherlands), comparing control, WT and PAFR KO groups.

ELISA for TGF-β

Total TGF-β1 protein was measured by ELISA (TGF-β1 Emax®, Promega). Kidney cells were lysed in RIPA buffer and protein levels quantified by DC Protein Assay (Bio-Rad, Hercules, USA). After overnight coating of a 96-well plate with a primary antibody, TGF-β1 was detected in cell lysates using a secondary antibody. The system uses horseradish peroxidase-conjugated secondary antibody and a single-component TMB substrate for the final chromogenic detection of bound TGF-β1. Using this assay, biologically active TGF-β1 can be detected in the range of 15.6–1000 pg/ml. Results are expressed as ng/mg of TGF-β protein.

Bioplex

Kidney cells were lysed in RIPA buffer with protease inhibitor. A Bio-Plex mouse Plex cytokine assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to test samples for the presence of several cytokines. The assay was read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager software version 4.0. Standard curves ranged from 32 000 to 1.95 pg/ml.

Assessment of Apoptosis

To detect apoptotic cells, the In situ Cell Death Detection Kit TMR red (Roche Diagnostics GmbH, Mannheim, Germany) was used (TUNEL Technology) in paraffin-fixed tissues.

Statistics

The data were described as mean ± s.e.m. Differences among groups were compared using ANOVA (with Tukey post-test) and Student's *t*-test. Significant differences were regarded as $P < 0.05$. All statistical analyses were performed with the aid of GraphPad PRISM®.

RESULTS

PAFR Is Expressed in Renal Tissue of Mice and Humans with CKD

First, we evaluated the time course of PAFR expression after induction of the UUO surgery. We observed that there is an early upregulation of this receptor within 1 day, and that its expression keeps elevated until later time points—with a peak on day 5 post surgery (Figure 1a). Also, to verify what cell type would be responsible for such expression, we performed IHC for PAFR in a time-course experiment. As observed in Figure 1b, at the early moments after UUO (1 and 3 days) there is a stronger staining for PAFR in the infiltrating cells. But, at later points (5 and 7 days) the renal cells also express higher levels of PAFR. As a control, we also performed the same staining in PAFR KO mice, and we did not see any PAFR expression in these groups.

To reinforce that PAFR is expressed during the development of CKD, we obtained human kidney slides and performed IHC for that receptor. As observed in Figure 1c, the healthy portion of the kidney from a nephrectomized patient with renal tumor shows no staining for PAFR. On the other hand, a biopsy sample of the kidney from an advanced diabetic patient, with diabetic nephropathy (one of the most common cause of CKD), shows a clear and strong staining for PAFR in the cortical tubular area. Together, these data indicate that PAFR is present during progressive renal disease.

UUO-Induced Renal Dysfunction Is Reduced in PAFR-Deficient Mice

Once PAFR seems to be involved in the obstructive nephropathy, we decided to evaluate whether the absence of PAFR (PAFR KO mice) would attenuate renal dysfunction following progressive kidney disease. For that, we submitted PAFR KO mice to UUO surgery, killing them after 7 days. As PAF results from the balance of its production and degradation, we decided to check the gene expression of two PAF-related key enzymes: the PAF-AT, responsible for its production, and PAF-AH, a mediator of its degradation. As the balance of these two molecules can indirectly presume PAF levels, we made a ratio regarding production/degradation. As observed in Figure 2a, WT mice submitted to UUO show higher levels of the PAF-AT/PAF-HA ratio than control

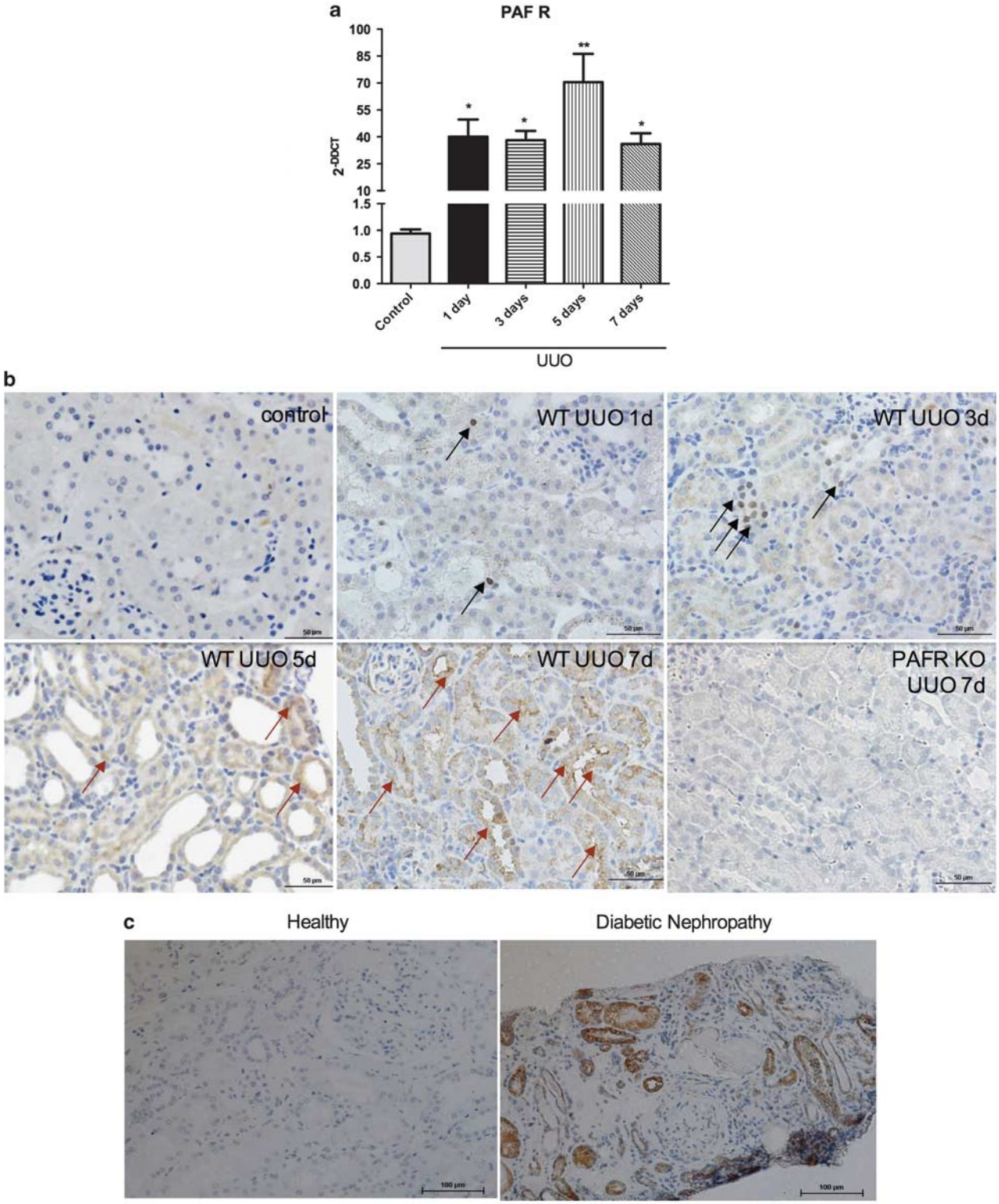

Figure 1 PAFR is present during progressive kidney disease. (**a**) PAFR kinectics after UUO (*$P<0.05$ *vs* Control and **$P<0.01$ *vs* Control). In (**b**), immunohistochemistry for PAFR was assessed in control mice and in WT animals at different time points after UUO. Also, the same staining was performed in PAFR KO animals 7 days after UUO. The balck arrows show PAFR-positive infiltrating cells and red arrows show PAFR-positive staining in renal cells. (**c**) Human slides were used for PAFR expression. The samples were obtained from the healthy portion of the kidney of a nephrectomized patient with renal tumor, and from a biopsy of a patient's kidney with diabetic nephropathy.

