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CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Role of Platelet-Derived Growth Factor-CC in Capillary Rarefaction in Renal Fibrosis

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We have identified platelet-derived growth factor (PDGF)-CC as a potent profibrotic mediator in kidney fibrosis and pro-angiogenic mediator in glomeruli. Because renal fibrosis is associated with progressive capillary rarefaction, we asked whether PDGF-CC neutralization in fibrosis might have detrimental anti-angiogenic effects leading to aggravated peritubular capillary loss. We analyzed capillary rarefaction in mice with and without PDGF-CC neutralization (using genetically deficient mice and neutralizing antibodies), in three different models of renal interstitial fibrosis, unilateral ureteral obstruction, unilateral ischemia-reperfusion, Col4a3-deficient (Alport) mice, and healthy animals. Independent of the effect of PDGF-CC neutralization on renal fibrosis, we found no difference in capillary rarefaction between PDGF-CC—neutralized mice and mice with intact PDGF-CC. We also found no differences in microvascular leakage (determined by extravasation of Evans Blue Dye) and in renal relative blood volume quantified using *in vivo* microcomputed tomography. PDGF-CC neutralization had no effects on renal microvasculature in healthy animals. Capillary endothelium did not express PDGF receptor-α, suggesting that potential PDGF-CC effects would have to be indirect. PDGF-CC neutralization or deficiency was not associated with preservation or accelerated loss of peritubular capillaries, suggesting no significant pro-angiogenic effects of PDGF-CC during renal fibrosis. From a clinical perspective, the profibrotic effects of PDGF-CC outweigh the pro-angiogenic effects and, thus, do not limit a potential therapeutic use of PDGF-CC inhibition in renal fibrosis. *(Am J Pathol 2015, 185: 2132—2142; http://dx.doi.org/10.1016/j.ajpath.2015.04.022)*

Renal fibrosis is the underlying pathological process of progressive kidney diseases. One of the hallmarks of renal fibrosis is the rarefaction of peritubular capillaries (PTCs). The loss of capillaries was proposed to be not only an important factor in progression of fibrosis (via aggravation of hypoxia), but was also suggested to be a trigger for fibrosis induction under certain conditions.[1,2] Molecules involved in this process are not well characterized. For example, one of the prototypic pro-angiogenic molecules, vascular endothelial growth factor (VEGF), was shown to have context-dependent effects, being antifibrotic or profibrotic depending on models and manipulations used.[3,4]

Platelet-derived growth factor (PDGF)-CC is a potent profibrotic factor in the kidney. Genetic PDGF-C deficiency or neutralizing antibodies reduced renal interstitial fibrosis in mice

with unilateral ureteral obstruction (UUO).[5] Several studies showed that PDGF-CC is also a potent, VEGF-independent, pro-angiogenic factor (eg, in the heart, muscle, or eye), and PDGF-CC inhibition was suggested as a novel anti-angiogenic therapy in pathological angiogenesis.[6–10] The pro-angiogenic role of PDGF-CC was confirmed in experimental models of

glomerular diseases in which PDGF-CC infusion promoted and PDGF-CC inhibition disrupted glomerular capillary healing.[11] Consistent with previous studies,[6-8] these pro-angiogenic effects were both direct, inducing endothelial cell proliferation, and indirect, inducing production of pro-angiogenic molecules in various cell types.[11]

Herein, we analyzed the question resulting from these experiments, namely whether PDGF-CC also exerts pro-angiogenic effects in the renal interstitium during renal fibrosis.

Materials and Methods

In Vivo Experiments

For these experiments, we reanalyzed the tissues from previously published experiments using mice with UUO (days 5 and 10), genetically deficient for PDGF-CC (PDGF-CC$^{-/-}$), versus wild-type littermates (PDGF-CC$^{+/+}$), or those treated with PDGF-CC–neutralizing antibodies (PDGF-CC antibody) versus isotype-matched IgG.[5]

Ischemia-reperfusion injury (I/R) was performed by left lateral incision. Both renal vein and renal artery were clamped with a microserrefine (Fine Science Tools GmbH, Heidelberg, Germany) for 30 minutes. The ischemia and subsequent reperfusion were checked macroscopically. All operations were performed on a heating pad set to 37°C (warm ischemia), and body temperature was measured rectally throughout the kidney ischemia (TC-1000 Temperature Controller; CWE, Ardmore, PA). Sham-operated on mice underwent the same procedure without clamping the vessels. Ischemia was induced in PDGF-CC$^{+/+}$ ($n = 12$) and PDGF-CC$^{-/-}$ ($n = 6$) mice or in mice treated with 10 mg/kg body weight PDGF-CC–neutralizing antibody ($n = 13$) or 10 mg/kg body weight isotype-matched control IgG ($n = 12$), on days 1, 3, and 5 after disease induction and twice weekly thereafter. Mice were sacrificed on day 21 after the surgery (unpublished data, T.N. and I.V.M.).

In addition, healthy PDGF-CC$^{-/-}$ mice ($n = 8$) and PDGF-CC$^{+/+}$ littermates ($n = 5$) and healthy C57Bl/6 mice treated with PDGF-CC–neutralizing antibody ($n = 3$, 10 mg/kg body weight, twice weekly i.p. for 3 weeks) versus un-specific IgG ($n = 3$, 10 mg/kg body weight, twice weekly i.p. for 3 weeks) were analyzed (all between 10 and 12 weeks old).

Alport mice were crossbred with PDGF-C$^{-/-}$ mice, and PDGF-C$^{-/-}$ or PDGF-C$^{+/+}$ Alport mice were sacrificed at the age of 10 weeks. Six-week-old male Alport mice were treated twice weekly via i.p. injection of PDGF-CC–neutralizing antibody or isotype-matched IgG (10 mg/kg body weight) for 3 weeks (unpublished data, C.R.C.vanR.).

For functional analysis of renal microvasculature [ie, extravasation of Evans Blue Dye and contrast-enhanced in vivo microcomputed tomography (CT) analysis of renal relative blood volume (rBV); see below], PDGF-CC$^{-/-}$ mice and PDGF-CC$^{+/+}$ littermates with UUO day 5 were compared (see below). The UUO was performed in a standardized manner, as previously described.[5,12] Briefly, after abdominal midline

incision, the left ureter was ligated and cut in between the ligatures to avoid ascending infection. The wound was sutured in two layers.

Measurement of Microvascular Permeability

Microvascular permeability was measured using a modified and optimized protocol (unpublished data, J.B.), as described by Yamaguchi et al.[13] This method is based on high-affinity binding of the Evans Blue Dye to plasma albumin,[14,15] which extravasates to the interstitial space only if the microvasculature becomes leaky and is a sensitive marker of microvascular leakage.[16] In brief, Evans Blue Dye (Sigma-Aldrich, Steinheim, Germany) was dissolved in 0.9% NaCl (5 mg/mL) and applied to anesthetized mice i.v. via the tail vein (10 µg/g body weight). After 35 minutes, the mice were perfused with 40 mL of 0.9% NaCl applied through the left ventricle of the heart, with particular focus on consistency and thoroughness of the perfusion. The kidneys were then extracted and cut into two halves longitudinally. Half of each kidney was weighed and incubated in 100% formamide (4 mL/g kidney weight) at 56°C for 12 hours. The extracted dye was measured spectrophotometrically at 620 nm. The results are expressed as µg/g kidney weight. The other half of the kidneys was used to confirm induction of fibrosis and capillary rarefaction, which, as expected, was observed in all mice (data not shown).

