CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Macrophages Regulate Unilateral Ureteral Obstruction-Induced Renal Lymphangiogenesis through C-C Motif Chemokine Receptor 2—Dependent Phosphatidylinositol 3-Kinase-AKT—Mechanistic Target of Rapamycin Signaling and Hypoxia-Inducible Factor-1 α /Vascular Endothelial Growth Factor-C Expression

Yan-Chao Guo,^{*,†} Meng Zhang,^{*,†} Fa-Xi Wang,^{*} Guang-Chang Pei,[†] Fei Sun,^{*} Ying Zhang,[†] Xiaoyu He,^{*} Yi Wang,^{*} Jia Song,^{*} Feng-Ming Zhu,[†] Nuruliazki S. Pandupuspitasari,^{*} Jing Liu,^{*} Kun Huang,[‡] Ping Yang,^{*} Fei Xiong,^{*} Shu Zhang,^{*} Qilin Yu,^{*} Ying Yao,^{*,†} and Cong-Yi Wang^{*}

From the Center for Biomedical Research,^{*} Key Laboratory of Organ Transplantation, Ministry of Education and Ministry of Health, Wuhan; the Department of Nephrology,[†] Tongji Hospital, Wuhan; and the Tongji School of Pharmacy,[‡] Tongji Medical College, Huazhong University of Science & Technology, Wuhan, China

Accepted for publication
April 18, 2017.

Address correspondence to
Cong-Yi Wang, M.D., Ph.D.,
the Center for Biomedical
Research, Tongji Hospital,
Tongji Medical College, Huazhong
University of Science and
Technology, 1095 Jiefang Ave.,
Wuhan 430030, China; or Ying
Yao, M.D., Ph.D., Department
of Nephrology, Tongji Hospital,
Tongji Medical College, Huazhong
University of Science and
Technology, 1095 Jiefang Ave.,
Wuhan 430030, China. E-mail:
wangcy@tjh.tjmu.edu.cn or
yaoyingkk@126.com.

Lymphangiogenesis occurs during renal fibrosis in patients with chronic kidney diseases and vascular endothelial growth factor (VEGF)-C is required for the formation of lymphatic vessels; however, the underlying mechanisms remain unclear. We demonstrate that macrophages can regulate unilateral ureteral obstruction (UUO)-induced renal lymphangiogenesis by expressing high levels of VEGF-C by C-C motif chemokine receptor 2 (CCR2)-mediated signaling. Mice deficient in *Ccr2* manifested repressed lymphangiogenesis along with attenuated renal injury and fibrosis after UUO induction. The infiltrated macrophages after UUO induction generated a microenvironment in favor of lymphangiogenesis, which likely depended on *Ccr2* expression. Mechanistic studies revealed that CCR2 is required for macrophages to activate phosphatidylinositol 3-kinase (PI3K)-AKT-mechanistic target of rapamycin (mTOR) signaling in response to its ligand monocyte chemoattractant protein 1 stimulation, whereas hypoxia-inducible factor (HIF)-1 α is downstream of PI3K-AKT-mTOR signaling. HIF-1 α directly bound to the VEGF-C promoter to drive its expression to enhance lymphangiogenesis. Collectively, we characterized a novel regulatory network in macrophages, in which CCR2 activates PI3K-AKT-mTOR signaling to mediate HIF-1 α expression, which then drives VEGF-C expression to promote lymphangiogenesis. (*Am J Pathol* 2017, 187: 1736–1749; <http://dx.doi.org/10.1016/j.ajpath.2017.04.007>)

Lymphangiogenesis, the development and proliferation of lymphatic vessels (LVs), is a necessary process in embryonic development.^{1,2} In adults, however, lymphangiogenesis also occurs under different pathologic conditions such as tumor metastasis, lymphedema, inflammation, wound healing, and transplant rejection.^{3–7} The binding of vascular endothelial growth factor (VEGF)-C/D to the corresponding tyrosine kinase VEGF receptor 3, expressed on the lymphatic endothelial cells (LECs), is crucial for the proliferation of LECs in