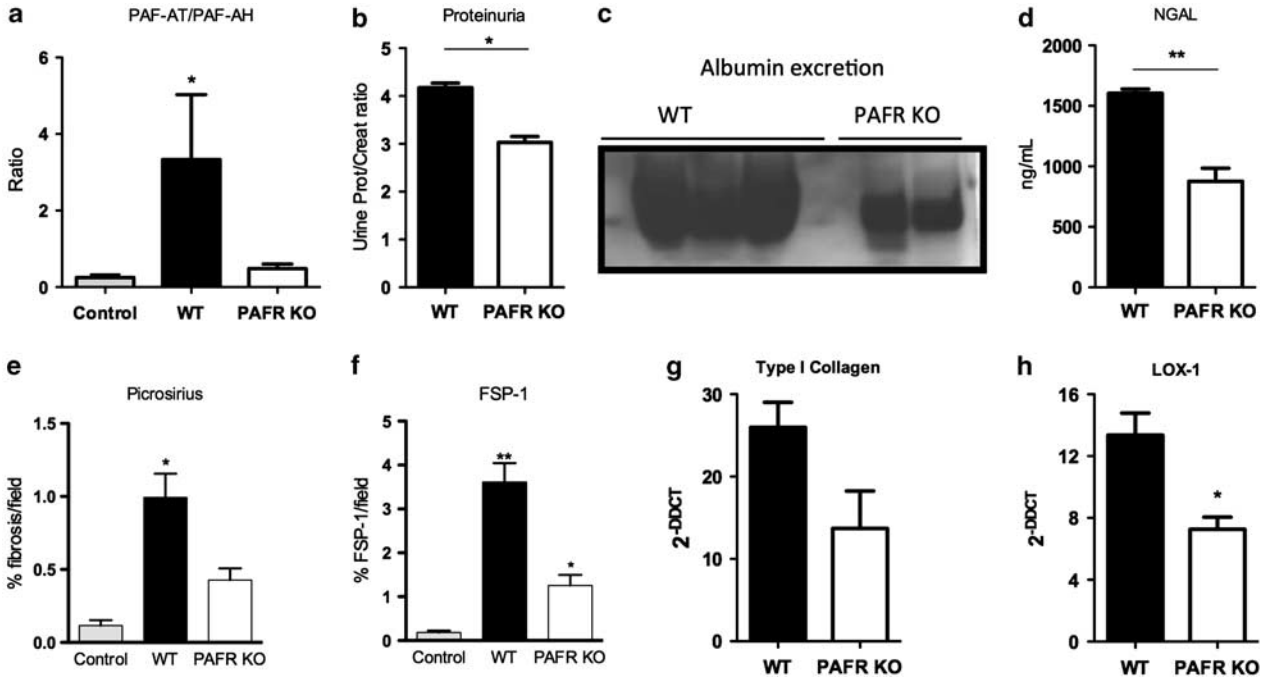

Figure 2 PAFR is important for fibrosis development during obstructive nephropathy. (**a**) Ratio between the mRNA levels of PAF-AT and PAF-AH in control WT and PAFR KO groups. Renal function in WT and PAFR KO animals was assessed for (**b**) proteinuria and (**c**) albumin excretion. (**d**) Levels of urinary NGAL, 7 days after UUO in WT and PAFR KO animals. Quantification of fibrosis deposition by Picrosirius staining is shown in (**e**) (*$P<0.05$ *vs* Control and PAFR KO). In (**f**), quantification of FSP-1 staining (*$P<0.05$ *vs* Control and **$P<0.01$ *vs* Control and PAFR KO). (**g**) Gene expression of type I collagen from WT and PAFR KO mice. (**h**) mRNA transcripts quantification of LOX-1 from the same animals stated before (*$P<0.05$ *vs* WT).

or PAFR KO animals. Also, PAFR KO mice, compared with WT animals, have less urinary protein excretion (Figure 2b), and, moreover, decreased levels of urinary albumin, a marker of kidney impairment (Figure 2c). Also, PAFR KO animals showed decreased levels of urinary NGAL, a more sensitive biomarker of kidney injury (Figure 2d). These data indicate that PAFR, engaged by endogenous PAF or PAF-like molecules produced during UUO, contributes to the development of renal dysfunction.

Lack of PAFR Decreases Collagen Deposition

Next, we aimed to investigate whether the protection observed in the renal function analysis in the PAFR KO mice was a consequence of less collagen deposition in these animals. As observed in Figure 3, WT mice presented increased collagen deposition after ureter obstruction, indicated by Picrosirius staining. On the other hand, PAFR KO mice showed less staining for collagen. We also evaluated the protein expression of FSP-1—a marker of EMT—and once more, in the PAFR KO group there is a marked reduction in this parameter (Figure 3). We were able to quantify both collagen deposition and FSP-1 staining, and we observed that WT animals had significantly higher levels of these fibrotic markers than control and PAFR KO (Figures 2e and f). Moreover, PAFR KO mice presented significantly lower levels of type I collagen mRNA when compared with WT littermates (Figure 2g). In addition, we also observed that gene expression of LOX-1, an important enzyme for collagen production,[20] is significantly reduced in KO animals (Figure 2h). Taken together, these results indicate that the deficiency of PAFR correlates with less fibrosis in kidneys submitted to UUO.

UUO-Induced Pro-Fibrotic Signaling Is Reduced in PAFR-Deficient Mice

The next step was to evaluate the role of PAFR in the molecular mechanisms involved in fibrosis development. For that, we quantified the levels of two known molecules implicated in fibrotic processes, TGF-β and BMP-7.[21] TGF-β was overexpressed in WT mice in both transcriptional and protein levels (active form), whereas in PAFR KO animals such values were similar to control (Figures 4a and d). When we analyzed BMP-7 expression, the opposite effect was observed, with a significant upregulation in PAFR KO animals (Figure 4b). As fibrosis results from an imbalance between pro- and anti-fibrotic stimuli, we did a ratio between TGF-β/BMP-7 expressions and found a striking difference between WT and PAFR KO groups, favoring an anti-fibrotic state in PAFR KO mice (Figure 4c).