For these experiments, 11 UUO mice [for PDGF-CC$^{-/-}$ ($n = 5$) and PDGF-CC$^{+/+}$ ($n = 6$)] and 8 healthy mice [PDGF-CC$^{-/-}$ ($n = 5$) and PDGF-CC$^{+/+}$ ($n = 3$)] were used.

In Vivo µCT Imaging

To quantify noninvasively the rBV in kidneys of PDGF-CC$^{-/-}$ ($n = 3$) and PDGF-CC$^{+/+}$ ($n = 3$) mice with UUO day 5, contrast-enhanced in vivo µCT was performed, as described previously,[17,18] and adopted for kidneys (unpublished data, J.E.). The accuracy and congruency of this novel contrast-enhanced in vivo µCT imaging technique were already proved previously for quantifying vascularization in tumor xenografts and experimental models for liver fibrosis in mice.[17,18] In brief, mice were anesthetized with 1.5% isoflurane in oxygen-enriched air and imaged before and after i.v. injection of 100 µL iodine-containing contrast agent eXIATM160XL (Binitio Biomedical, Ottawa, ON, Canada) using a TomoScope 30s Duo µCT scanner (CT Imaging, Erlangen, Germany) at 41 and 65 kV. A Feldkamp-type reconstruction algorithm (CT Imaging), including ring artifact correction, was performed, resulting in a voxel side length of 35 µm. Reconstructed data were analyzed using Imalytics Preclinical software version 3.2 (Philips Research, Aachen, Germany).

The rBV was calculated as the average brightness of the renal cortex after contrast agent injection, the aorta as a reference blood vessel after contrast agent injection (100% rBV), and the renal cortex before contrast agent administration (0% rBV).[19]

Table 1   List of Primers Used for Real-Time RT-PCR Using SYBR Green in the Study

Gene	Forward primer	Reverse primer
Vegfa	5′-TCTTCAAGCCGTCCTGTGTG-3′	5′-CTCCAGGGCTTCATCGTTACA-3′
Flt1	5′-CTGTCCATGAAAGTGAAGGCC-3′	5′-CATGTACCAAATAGCGAGCAGACT-3′
Kdr	5′-TGGATTCCTACCAGTATGGGACC-3′	5′-TCTAGCTGCCAGTACCACTGGA-3′
Fgf2	5′-CCGCGTGGATGGCGTC-3′	5′-CCTCTCTCTTCTGCTTGGAGTTG-3′
Angpt1	5′-CTGAATGGGATAAAGTGGCACTACT-3′	5′-TCCAAGGGCCGGATCAT-3′
Angpt2	5′-ATGACCTAATGGAGACCGTCAAC-3′	5′-AGTCTCTGAAGGTGGTTTGCTCTT-3′
Gapdh	5′-GGCAAATTCAACGGCACAGT-3′	5′-AGATGGTGATGGGCTTCCC-3′

For this purpose, five representative volumes of interest (each 500 μm³) were analyzed per kidney cortex.

Renal Morphology and Immunohistochemistry

Tissues for light microscopy and immunohistochemistry were fixed in methyl Carnoy's solution and embedded in paraffin, and sections (1 μm thick) were stained and processed as previously described.[5,11,12,20] All histomorphological analyses were performed in a blinded manner (P.B., J.B., P.H., and F.S.).

Monoclonal rat anti-mouse MECA-32 antibody was purchased from Developmental Studies Hybridoma Bank (The University of Iowa, Iowa City, IA) and used in a dilution of 1:250, and rabbit monoclonal anti-human PDGFR receptor (PDGFR)-β antibody, clone Y92 (Abcam, Cambridge, MA) was purchased and used in a dilution of 1:100. Biotinylated secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). For negative controls, instead of primary antibodies, phosphate-buffered saline (PBS) buffer or irrelevant matched IgG was used, and no un-specific staining was observed (data not shown).

To analyze renal microvasculature, we first tested several immunohistochemical protocols using CD31, VEGF receptor-2, and MECA-32 antibodies. All antibodies marked specifically PTCs, but because the latter performed best (data not shown), for all the following experiments, we have used MECA-32 staining for PTC analyses. The analysis of PTC was performed as described previously, and the data are given as fold change compared with untreated or wild-type mice.[20] Morphometry was performed using an image processing and analysis system (Qwin Leica; Leica, Cambridge, UK), linked to a Leica DML3000 light microscope. Images were obtained with a 40× objective by one blinded observer (P.H.). Per slide, 10 to 20 images of cortex were analyzed, and the number of PTCs was counted by two blinded observers (P.H. and F.S.).

Immunofluorescence Double Staining

Double-immunofluorescence staining for MECA-32 and PDGFR-α as well as CD31 and Ki-67 was performed in methyl Carnoy's solution-fixed, paraffin-embedded, xylene-deparaffinized, and graded ethanol-dehydrated sections. Monoclonal rabbit anti-human PDGFR-α, clone D1E1E, was purchased from Cell Signaling Technology Inc. (Danvers, MA; 1:50 dilution). For the analyses of proliferating endothelial cells, instead of MECA-32, we used anti-CD31 antibody to stain the endothelium because this was compatible with Ki-67 antibody for the double immunofluorescence. Rat monoclonal anti-mouse CD31 antibody, clone SZ31, was obtained from Dianova (Hamburg, Germany; 1:20 dilution), and rabbit monoclonal anti-human Ki-67 antibody, clone SP6, was purchased from Abcam (1:150 dilution). Primary antibodies were diluted in PBS containing 1% bovine serum albumin (Sigma-Aldrich), applied simultaneously, and incubated for 1 hour. After washing with PBS (2×), secondary antibodies (goat anti-rat Alexa-647 and goat anti-rabbit Alexa-488; Molecular Probes, Leiden, the Netherlands) were applied simultaneously and incubated for 30 minutes. After washing with PBS (2×), 1 μg/mL DAPI was applied for 10 minutes, washed with PBS (2×), and mounted with Immu-Mount (Thermo Fischer Scientific, Rockford, IL). The immunofluorescence was analyzed using confocal laser-scanning microscope LSM710 from Leica and immunofluorescence microscope BZ9000 from Keyence (Osaka, Japan). PBS buffer or irrelevant matched IgG, instead of primary antibodies, was used as a negative control; both were negative.