Supported by the National Natural Science Foundation of China grants 81530024 (C.-Y.W.), 81471046 (P.Y.), 81500637 (F.X.), and 81470988 (S.Z.); Program Project for Type 1 Diabetes from the Chinese Ministry of Science & Technology grant 2016YFC1305002 (C.-Y.W.); Graduate School of Huazhong University of Science and Technology innovative research grant 0118650021 (Y.-C.G.); the Integrated Innovative Team for Major Human Disease Programs of Tongji Medical College, Huazhong University of Science & Technology (C.-Y.W.); and the Innovative Funding for Translational Research from Tongji Hospital (C.-Y.W.).

Disclosures: None declared.

the process of lymphangiogenesis.^{8–10} Interestingly, lymphangiogenesis is promoted by macrophages through stimulation of local preexisting LEC division by secretion of VEGF-C/D or *trans*-differentiating into LECs.^{11–13} Recent studies have demonstrated that inflammation-associated lymphangiogenesis also occurs in chronic kidney disease and renal fibrosis induced in animal models of unilateral ureteral obstruction (UUO), in which tubular epithelial cells (TECs) and macrophages are found to be the main source of VEGF-C in the kidneys.^{14–16} Therefore, depletion of macrophages or blockade of VEGF-C signaling in macrophages attenuates UUO-induced lymphangiogenesis.¹⁶ Nevertheless, the mechanisms by which macrophages regulate renal lymphangiogenesis are yet to be clarified.

The C-C motif chemokine receptor 2 (CCR2) is a seven transmembrane G-protein–coupled receptor expressed primarily on monocytes/macrophages.^{17,18} Previous studies revealed that CCR2 on macrophages plays an essential role in mediating angiogenesis during tissue repair and tumor metastasis.^{19,20} We thus hypothesized that CCR2 also plays a critical role for macrophages to mediate renal lymphangiogenesis in diseased condition. To address this assumption, we conducted UUO induction of renal injury and fibrosis in *Ccr2*^{−/−} mice and demonstrated that loss of *Ccr2* repressed the expression of VEGF-C in macrophages, which then attenuated renal lymphangiogenesis.

Materials and Methods

Animals and UUO Model

Ccr2 knockout (*Ccr2*^{−/−}) mice in C57BL/6 background were originally purchased from The Jackson Laboratory (Bar Harbor, ME), whereas the wild-type (WT) C57BL/6 control mice were purchased from the Beijing HFK Bioscience Co. Ltd. (Beijing, China). All mice were housed in the Tongji Medical College Animal Center on a 12/12 hour light/dark cycle. Eight-week-old male mice were selected and subjected to UUO induction or sham operation as previously reported.²¹ All mice were sacrificed on days 5 and 14 after UUO induction, and kidneys were harvested for experimental purpose. All animal care and experimental procedures were approved by the Animal Care and Use Committee of Tongji Hospital and performed in accordance with NIH guidelines.²²

Histologic and Immunohistochemical Analysis

Renal tissues were fixed with 4% paraformaldehyde (24 hours), embedded in paraffin, and sectioned into 4-μm sections. After deparaffination in xylene and rehydration in a graded alcohol series, all sections were stained with periodic acid-Schiff and Masson's trichrome using the established techniques and scoring methods.²³ For immunohistologic analysis, antigens were retrieved in a citric acid solution (pH 6.0, 95°C–100°C) for 20 minutes.