To make sure that an overall pro-fibrotic signaling is attenuated in PAFR KO animals, we performed a PCR array for ECM and adhesion molecules. As observed in Figure 5a,

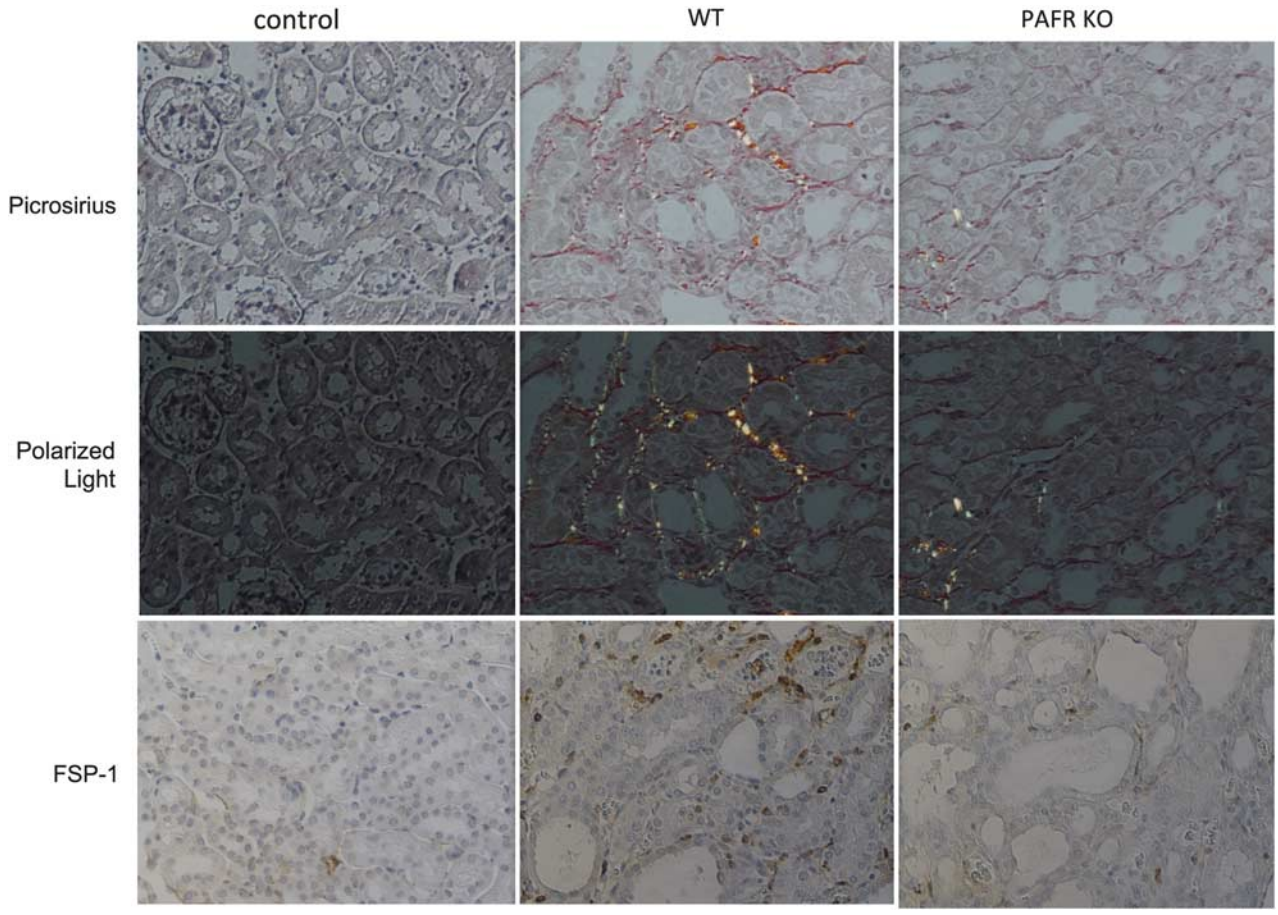

Figure 3 PAFR KO animals submited to UUO show less histologic changes than WT counterparts. Representative images of Picrosirius staining, under normal and polarized light, and FSP-1 protein expression from control mice and WT and PAFR KO mice submitted to UUO.

the heat map of gene expression is quite similar between control and PAFR KO mice, but striking different from WT animals. Furthermore, when we compare UUO-submitted WT or PAFR KO groups *vs* control group, we can observe that the number of upregulated genes in the first group is much more pronounced than in the PAFR KO one, suggesting once again that the absence of PAFR promotes a global gene profile more similar to the control group (Figures 5b and c). More interestingly, when we compare WT and PAFR KO animals we can see that ECM and adhesion molecules machinery genes are greatly overexpressed in the first group (Figure 5d). The list of downregulated and upregulated genes in each condition can be checked in Supplementary Information, in the Supplementary Tables 1, 2 and 3. All these data suggest that PAFR engagement is important for a proper fibrogenic signaling.

PAFR KO Mice Have Less Prominent Inflammatory Response

As PAF has been widely linked to inflammation and since this process can accelerate fibrosis,[22] we decided to evaluate the inflammatory profile in kidney tissue homogenates after 7 days of UUO induction. Initially, we observed that the renal tissue of WT animals showed an increase in the chemoattractant molecules KC and monocyte chemotactic protein-1 (MCP-1), a fact that was noted in a significantly less extent in PAFR KO mice (Figures 6a and b). Also, when we looked for CD68 expression—a marker of macrophage population—in kidney tissue, we observed that such molecule was more than three-fold higher in WT mice, compared with PAFR KO (Figure 6c).

The adaptive immune response can be activated as a consequence of the inflammatory process. So, we decided to take a look at some lymphocyte-related molecules. We observed that PAFR KO mice have significantly less protein expression for the lymphoproliferative molecule interleukin 2 (IL-2), as well as a tendency to switch to a Th1 pattern, favoring an anti-fibrotic signaling (Figures 6d–f). Still, we were able to verify that the established pro-inflammatory molecules TNF-α, IL-1β and IL-6 were significantly downregulated in PAFR KO mice, when compared with WT animals (Figures 7a–c). In counterpart, we noticed that the expression of the immunosuppressive molecule IL-10 was significantly increased in the

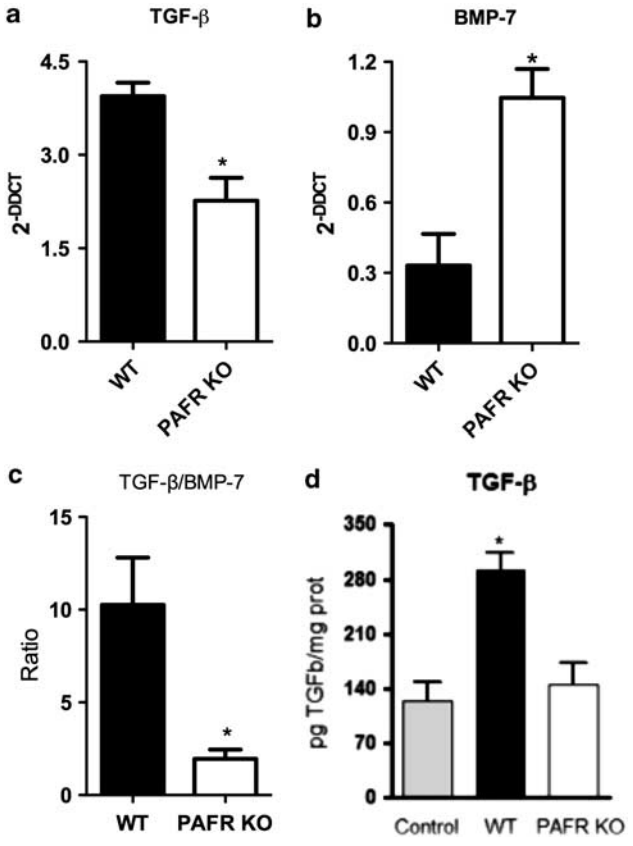

Figure 4 Pro-fibrotic signaling is downregulated in PAFR KO animals. Gene expression of TGF-β and BMP-7 is shown in (**a** and **b**), respectively (*$P<0.05$ *vs* WT). In (**c**), it is shown the ratio TGF-β/BMP-7 (*$P<0.05$ *vs* WT). (**d**) Indicates the protein expression of TGF-β in renal tissue of control animals and WT and PAFR KO mice submitted to UUO (*$P<0.05$ *vs* control and PAFR KO groups).