RNA Extraction and Analysis

Total RNA was extracted from isolated murine renal cortex using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA syntheses and real-time quantitative PCRs were performed as previously described.[5,11] In short, the concentration and the purity of RNA were measured spectrophotometrically using Nanodrop (NanoDrop Technologies, Wilmington, DE). For real-time PCR, the Qiagen QuantiFast SYBR Green RT-PCR kit (Qiagen) was used according to the manufacturer's instructions. For normalization of the data, the standard ΔCt method was used with Gapdh as the housekeeping gene. The expression of the genes of interest was calculated as relative fold change in expression in comparison to the appropriate control group, arbitrarily set as 1. Sequences of primers and probes are given in Table 1.

Statistical Analysis

Data are presented as means ± SD. By using GraphPad Prism Version 5.03 (La Jolla, CA), one-way analysis of variance with Bonferroni post hoc analysis or t-test was used, where appropriate, to evaluate the results. $P < 0.05$ was considered significant.
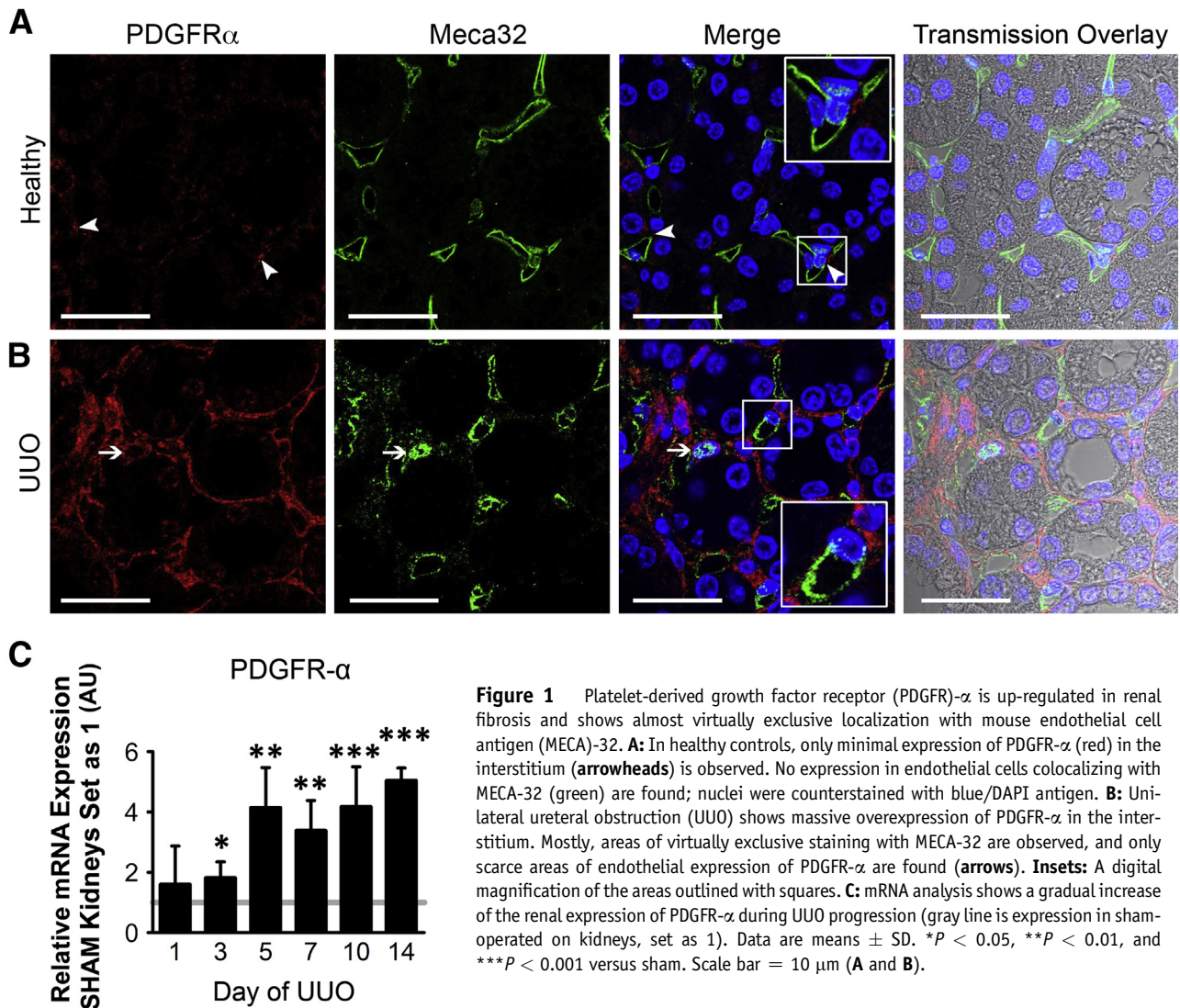
Figure 1   Platelet-derived growth factor receptor (PDGFR)-α is up-regulated in renal fibrosis and shows almost virtually exclusive localization with mouse endothelial cell antigen (MECA)-32. **A:** In healthy controls, only minimal expression of PDGFR-α (red) in the interstitium (**arrowheads**) is observed. No expression in endothelial cells colocalizing with MECA-32 (green) are found; nuclei were counterstained with blue/DAPI antigen. **B:** Unilateral ureteral obstruction (UUO) shows massive overexpression of PDGFR-α in the interstitium. Mostly, areas of virtually exclusive staining with MECA-32 are observed, and only scarce areas of endothelial expression of PDGFR-α are found (**arrows**). **Insets:** A digital magnification of the areas outlined with squares. **C:** mRNA analysis shows a gradual increase of the renal expression of PDGFR-α during UUO progression (gray line is expression in sham-operated on kidneys, set as 1). Data are means $\pm$ SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus sham. Scale bar $= 10$ μm (**A** and **B**).

Results

PDGFR-α Expression on Endothelial Cells of PTCs

We first analyzed the expression of PDGF receptor (PDGFR)-α, the receptor of PDGF-CC, by immunofluorescence using confocal laser-scanning microscopy (Figure 1, A and B). As expected, it was specifically expressed in the interstitium and markedly up-regulated in mice with UUO. Colocalization of PDGFR-α and MECA-32 showed mutually exclusive staining in the healthy kidneys (Figure 1A) and also in the fibrotic kidneys (Figure 1B). However, in small areas, some endothelial PDGFR-α expression could be observed (Figure 1B). Expression of cortical PDGFR-α was significantly increased after induction of UUO on the mRNA level (Figure 1C).

PDGF-CC Neutralization Does Not Alter the Number of PTCs in Healthy Mice

In healthy animals, no significant differences in the number of PTCs were observed between IgG-treated and PDGF-CC

antibody−treated mice (Figure 2A). Similarly, no significant differences were observed between PDGF-CC$^{+/+}$ and PDGF-CC$^{-/-}$ mice (Figure 2B). In addition, a comparison of the contralateral nonfibrotic kidneys revealed no differences in number of PTCs between IgG-treated and PDGF-CC antibody−treated mice neither in the UUO day 5 group (1.00 ± 0.02 versus 0.96 ± 0.08) nor in the UUO day 10 group (1.00 ± 0.05 versus 0.99 ± 0.07) or the I/R day 21 group (1.00 ± 0.10 versus 0.92 ± 0.06).

PDGF-CC Neutralization Does Not Alter the Number of PTCs in Three Different Models of Renal Fibrosis

As previously demonstrated, genetic deficiency of PDGF-CC resulted in a significant reduction of renal fibrosis on both days 5 and 10 of UUO, whereas treatment with PDGF-CC−neutralizing antibody resulted in a significant reduction of renal fibrosis only on day 5, but not day 10.[5] Expression of cortical PDGFR-β, a marker of interstitial fibroblasts and myofibroblasts, was reduced in mice treated with PDGF-CC antibody versus IgG on
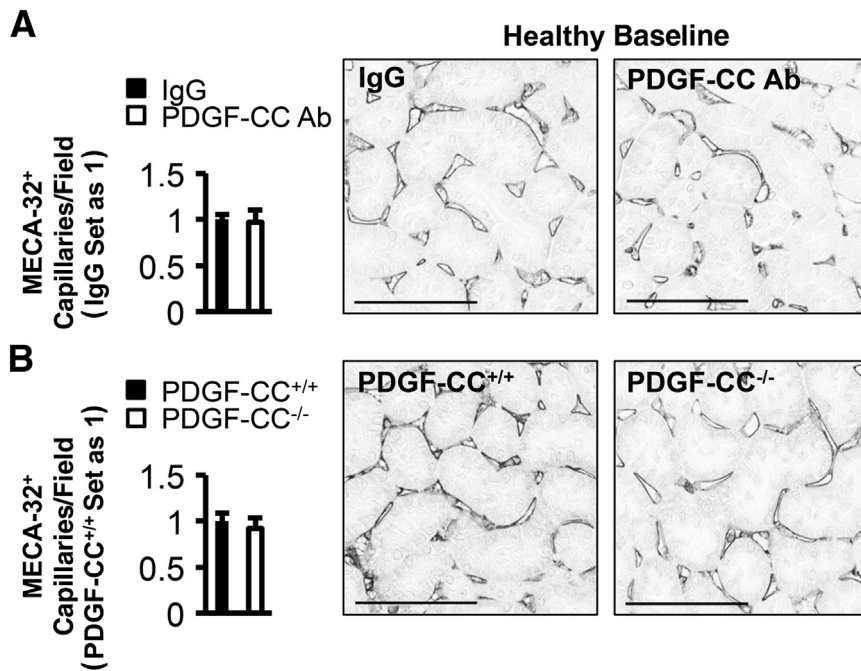
Figure 2 Deficiency of platelet-derived growth factor (PDGF)-CC does not alter the number of peritubular capillaries (PTCs) in healthy mice. The number of PTCs was analyzed in healthy IgG ($n = 3$) or PDGF-CC antibody (Ab)—treated ($n = 3$) and healthy wild-type PDGF-CC$^{+/+}$ ($n = 8$) and PDGF-CC$^{-/-}$ ($n = 5$) mice. No significant differences in the number of PTCs are found between control IgG and PDGF-CC antibody—treated mice (**A**), or between PDGF-CC$^{+/+}$ and PDGF-CC$^{-/-}$ mice (**B**). Data are means $\pm$ SD. Scale bar $= 100$ μm (**A** and **B**). MECA, mouse endothelial cell antigen.

day 5, but not on day 10, of UUO, confirming our previous results in these mice (Supplemental Figure S1). The quantification of PTCs showed no significant difference between IgG and PDGF-CC antibody—treated mice on day 5 of UUO (Figure 3A). Compared with PDGF-CC$^{+/+}$ mice, obstructed kidneys of PDGF-CC$^{-/-}$ mice had marginally, but significantly, more PTCs (19%) (Figure 3B). On day 10 of UUO, however, no significant differences in the number of PTCs in the obstructed kidneys were found in either experiment (ie, PDGF-CC antibody versus IgG-treated mice) (Figure 3C) or PDGF-CC$^{+/+}$ versus PDGF-CC$^{-/-}$ mice (Figure 3D). In addition, we found no significant differences in proliferation of endothelial cells of PTCs on day 5 of UUO between PDGF-CC—deficient and antibody-treated mice versus WT or IgG-treated mice, respectively (Figure 3, E and F).

In a model of severe unilateral I/R injury leading to fibrosis (day 21), both genetic deficiency of PDGF-CC and treatment with neutralizing antibodies had no effect on renal fibrosis (unpublished data, T.N. and I.V.M.). In these mice, no significant differences in PTCs were observed between PDGF-CC$^{+/+}$ and PDGF-CC$^{-/-}$ (Figure 4A) and PDGF-CC antibody versus IgG-treated mice (Figure 4B).

In a third model of Alport mice with progressive renal injury, both genetic deficiency of PDGF-CC and treatment with neutralizing antibodies significantly reduced renal fibrosis and improved renal function (unpublished data, C.R.C.vanR.). Compared with IgG-treated Alports, PDGF-CC antibody—treated Alport mice had significantly higher PTC numbers (Figure 4C), whereas quantification of PTC yielded similar numbers in PDGF-CC$^{+/+}$ versus PDGF-CC$^{-/-}$ Alport mice (Figure 4D).

We did not observe a significant correlation between the PTC numbers and fibrosis markers in any of the experiments (Table 2).

PDGF-CC Neutralization Does Not Alter Functional Characteristics of PTCs

In healthy mice, no significant differences in the extravasation of Evans Blue Dye, a well-established marker of microvascular leakage, were observed between PDGF-CC$^{+/+}$ and PDGF-CC$^{-/-}$ mice (Figure 5A). On day 5 of UUO, compared with contralateral kidneys, the obstructed fibrotic kidneys showed a more than threefold increase in microvascular leakiness. However, no significant difference between PDGF-CC$^{+/+}$ and PDGF-CC$^{-/-}$ mice was observed, although the PDGF-CC$^{-/-}$ mice showed a tendency toward increased leakage (Figure 5B).

PDGF-CC Neutralization Does Not Alter the Functional Kidney Vascularization *in Vivo*

To noninvasively quantify the functional kidney vascularization *in vivo*, contrast-enhanced *in vivo* microcomputed tomography (μCT) imaging was performed in mice with UUO. On day 5 of UUO, obstructed fibrotic kidneys of both PDGF-CC$^{+/+}$ and PDGF-CC$^{-/-}$ mice exhibited a significantly lower rBV compared with contralateral kidneys (-49% and -43%, respectively) (Figure 6), indicating that renal fibrosis results in reduced tissue vascularization. However, no differences in rBV were observed between PDGF-CC$^{+/+}$ and PDGF-CC$^{-/-}$ mice in either contralateral or fibrotic kidneys (Figure 6).