PAFR deleted gene group (Figure 7d). As a result, the reduction in the inflammatory process led to less apoptosis in the renal tissue of PAFR KO animals submitted to obstructive nephropathy (Figure 7e). Together, these results show that engagement of PAFR by PAF or PAF-like molecules produced during UUO induces a pro-inflammatory profile with consequent more cell death, which will later favor a pro-fibrotic environment.

Early Pharmacological Blockade of PAFR Reduces Renal Fibrosis

To go further regarding the link inflammation–fibrosis, we decided to block PAFR during the onset of the disease. For that, we treated WT mice with the PAFR antagonist WEB2170 at the first 3 days after UUO. As observed in Figure 8, the treatment led to a decreased loss of urinary protein, lower levels of urinary NGAL, despite less fibrosis deposition and reduced expression of pro-fibrotic markers TGF-β and type I collagen (Figure 8). These results confirm that early modulation of the inflammatory process can attenuate the fibrotic status of chronic diseases.

DISCUSSION

Renal fibrosis has become a global threat for public health, once the number of patients with this problem is increasing all over the world. The presence of a fibrotic kidney, and consequently the establishment of CKD lead to a worst prognosis with reduction in the quality of life and consumption of huge amounts of money for treatment and/or renal replacement therapies. Although there is an important advance on the knowledge of the molecular pathways involved in this fibrotic process, there is still a long road to achieve, and the search for new therapeutic targets is of extreme importance. Here, we provide evidence that PAFR activation is involved in renal fibrosis, through a mechanism that is, at least in part, due to EMT.

In this work, we showed that PAFR is upregulated early after UUO induction and that its expression was kept elevated until late time points, suggesting the importance of such receptor during CKD. This corroborates other studies that showed an increase of PAFR in the onset of different diseases, like systemic infection, necrotizing enterocolitis, graft-*vs*-host disease and peripheral nerve injury.[23–26] As expected, initially, the infiltrating immune cells must be responsible for PAFR expression upregulation, and at later time points the renal cells are also responsible for the maintenance of higher PAFR expression. PAF and a wide range of oxidatively modified phospholipids are recognized by PAFR that is constitutively expressed by cells participating in innate immunity. Pathologic inflammatory responses can be triggered if mechanisms that generate PAF or other ligands recognized by the PAF receptor are dysregulated or impaired.[27]

In a previous study, we found that oxidized lipids induce the formation of a complex comprising PAFR and the scavenger receptor CD36 within lipid raft platforms in macrophages and this is required for gene expression and production of TGF-β, Arg-1 and mannose receptors. Thus, activate macrophage toward an alternative M2 phenotype,[28,29] which are related to the collagen production and fibrosis. These mechanisms may be applied to the CKD, since oxidized lipids are formed in during its pathogenesis.[30] Besides, another pathway that is downstream PAFR activation and that is involved with TGF-β production is the MAPK ERK.[31,32] Future studies linking these molecules should better elucidate this issue.

Moreover, renal dysfunction has already been linked to an increase in PAF levels. Mariano *et al*[33] showed that patients with sepsis-associated acute renal failure presented an increase in serum and urinary PAF levels. Also, this increase was associated with higher levels of the inflammatory cytokines TNF, IL-1, IL-6 and IL-8.

As proteinuria is considered as a good marker of progressive renal dysfunction,[34] we measured this parameter, as well as albumin excretion, to compare WT and PAFR KO animals. The reduction of protein excretion in PAFR KO group indicates that endogenous PAF contributes to renal