Regulation of Genes Involved in Angiogenesis during Renal Fibrosis and PDGF-CC Neutralization

In healthy animals, the relative gene expression of major genes involved in angiogenesis [ie, *Vegfa*, *Flt1* (encodes VEGF

receptor-1), *Kdr* (encodes VEGF receptor-2), *Fgf2* (fibroblast growth factor 2), *Angpt1*, and *Angpt2*] was not significantly different in PDGF-CC$^{-/-}$ mice or mice treated with PDGF-CC antibody, compared with PDGF-CC$^{+/+}$ or IgG-treated mice, respectively (Figure 7A). On day 5 of UUO, *Fgf2* was significantly reduced both by PDGF-CC antibody treatment (−40% versus IgG group) and in PDGF-CC$^{-/-}$ mice (−80%

versus PDGF-CC$^{+/+}$) (Figure 7B). The expression of all other analyzed angiogenic genes was similar between IgG and PDGF-CC antibody−treated mice and PDGF-CC$^{+/+}$ and PDGF-CC$^{-/-}$ mice (Figure 7B).

On day 21 of I/R, compared with PDGF-CC$^{+/+}$ mice, PDGF-CC$^{-/-}$ mice had significantly higher expression of *Vegfa* (14%) and *Flt1* (23%), whereas all of the other genes were comparable between the two groups (Figure 7C).

Discussion

The major novel finding of our study is that despite the proangiogenic character of PDGF-CC in glomerular diseases, PDGF-CC neutralization had no significant effects on the peritubular microvasculature in healthy animals and, more important, did not significantly aggravate capillary rarefaction during renal fibrosis. These findings from histological and functional analyses were consistent in almost all of the scenarios analyzed (ie, in both genetically PDGF-CC−deficient mice or using a neutralizing PDGF-CC antibody). They were also consistent in all models analyzed: the UUO, the unilateral I/R, and the Alport mice as models of tubulointerstitial fibrosis.

We previously showed that healthy mice genetically deficient for PDGF-CC have an aberrant cerebral microvasculature.[21] We, therefore, first analyzed whether the renal interstitial microvasculature is affected by PDGF-CC neutralization in healthy mice. Our histomorphological and functional analyses failed to detect any such effects. Similarly, no effects of PDGF-CC deficiency or inhibition on interstitial capillaries in the contralateral unaffected kidneys of the UUO or I/R mice were observed. These data are further supported by our previous findings in acute self-limited rat mesangioproliferative glomerulonephritis, which does not display any overt tubulointerstitial damage. In that model, we also found no effect of PDGF-CC−neutralizing antibody on the interstitial microvasculature.[11] Taken together, PDGF-CC does not influence the physiological renal microvascular homeostasis in healthy rodents. This supports the notion that in adult mice,
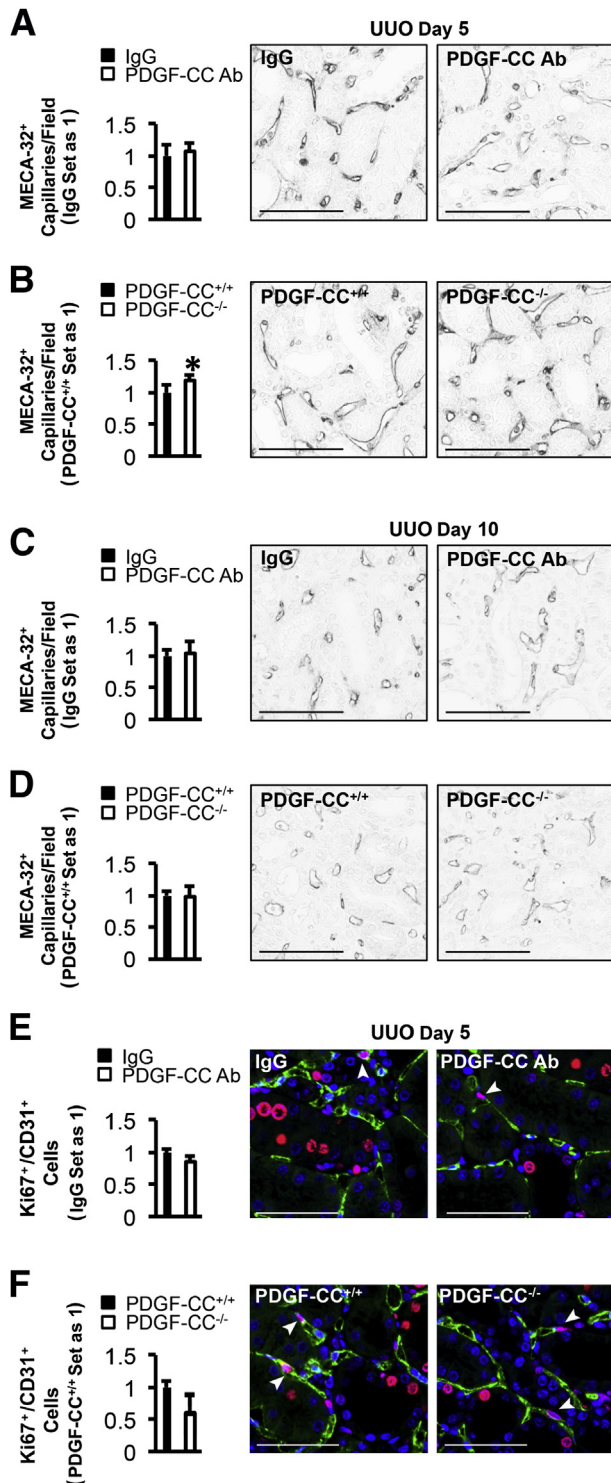