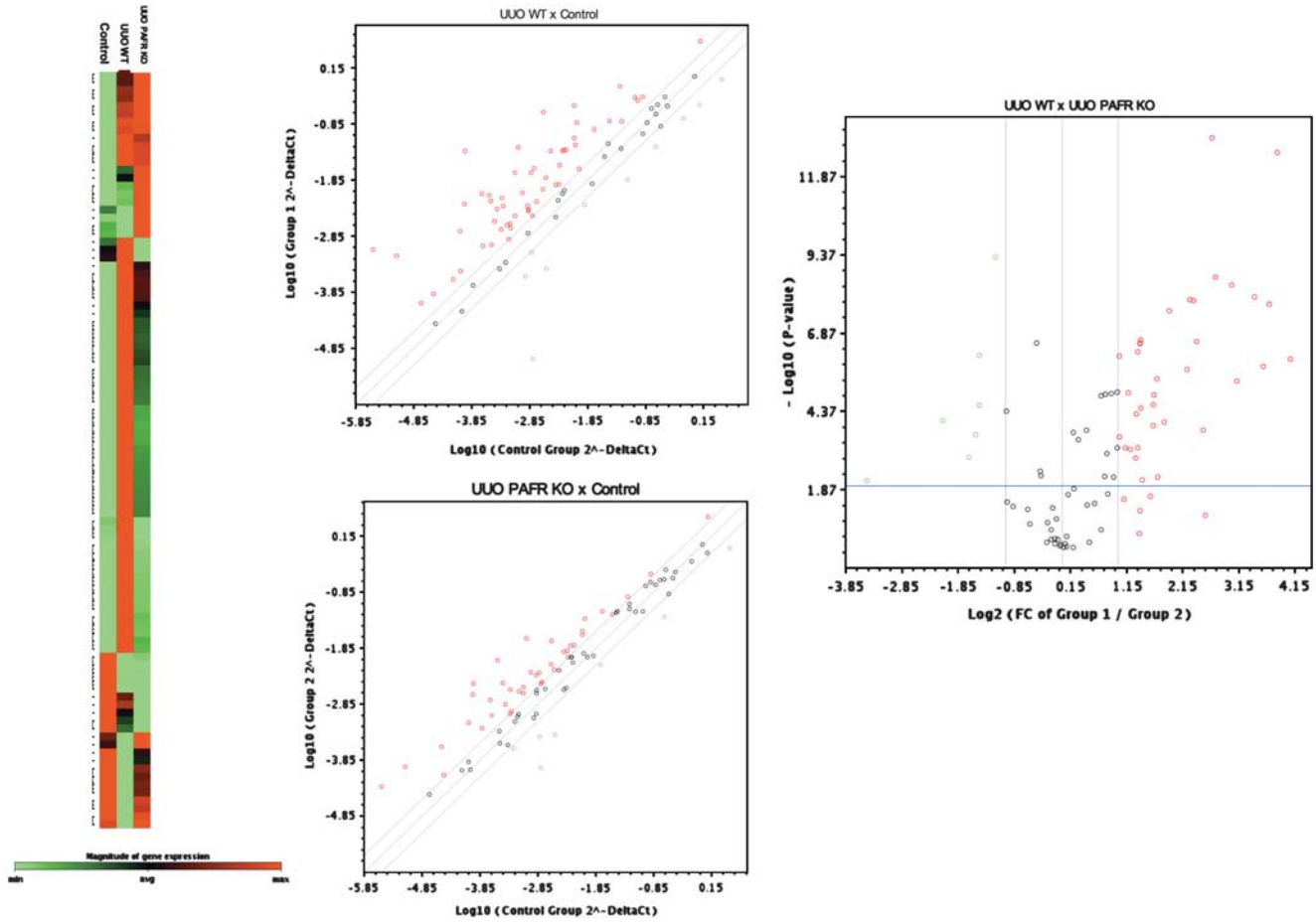

Figure 5 PAFR engagement increases ECM and adhesion molecules gene machinery. (**a**) Heat map comparing Control, WT and PAFR KO groups. Red and green colors represent upregulated and downregulated genes, respectively. The color intensity indicates the extent of gene expression. (**b**) Dot plot representation of upregulated (red dots) and downregulated (green dots) genes comparing UUO-submitted WT animals (group 1) *vs* control mice. (**c**) Dot plot representation of upregulated (red dots) and downregulated (green dots) genes comparing UUO-submitted PAFR KO animals (group 2) *vs* control mice. (**d**) Volcano graphic representation of upregulated and downregulated genes—red and green dots, respectively—comparing WT (group 1) and PAFR KO mice (group 2) submitted to UUO.

dysfunction. This is in accordance with previous studies that showed, in isolated perfused rat kidney, that the addition of PAF induced proteinuria, at similar levels to that induced by angiotensin II.[35] In addition, Torras *et al*[36] showed that long-term pharmacological blockade of PAFR protects from progressive renal injury. On the other hand, another work did not observe any difference concerning albuminuria levels between animals treated or not with PAFR antagonist in the ablation model of CKD.[37] However, they used the 5/6 nephrectomy model, whereas we used the UUO model which can explain the different results. The progression of renal disease is complex and multifactorial, and an effect on the filtration barrier could not be excluded to explain the proteinuria reduction in PAFR KO animals.[38] But, we believe that fibrosis progression is also an important mediator of the urinary protein loose. Moreover, renal fibrosis also has an effect on the filtration barrier, facilitating the loss of protein within the glomerular filtrate. There is evidence of PAF involvement in fibrosis in different organs, like liver and lung.[39,40] In our study, the attenuated renal dysfunction observed in PAFR KO mice correlated with lower levels of fibrosis. This is in accordance with results from Doi *et al*,[41] using the acid folic model of renal injury. In addition, a classic study showed that stimulation of PAFR promoted overexpression of ECM proteins in renal tubule-interstitial cells.[42]

Recently, we have shown that inflammation is a pivotal mediator of renal fibrosis, and that its modulation could bring beneficial effects.[19,43,44] In this current study, we were able to confirm that PAFR is important for the inflammatory process, not only contributing to chemotaxis, but also activating innate and adaptive immune responses. PAF and its receptor have been widely linked to inflammation,[27,45–48] and is interesting to highlight that not only immune cells, but also a great variety of cell types, from different organs, show an increase in inflammatory signaling as a response to PAF, for example, activating NF-κB, promoting superoxide

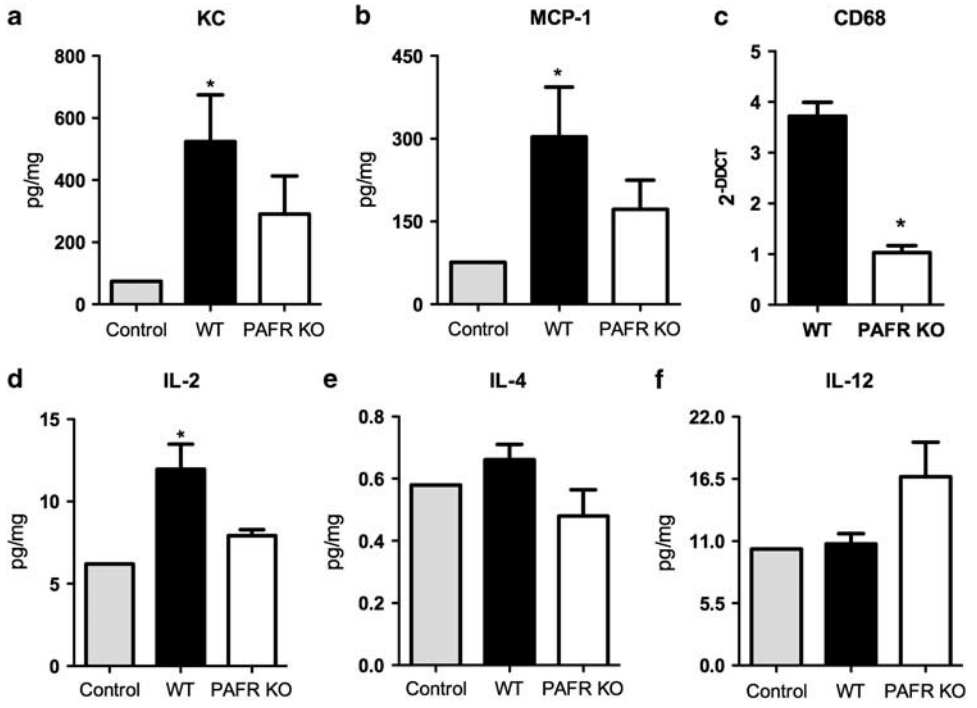

Figure 6 Lack of PAFR decreases immune infiltrating cells signaling. Protein expression of (**a**) KC and (**b**) MCP-1 was measured in kidney lysates from control and WT and PAFR KO animals submitted to UUO (*$P<0.05$ *vs* control and PAFR KO groups). (**c**) Gene expression of CD68, a macrophage marker, evaluated in renal tissue (*$P<0.05$ *vs* WT). Kidney protein expression of the lymphocyte-related molecules IL-2, IL-4 and IL-12 is shown in (**d**–**f**) (*$P<0.05$ *vs* control and PAFR KO).