Figure 3 Deficiency of platelet-derived growth factor (PDGF)-CC does not alter the number of peritubular capillaries (PTCs) in ureteral obstruction−induced renal fibrosis. PTCs were analyzed at two different time points of unilateral ureteral obstruction (UUO), days 5 and 10, in IgG ($n = 4$ for day 5 and $n = 4$ for day 10) versus PDGF-CC antibody−treated mice ($n = 4$ for day 5 and $n = 4$ for day 10) and in PDGF-CC$^{+/+}$ ($n = 7$ for day 5 and $n = 5$ for day 10) versus PDGF-CC$^{-/-}$ ($n = 7$ for day 5 and $n = 6$ for day 10) mice. No significant differences in the number of PTCs between IgG and PDGF-CC antibody−treated mice are observed on day 5 (**A**) or on day 10 (**C**) of UUO. PDGF-CC$^{-/-}$ mice have significantly more PTCs compared with PDGF-CC$^{+/+}$ mice on day 5 (**B**) but not on day 10 (**D**) of UUO. The analyses of proliferation of microvascular cortical interstitial endothelial cells using CD31−Ki-67 double immunofluorescence on day 5 of UUO reveal no significant differences between mice with PDGF-CC antibody treatment (**E**) or genetic deficiency (**F**) compared with control IgG treatment or wild-type mice, respectively. Data are means ± SD. *$P < 0.05$ versus PDGF-CC$^{+/+}$ or respective contralateral (CONT). Scale bar = 100 μm (**A**−**F**).
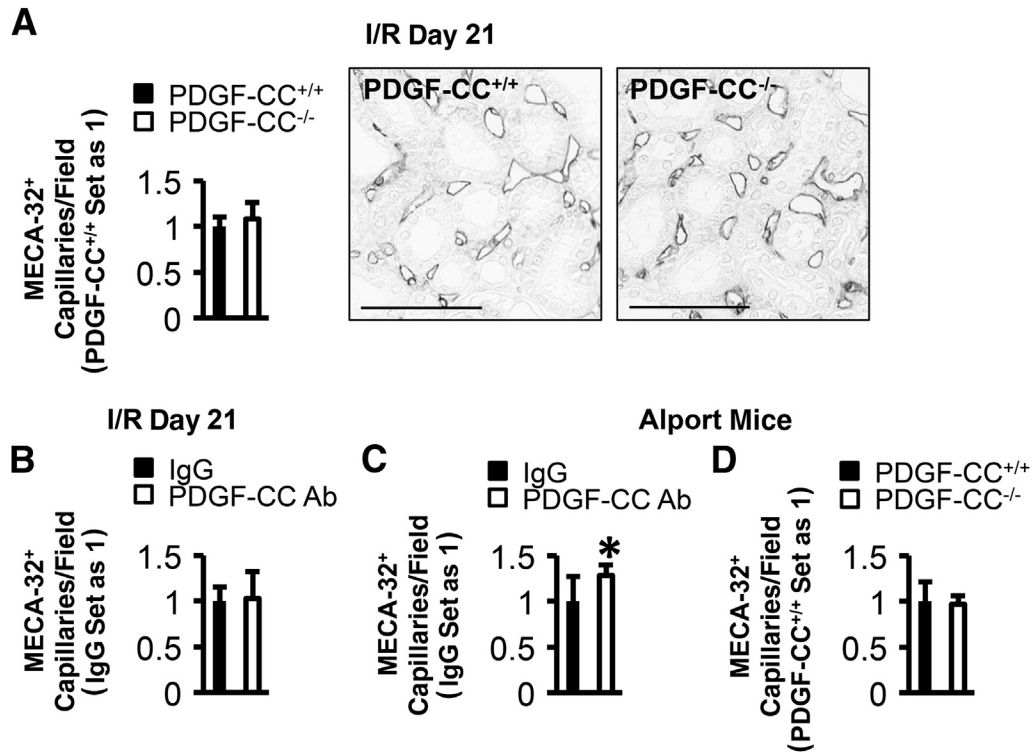
Figure 4   Deficiency of platelet-derived growth factor (PDGF)-CC does not alter the number of peritubular capillaries (PTCs) in postischemic renal fibrosis or in Alport mice. PTCs were also analyzed in postischemic renal fibrosis on day 21 after ischemia-reperfusion injury (I/R) in PDGF-CC$^{+/+}$ ($n = 12$) versus PDGF-CC$^{-/-}$ ($n = 6$) (**A**) or in mice treated with neutralizing PDGF-CC antibody (Ab; $n = 10$) versus IgG control ($n = 9$) (**B**). No significant differences in the number of PTCs are observed between these groups. In addition, we also analyzed the Alport mice treated with neutralizing PDGF-CC antibody ($n = 6$) versus IgG control ($n = 4$) (**C**) or in PDGF-CC$^{+/+}$ ($n = 7$) versus PDGF-CC$^{-/-}$ ($n = 4$) (**D**). Although PDGF-CC$^{-/-}$ and PDGF-CC$^{+/+}$ Alport mice have a similar number of PTCs, the antibody-treated mice have significantly higher PTC numbers compared with IgG-treated Alport mice. *$P < 0.05$ versus IgG-treated control. Scale bar = 100 μm (**A**). MECA, mouse endothelial cell antigen.

PDGF-CC is involved in angiogenesis in pathological, but not in physiological, instances.[7]

We and others have shown that PDGF-CC is up-regulated in renal fibrosis, in both human biopsy specimens and animal models, including UUO.[5,22–25] Herein, we have analyzed the expression of the PDGF-CC receptor, PDGFR-α, with a particular focus on its potential localization on the endothelium of PTCs. Consistent with previous results, we confirmed the marked up-regulation of PDGFR-α specifically in the tubulointerstitial compartment in fibrotic murine kidneys.[24,26–28] Our colocalization studies showed that immunoreactivity of the endothelial marker MECA-32 and PDGFR-α was not observed in the same cells, and both markers were mutually exclusive. PDGFR-α only rarely colocalized with MECA-32, suggesting a possible, but low-level, expression of PDGFR-α on endothelium of the PTC (Figure 1), consistent with previous reports in other vascular beds, including our own data in the glomeruli.[7,8,11] However, these signals could also result from an overlap of endothelium with fibroblasts or pericytes, and further experiments are required to clearly address this issue. Taken together, both the ligand and the receptor are highly up-regulated during renal fibrosis, but because PDGFR-α was not expressed by PTC endothelial cells, any potential pro-angiogenic effect would, therefore, have to be indirect.

PDGF-CC exerts its actions on several cellular targets, and three major mechanisms underlying its pro-angiogenic actions were proposed: i) direct effects on the renal vasculature and microvascular endothelium; ii) effects on stroma

Table 2   Summary of the Animal Experiments and the Effects of PDGF-CC Neutralization on Renal Fibrosis and the Number of PTCs

Model	Groups	Fibrosis	PTC numbers
UUO day 5	KO vs WT	↓	↑
UUO day 5	Ab vs IgG	↓	=
UUO day 10	KO vs WT	↓	=
UUO day 10	Ab vs IgG	=	=
I/R day 21	KO vs WT	=	=
I/R day 21	Ab vs IgG	=	=
Alport	KO vs WT	↓	=
Alport	Ab vs IgG	↓	↑

Renal fibrosis was analyzed using computer-based morphometry of immunohistochemical staining for fibrosis markers (eg, collagen type I, fibronectin, and α-smooth muscle actin) and mRNA expression of the same markers (unpublished studies, T.N., I.V.M., C.R.C.vanR.).[5] Correlation between the fibrosis markers and PTC numbers was performed using univariate regression analyses, and was not significant in any of the experiments.