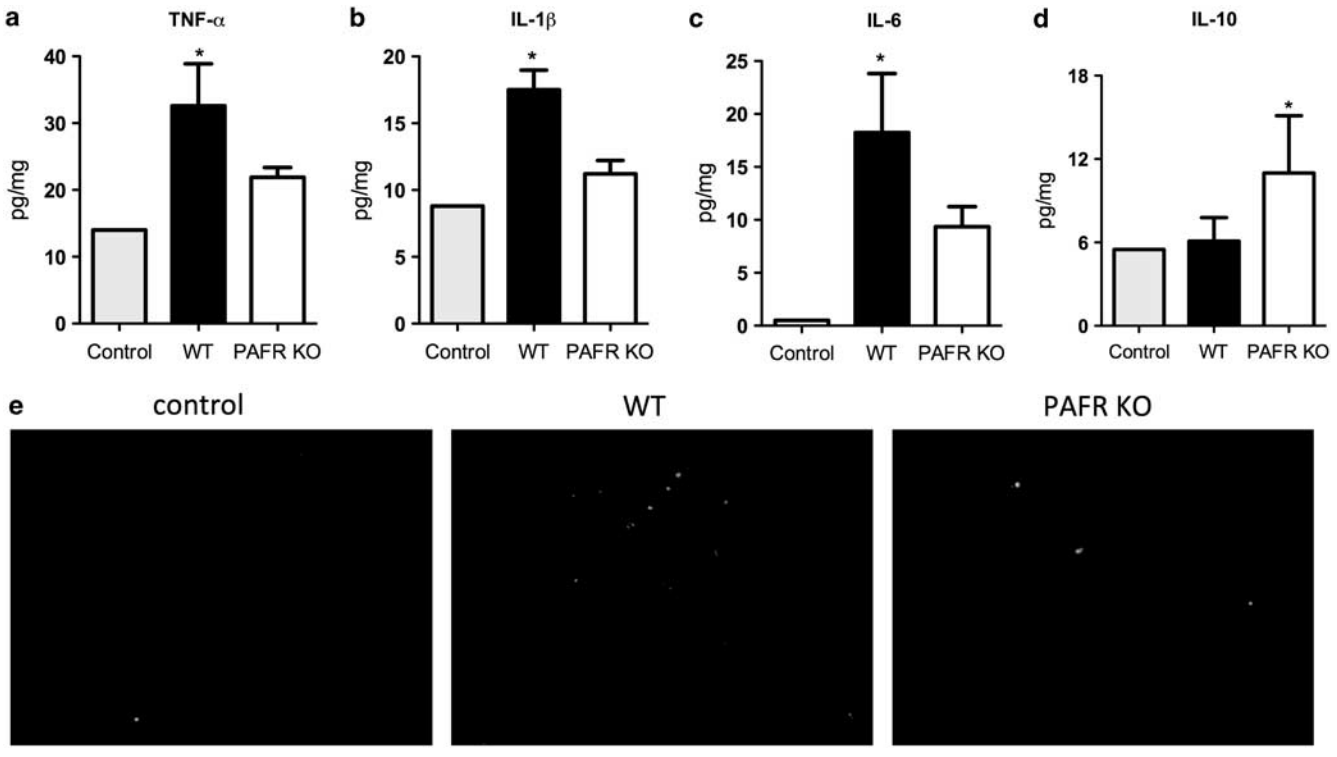

Figure 7 UUO-induced inflammation is reduced in PAFR KO animals. Protein levels of the pro-inflammatory molecules TNF-α (**a**), IL-1β (**b**) and IL-6 (**c**) were measured in renal tissue (*$P<0.05$ *vs* control and PAFR KO mice). The immune regulatory molecule IL-10 also evaluated in kidney lysates (**d**—*$P<0.05$ *vs* control and WT groups) was evaluated in renal tissue. Also, apoptosis was assessed and a representative image is shown in (**e**).

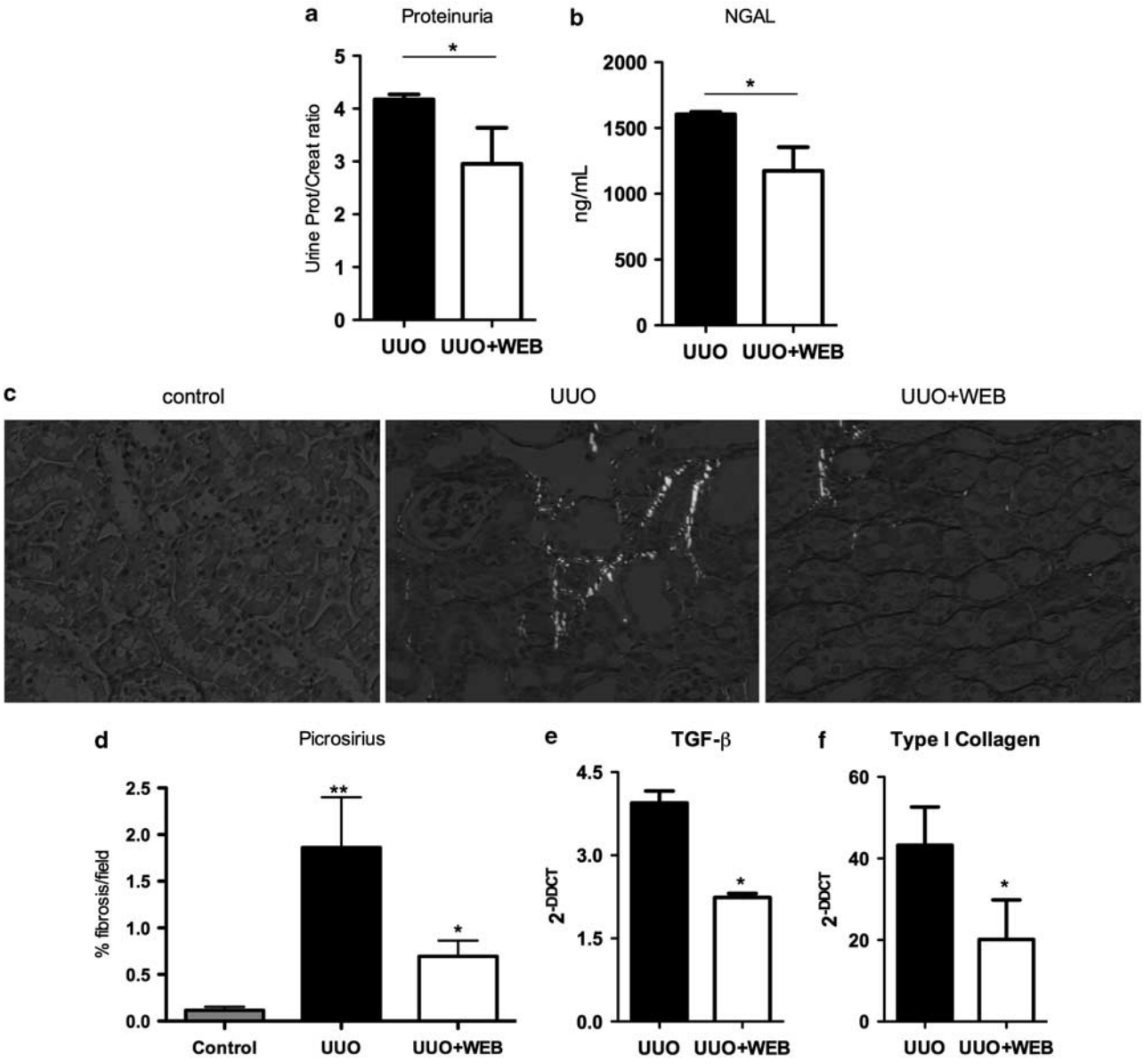

Figure 8 Early blockade of PAFR attenuates renal progressive disease. (**a**) Proteinuria levels were evaluated in animals submitted to UUO and that were treated or not with WEB2170. (**b**) Levels of urinary NGAL, 7 days after UUO in mice that were treated or not with WEB2170. In (**c**), we can see representative images of the indicated groups after Picrosirius staining and polarized light evaluation. The quantification of fibrosis deposition can be seen in (**d**) (*$P<0.05$ *vs* Control and **$P<0.01$ *vs* Control and UUO + WEB). The gene expression of type I collagen and TGF-β can be observed in (**e** and **f**), respectively. *$P<0.05$ *vs* UUO.

production, vasodilation and enhancement of cell migration.[49] In addition, Venkatesha *et al*[50] demonstrated that PAF-induced CCL2 (also known as MCP-1) gene expression requires NF-κB activation and Ca2 + /calcineurin signaling pathways, showing a loop of amplification of the inflammatory process. Such information reinforces our MCP-1 data, suggesting that lack of PAFR decreases the production of this chemokine. There is also evidence that engagement of PAFR in macrophages induces regulatory phenotype.[51] These cells, also called M2 macrophages, have an important role in tissue remodeling and repair but excessive activity of these macrophages can induce fibrosis[52]

One more feature that we could observe during the experiments was the reduced presence of apoptotic cells in PAFR KO mice. Apoptosis is one of the hallmarks in UUO model,[53] which was also noticed in our WT group data. Furthermore, PAFR signaling *per se* have been associated with apoptosis.[45,54,55] On the other hand, Ryan *et al*[56] showed that, in a model of neuronal toxicity, PAF could induce apoptosis independent of its receptor.

Finally, our results suggest that PAFR signaling must be involved in EMT, as KO mice showed less gene and protein expression for mesenchymal markers, maintaining an epithelial phenotype. So far, no other publication has linked PAFR to renal EMT, although some papers have shown that PAF and/or PAFR have a role in the production of ECM molecules.[41,42] Some consolidated data indicate that the balance of TGF-β/BMP-7 dictates the fibrotic state of an organ, where the more production of the first molecule may direct to a pro-fibrotic signaling. In counterpart, favoring the BMP-7 production may generate a cytoprotective state.[10,21,57–64] This may provide another explanation for the protection observed in PAFR KO mice, since in this group there was a striking reduction in TGF-β/BMP-7 ratio, compared with WT.

In conclusion, we postulate that PAF or PAF-like molecules generated during UUO could engage PAFR and contribute to renal fibrosis through induction of a pro-inflammatory microenvironment. Also, we reinforce the involvement of EMT in the development of CKD.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

ACKNOWLEDGMENTS

This study was supported by the São Paulo State Funding Agency (FAPESP) (grants numbers: 2009/54474-8 and 2012/02270-2), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Instituto Nacional de Ciência e Tecnologia de Fluídos Complexos (INCT Complex Fluids).

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Prunotto M, Budd DC, Gabbiani G, *et al.* Epithelial-mesenchymal crosstalk alteration in kidney fibrosis. J Pathol 2012.
2. Coresh J, Selvin E, Stevens LA, *et al.* Prevalence of chronic kidney disease in the United States. JAMA 2007;298:2038–2047.
3. Liu Y. Cellular and molecular mechanisms of renal fibrosis. Nat Rev Nephrol 2011;7:684–696.
4. Oliveira de Lima A, Kesrouani S, Gomes RA, *et al.* Population screening for chronic kidney disease: a survey involving 38 721 Brazilians. Nephrol Dial Transplant 2012;(Suppl 3):iii 135–138.
5. Sharma SK, Zou H, Togtokh A, *et al.* Burden of CKD, proteinuria, and cardiovascular risk among Chinese, Mongolian, and Nepalese participants in the International Society of Nephrology screening programs. Am J Kidney Dis 2010;56:915–927.
6. Prunotto M, Budd DC, Meier M, *et al.* From acute injury to chronic disease: pathophysiological hypothesis of an epithelial/mesenchymal crosstalk alteration in CKD. Nephrol Dial Transplant 2012;(Suppl 3):iii 43–50.
7. Lopez-Novoa JM, Nieto MA. Inflammation and EMT: an alliance towards organ fibrosis and cancer progression. EMBO Mol Med 2009;1:303–314.
8. Strutz F, Okada H, Lo CW, *et al.* Identification and characterization of a fibroblast marker: FSP1. J Cell Biol 1995;130:393–405.
9. Chandrasekaran K, Karolina DS, Sepramaniam S, *et al.* Role of microRNAs in kidney homeostasis and disease. Kidney Int 2012;81: 617–627.
10. Dooley S, ten Dijke P. TGF-beta in progression of liver disease. Cell Tissue Res 2012;347:245–256.
11. Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. Curr Opin Allergy Clin Immunol 2012;12:53–59.
12. Kovacic JC, Mercader N, Torres M, *et al.* Epithelial-to-mesenchymal and endothelial-to-mesenchymal transition: from cardiovascular development to disease. Circulation 2012;125:1795–1808.
13. Sipos F, Galamb O. Epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions in the colon. World J Gastroenterol 2012;18: 601–608.
14. Schlondorff D, Neuwirth R. Platelet-activating factor and the kidney. Am J Physiol 1986;251(1 Pt 2):F1–11.
15. Ishii S, Nagase T, Shimizu T. Platelet-activating factor receptor. Prostaglandins Other Lipid Mediat 2002;68-69:599–609.
16. Rios FJ, Koga MM, Ferracini M, *et al.* Co-stimulation of PAFR and CD36 is required for oxLDL-induced human macrophages activation. PLoS ONE 2012;7:e36632.
17. Lopez-Novoa JM. Potential role of platelet activating factor in acute renal failure. Kidney Int 1999;55:1672–1682.
18. Ishii S, Kuwaki T, Nagase T, *et al.* Impaired anaphylactic responses with intact sensitivity to endotoxin in mice lacking a platelet-activating factor receptor. J Exp Med 1998;187:1779–1788.
19. Correa-Costa M, Semedo P, Monteiro AP, *et al.* Induction of heme oxygenase-1 can halt and even reverse renal tubule-interstitial fibrosis. PLoS ONE 2010;5:e14298.
20. Rodriguez C, Rodriguez-Sinovas A, Martinez-Gonzalez J. Lysyl oxidase as a potential therapeutic target. Drug News Perspect 2008;21:218–224.
21. Zeisberg M, Hanai J, Sugimoto H, *et al.* BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med 2003;9:964–968.
22. Lee SB, Kalluri R. Mechanistic connection between inflammation and fibrosis. Kidney Int Suppl 2010;119:S22–S26.
23. Duitman J, Schouten M, Groot AP, *et al.* CCAAT/enhancer-binding protein delta facilitates bacterial dissemination during pneumococcal pneumonia in a platelet-activating factor receptor-dependent manner. Proc Natl Acad Sci US A 2012;109:9113–9118.
24. Hasegawa S, Kohro Y, Shiratori M, *et al.* Role of PAF receptor in proinflammatory cytokine expression in the dorsal root ganglion and tactile allodynia in a rodent model of neuropathic pain. PLoS ONE 2010;5:e10467.
25. Lu J, Jilling T, Li D, *et al.* Polyunsaturated fatty acid supplementation alters proinflammatory gene expression and reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Pediatr Res 2007;61:427–432.
26. Castor MG, Rezende BM, Resende CB, *et al.* Platelet-activating factor receptor plays a role in the pathogenesis of graft-versus-host disease by regulating leukocyte recruitment, tissue injury, and lethality. J Leukoc Biol 2012;91:629–639.
27. Zimmerman GA, McIntyre TM, Prescott SM, *et al.* The platelet-activating factor signaling system and its regulators in syndromes of inflammation and thrombosis. Crit Care Med 2002;30(5 Suppl): S294–S301.
28. Rios FJ, Ferracini M, Pecenin M, *et al.* Uptake of oxLDL and IL-10 production by macrophages requires PAFR and CD36 recruitment into the same lipid rafts. PLoS ONE 2013;8:e76893.
29. Rios FJ, Koga MM, Pecenin M, *et al.* Oxidized LDL induces alternative macrophage phenotype through activation of CD36 and PAFR. Mediators Inflamm 2013;2013:198193.
30. Ribeiro S, Faria Mdo S, Silva G, *et al.* Oxidized low-density lipoprotein and lipoprotein(a) levels in chronic kidney disease patients under hemodialysis: influence of adiponectin and of a polymorphism in the apolipoprotein(a) gene. Hemodial Int Int Symp Home Hemodial 2012;16:481–490.
31. Chen Z, Rola-Pleszczynski M, Stankova J. Activation of ERK1/2 by platelet-activating factor receptor is independent of receptor internalisation and G-protein activation. Cell Signal 2003;15:843–850.
32. Gonzalez-Ramos M, Calleros L, Lopez-Ongil S, *et al.* HSP70 increases extracellular matrix production by human vascular smooth muscle through TGF-beta1 up-regulation. Int J Biochem Cell Biol 2013;45:232–242.
33. Mariano F, Guida G, Donati D, *et al.* Production of platelet-activating factor in patients with sepsis-associated acute renal failure. Nephrol Dial Transplant 1999;14:1150–1157.
34. Cravedi P, Ruggenenti P, Remuzzi G. Proteinuria should be used as a surrogate in CKD. Nat Rev Nephrol 2012;8:301–306.
35. Perico N, Lapinski R, Konopka K, *et al.* Platelet-activating factor mediates angiotensin II-induced proteinuria in isolated perfused rat kidney. J Am Soc Nephrol 1997;8:1391–1398.