↑, increased; ↓, decreased; Ab, neutralizing platelet-derived growth factor (PDGF)-CC antibody; Alport, Col4a3-deficient mice; I/R, unilateral ischemia/reperfusion injury; KO, knockout for PDGF-CC; UUO, unilateral ureteral obstruction; WT, wild type.
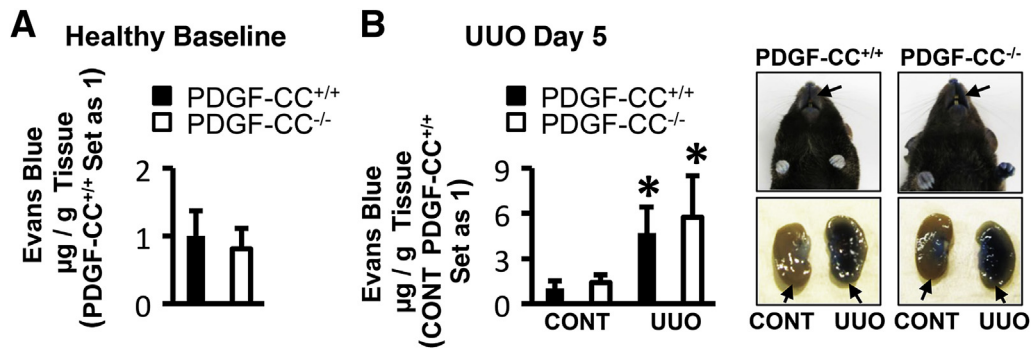
Figure 5 Vascular leakage measured by Evans Blue Dye extravasation is not altered by platelet-derived growth factor (PDGF)-CC deficiency. **A:** Healthy PDGF-CC^{+/+} ($n = 3$) and PDGF-CC^{-/-} ($n = 5$) mice were analyzed, and no significant differences in vascular leakage were found. **B:** Macroscopically, mice injected with Evans Blue Dye have a blue nose (**arrows**), skin, and paws. On day 5 of unilateral ureteral obstruction (UUO), a marked increase of Evans Blue Dye extravasation was found in fibrotic kidneys of both PDGF-CC^{+/+} ($n = 6$) and PDGF-CC^{-/-} ($n = 5$) mice, when compared with contralateral kidneys ($P < 0.05$; macroscopic comparison of contralateral versus UUO kidneys). No significant differences in vascular leakage are observed in fibrotic kidneys between PDGF-CC^{+/+} and PDGF-CC^{-/-} mice. Data are means ± SD. *$P < 0.05$ versus respective contralateral (CONT).

and, in particular, fibroblasts indirectly influencing angiogenesis; and iii) via a switch of inflammatory macrophages toward a pro-angiogenic phenotype.[7,11] Given the cellular expression and regulation of PDGF-CC and PDGFR-α discussed above, and the prominent inflammatory infiltrates in renal fibrosis, all these mechanisms are plausible to take place during renal fibrosis. In most of the models studied herein, PDGF-CC inhibition or deficiency significantly reduced renal fibrosis.[5] One might, therefore, expect to find a significantly better preserved amount of capillaries in mice with PDGF-CC neutralization, as was the case at a single time point after UUO in PDGF-CC–deficient mice. On the other hand, because of the general pro-angiogenic activity of PDGF-CC, neutralization or deficiency of PDGF-CC could have led to a stronger decline of interstitial capillaries in renal fibrosis. However, in all other models, we found similar numbers of capillaries between the groups. Taken together, these data suggest that the potential pro-angiogenic properties of endogenous PDGF-CC on PTC are negligible and are surpassed by its profibrotic properties.

The exact mechanisms triggering capillary dropout in renal fibrosis are not well understood. One hypothesis suggests that, despite hypoxia, dysbalance of angiogenic molecules in favor of anti-angiogenic factors results in microvascular rarefaction in kidney fibrosis.[1,2,29] On PDGF-CC neutralization, we found no indication of a consistent regulation of angiogenic factors, and found only a model-specific down-regulation of pro-angiogenic *Fgf2* in UUO and *Vegfa* in I/R (Figure 7). In addition, we previously found no regulation of *Pdgfa* or *Pdgfb* in the PDFG-CC–deficient or antibody-treated mice in the UUO model, whereas both PDGF receptors were down-regulated and exhibited reduced phosphorylation.[30] These data argue against a compensatory up-regulation of pro-angiogenic or down-regulation of anti-angiogenic factors that could have masked the potential anti-angiogenic effects of PDGF-CC neutralization in our study. Our data showed that the effects of PDGF-CC neutralization on PTC loss and fibrosis were largely independent and did not correlate (Table 2). This, in turn, might suggest that renal microvascular rarefaction is a
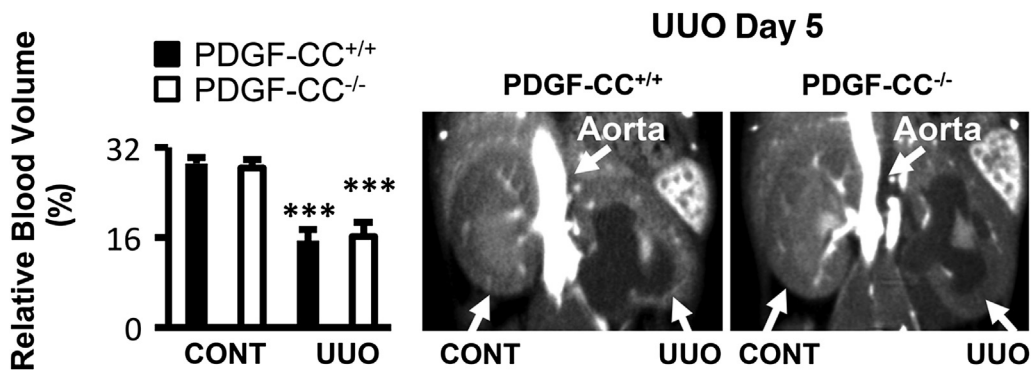