36. Torras J, Cruzado JM, Riera M, *et al.* Long-term protective effect of UR-12670 after warm renal ischemia in uninephrectomized rats. Kidney Int 1999;56:1798–1808.
37. Thaiss F, Mihatsch MJ, Oberle G, *et al.* Platelet-activating factor receptor antagonist improves renal function and glomerular lesions in renal ablation. J Lab Clin Med 1994;124:775–781.
38. Camussi G, Tetta C, Coda R, *et al.* Platelet-activating factor-induced loss of glomerular anionic charges. Kidney Int 1984;25:73–81.
39. Chen J, Ziboh V, Giri SN. Up-regulation of platelet-activating factor receptors in lung and alveolar macrophages in the bleomycin-hamster model of pulmonary fibrosis. J Pharmacol Exp Therap 1997;280: 1219–1227.
40. Karantonis HC, Gribilas G, Stamoulis I, *et al.* Platelet-activating factor involvement in thioacetamide-induced experimental liver fibrosis and cirrhosis. Dig Dis Sci 2010;55:276–284.
41. Doi K, Okamoto K, Negishi K, *et al.* Attenuation of folic acid-induced renal inflammatory injury in platelet-activating factor receptor-deficient mice. Am J Pathol 2006;168:1413–1424.
42. Ruiz-Ortega M, Bustos C, Plaza JJ, *et al.* Overexpression of extracellular matrix proteins in renal tubulointerstitial cells by platelet-activating-factor stimulation. Nephrol Dial Transplant 1998;13:886–892.
43. Braga TT, Correa-Costa M, Guise YF, *et al.* MyD88 signaling pathway is involved in renal fibrosis by favoring a TH2 immune response and activating alternative M2 macrophages. Mol Med (Cambridge, MA) 2012;18:1231–1239.
44. Correa-Costa M, Braga TT, Semedo P, *et al.* Pivotal role of Toll-like receptors 2 and 4, its adaptor molecule MyD88, and inflammasome complex in experimental tubule-interstitial nephritis. PLoS ONE 2011;6:e29004.
45. Lacerda-Queiroz N, Rodrigues DH, Vilela MC, *et al.* Platelet-activating factor receptor is essential for the development of experimental cerebral malaria. Am J Pathol 2012;180:246–255.
46. Rodrigues DH, Lacerda-Queiroz N, de Miranda AS, *et al.* Absence of PAF receptor alters cellular infiltrate but not rolling and adhesion of leukocytes in experimental autoimmune encephalomyelitis. Brain Res 2012;1385:298–306.
47. Sharif NA, Wiernas TK. Platelet-activating factor-induced intracellular signaling and release of cytokines and prostaglandin E2 in immortalized human corneal epithelial cells. J Ocul Pharmacol Ther 2010;26:21–29.
48. Sharma J, Young DM, Marentette JO, Rastogi P, Turk J, McHowat J. Lung endothelial cell platelet-activating factor production and inflammatory cell adherence are increased in response to cigarette smoke component exposure. Am J Physiol Lung Cell Mol Physiol 2012;302:L47–L55.
49. Ishii S, Shimizu T. Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. Prog Lipid Res 2000;39:41–82.
50. Venkatesha RT, Ahamed J, Nuesch C, Zaidi AK, Ali H. Platelet-activating factor-induced chemokine gene expression requires NF-kappaB activation and Ca2 + /calcineurin signaling pathways. Inhibition by receptor phosphorylation and beta-arrestin recruitment. J Biol Chem 2004;279:44606–44612.
51. Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. J Clin Invest 2001;108: 957–962.
52. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol 2005;5:953–964.
53. Bascands JL, Schanstra JP. Obstructive nephropathy: insights from genetically engineered animals. Kidney Int 2005;68:925–937.
54. Bennett SA, Chen J, Pappas BA, Roberts DC, Tenniswood M. Platelet activating factor receptor expression is associated with neuronal apoptosis in an in vivo model of excitotoxicity. Cell Death Differ 1998;5:867–875.
55. Chen CH, Jiang T, Yang JH, Jiang W, Lu J, Marathe GK, *et al.* Low-density lipoprotein in hypercholesterolemic human plasma induces vascular endothelial cell apoptosis by inhibiting fibroblast growth factor 2 transcription. Circulation 2003;107:2102–2108.
56. Ryan SD, Harris CS, Mo F, Lee H, Hou ST, Bazan NG, *et al.* Platelet activating factor-induced neuronal apoptosis is initiated independently of its G-protein coupled PAF receptor and is inhibited by the benzoate orsellinic acid. J Neurochem 2007;103:88–97.
57. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest 2005;115:209–218.
58. Bedi S, Vidyasagar A, Djamali A. Epithelial-to-mesenchymal transition and chronic allograft tubulointerstitial fibrosis. Transplant Rev (Orlando) 2008;22:1–5.
59. Chung AC, Huang XR, Zhou L, Heuchel R, Lai KN, Lan HY. Disruption of the Smad7 gene promotes renal fibrosis and inflammation in unilateral ureteral obstruction (UUO) in mice. Nephrol Dial Transplant 2009;24:1443–1454.
60. Hwang M, Kim HJ, Noh HJ, Chang YC, Chae YM, Kim KH, *et al.* TGF-beta1 siRNA suppresses the tubulointerstitial fibrosis in the kidney of ureteral obstruction. Exp Mol Pathol 2006;81:48–54.
61. Wang S, Chen Q, Simon TC, Strebeck F, Chaudhary L, Morrissey J, *et al.* Bone morphogenic protein-7 (BMP-7), a novel therapy for diabetic nephropathy. Kidney Int 2003;63:2037–2049.
62. Zeisberg M, Bottiglio C, Kumar N, Maeshima Y, Strutz F, Muller GA, *et al.* Bone morphogenic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models. Am J Physiol 2003;285:F1060–F1067.
63. Zeisberg M, Kalluri R. The role of epithelial-to-mesenchymal transition in renal fibrosis. J Mol Med (Berlin, Germany) 2004;82:175–181.
64. Zeisberg M, Shah AA, Kalluri R. Bone morphogenic protein-7 induces mesenchymal to epithelial transition in adult renal fibroblasts and facilitates regeneration of injured kidney. J Biol Chem 2005;280: 8094–8100.