Figure 6 Deficiency of platelet-derived growth factor (PDGF)-CC does not alter renal relative blood volume (rBV), assessed by *in vivo* microcomputed tomography (μCT). To noninvasively quantify the rBV in contralateral (CONT) and fibrotic kidneys in mice with unilateral ureteral obstruction (UUO) on day 5, contrast-enhanced *in vivo* μCT was applied to PDGF-CC^{+/+} ($n = 3$) and PDGF-CC^{-/-} ($n = 3$) mice. Obstructed kidneys of both PDGF-CC^{+/+} and PDGF-CC^{-/-} mice have significantly lower rBV values compared with contralateral kidneys ($P < 0.001$). No significant differences between PDGF-CC^{+/+} and PDGF-CC^{-/-} in either contralateral or obstructed fibrotic kidneys are observed (**arrows** pointing to the kidneys or aorta). Data are presented as means ± SD. ***$P < 0.001$ versus respective CONT.
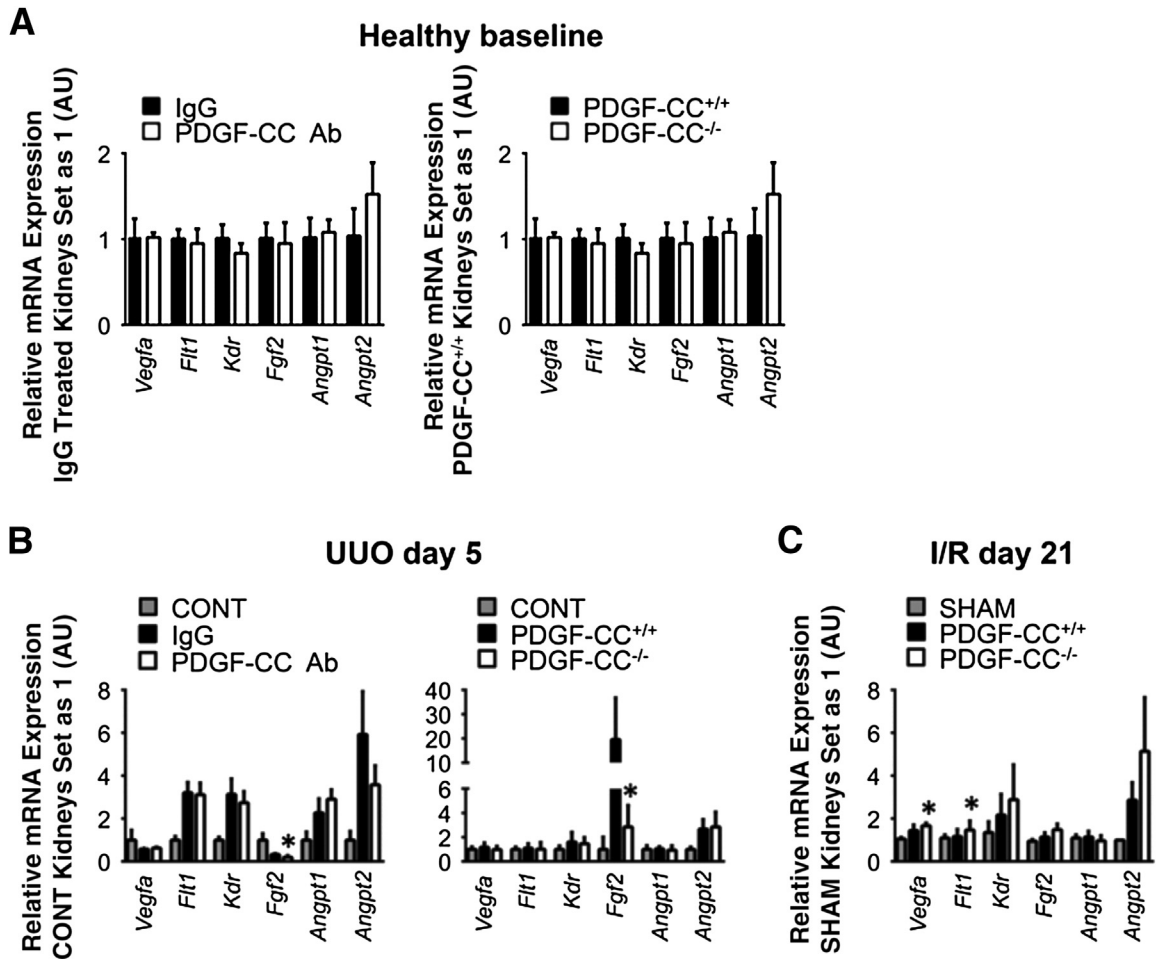
Figure 7 Transcript expression of angiogenesis-related molecules. **A:** In healthy mice, neither platelet-derived growth factor (PDGF)-CC antibody treatment nor genetic deficiency of PDGF-CC (PDGF-CC$^{-/-}$) alters the relative gene expression of any of the analyzed genes ($n = 4$). **B:** On day 5 of unilateral ureteral obstruction (UUO), fibroblast growth factor (FGF)-2 is significantly lower in both PDGF-CC antibody—treated and in PDGF-CC$^{-/-}$ mice, compared with IgG-treated or PDGF-CC$^{+/+}$ mice, respectively ($n = 4$). All of the other genes show similar expression levels between the groups. **C:** On day 21 of ischemia-reperfusion injury (I/R), the expression levels of vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR)-2 are significantly higher in PDGF-CC$^{-/-}$ compared with PDGF-CC$^{+/+}$ mice ($n = 5$, PDGF-CC$^{+/+}$; $n = 6$, PDGF-CC$^{-/-}$). Data are means ± SD. *$P < 0.05$ versus IgG or PDGF-CC$^{+/+}$ (**A**) or sham-operated on kidneys (**B**). AU, arbitrary unit; CONT, contralateral.

process running in parallel with fibrosis and is rather a trigger for, than a consequence of, fibrosis. Taken together, all available data point to PDGF-CC as a selective factor influencing interstitial fibrosis, but not the associated processes such as PTC loss.

These findings might be kidney specific, because in the liver, PDGF-CC overexpression leads to liver fibrosis plus neoangiogenesis, followed by development of hepatocellular adenomas and carcinomas.[10,31,32] The exact mechanisms of these divergent organ-specific effects in fibrosis, associated with capillary rarefaction in kidneys but neoangiogenesis in livers, are not yet clear. Differences in regulation of PDGFs and their receptors might be one possible explanation, as we have shown previously between fibrotic kidneys and livers.[30] It might, however, be misleading to draw parallels, because in the mentioned liver studies, excessive PDGF-CC overexpression was used, whereas herein we interfered only with the endogenous PDGF-CC, which is more relevant.

Vessel leakage is associated with vascular dysfunction. Although the pro-angiogenic effects of VEGF are associated with the induction of leaky vessels, PDGF-CC promotes the formation of nonleaky capillaries.[7,33] On the other hand, we found that tissue plasminogen activator—induced PDGF-CC activation significantly impaired the blood-brain barrier integrity during stroke.[16,34] Our analyses of both healthy and fibrotic kidneys did not reveal any significant difference in microvascular leakiness between PDGF-CC—deficient and wild-type mice. This suggests that in the renal tubulointerstitium, PDGF-CC might not be a crucial factor influencing vascular leakage.

The pro-angiogenic effects of PDGF-CC are largely VEGF independent.[6–8,35] Although we found a slight increase in *Vegfa* and *Flt1* mRNA expression in mice lacking PDGF-CC in the I/R model, in all other instances, our data did not reveal any regulation of VEGF or its receptors. On the other hand, we have shown previously in glomeruli that PDGF-CC induced another

pro-angiogenic factor, FGF-2.[11] Interestingly, fibrotic kidneys of mice with PDGF-CC deficiency or inhibition contained low *Fgf2* mRNA levels compared with wild-type mice (Figure 7). As shown previously, this regulation of *Fgf2* was only observed during disease, but not in healthy animals.[11]

Capillary rarefaction is a common finding in both experimental models and patients with renal fibrosis, in particular in the advanced stages.[1,2,4] Capillary rarefaction in fibrosis was proposed to aggravate tissue hypoxia, thereby further driving renal fibrosis in a circulus vitiosus.[1,2,4] It might also be an initiating factor in some instances (eg, in chronic vascular rejection of kidney allografts or in primary vascular diseases). Aggravation of capillary rarefaction during renal fibrosis could be detrimental for the progression of kidney diseases. In this respect, our data suggest that PDGF-CC neutralization does not significantly aggravate capillary rarefaction in experimental renal interstitial fibrosis and does not limit its therapeutic use as a novel potential antifibrotic treatment approach.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2015.04.022*.

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