The sections were next incubated with 3% H₂O₂ for 20 minutes, and then 5% goat serum for 30 minutes. The sections were probed with primary antibodies against Aquaporin 1 (dilution 1:250; catalog number ab168387; Abcam, Cambridge, MA), VEGF-C (dilution 1:200; catalog number 22601-1-AP; Proteintech, Chicago, IL), F4/80 (dilution 1:50; MCA497RT; ABD Serotec, Oxford, UK), lymphatic vascular endothelial hyaluronan receptor 1 (LYVE-1; dilution 1:1000; ab14917; Abcam), CD45 (dilution 1:1000; GB11066; Goodbio Technology, Wuhan, China), CD4 (dilution 1:1500; GB11064; Goodbio Technology), CD8α (dilution 1:1500; Goodbio Technology), or myeloperoxidase (dilution 1:100; PA5-16672; Thermo Scientific, Waltham, MA) overnight at 4°C. The sections were then developed using a Vector stain ABC kit (Vector Laboratories Inc., Burlingame, CA) and further counterstained with Harris' hematoxylin as reported.²⁴ All sections were assessed under a microscope by two pathologists (Y.Z. and G.-C.P.) in a blinded fashion.

Immunofluorescence

Frozen renal sections (5 μm) were first fixed in 4% paraformaldehyde and then blocked with 5% normal donkey serum as previously described.²⁵ The sections were then incubated with primary antibodies against LYVE-1 (dilution 1:800; ab14917; Abcam), Ki-67 (dilution 1:50; sc-7846; Santa Cruz Biotechnology, Santa Cruz, CA), F4/80 (dilution 1:50; sc-59171; Santa Cruz Biotechnology), VEGF-C (dilution 1:50; catalog number 22601-1-AP; Proteintech), CCR2 (dilution 1:50; sc-6228; Santa Cruz Biotechnology), or hypoxia-inducible factor (HIF)-1α (dilution 1:50; catalog number 20960-1-AP; Proteintech) overnight at 4°C. After washes, the sections were next incubated with an appropriate secondary antibody (Alexa 488-labeled donkey anti-rabbit IgG, or cyanine 3-labeled donkey anti-goat IgG, or Rhodamine-Red donkey anti-rat IgG; dilution 1:400; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at 37°C and counterstained with DAPI.

Western Blot Analysis

Renal or cell lysates were prepared using the RIPA lysis buffer (Beyotime, Shanghai, China) containing the protease inhibitor cocktail (Roche, Indianapolis, IN). Forty micrograms of extracted proteins was electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk for 1 hour and then incubated with primary antibodies against fibronectin (dilution 1:1000; ab23750; Abcam), collagen I (dilution 1:500; ab765p; Millipore, Billerica, MA), α-smooth muscle actin (dilution 1:1000; ab7817; Abcam), VEGF-C (dilution 1:500; sc-374628; Santa Cruz Biotechnology), phosphorylated phosphatidylinositol 3-kinase (PI3K)p85 (catalog number 4228), PI3Kp85 (no. 4257s), phosphorylated AKT (catalog number 4060), AKT (catalog

number 4228), phosphorylated mechanistic target of rapamycin (mTOR; catalog number 2971), and mTOR (catalog number 2983; dilution 1:1000; Cell Signaling Technology, Danvers, MA), and HIF-1 α (dilution 1:500; catalog number 20960-1-AP; Proteintech) overnight at 4°C. After exposure to a horseradish peroxidase–conjugated secondary antibody, the reactive bands were visualized with enhanced chemiluminescence plus reagents, and the relative intensity of reactive bands was analyzed using the ImageJ software version 1.46r (<http://imagej.nih.gov/ij/>, last accessed February 7, 2017) as previously described.²⁶

Real-Time RT-PCR

The RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) was used to extract RNA from kidneys and to generate cDNAs according to the manufacturer's instructions. RT-PCR analysis was performed using the SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) in an ABI prism 7500 Sequence Detection System (Applied Biosystems, South San Francisco, CA) with primers specific for VEGF-C (forward: 5'-AGCAGCCACAAACACCTTCTT-3', reverse: 5'-TCAAACAACGTCTTGCTGAGG-3'). The relative expression level of target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase expression (forward: 5'-TGGCATTGTGGAAGGGCTCA-3', reverse: 5'-GCAC-CAGTGGATGCAGGGAT-3') and calculated with the $2^{-\Delta\Delta Ct}$ method.²⁷

Studies in BMDMs

Bone marrow–derived macrophages (BMDMs) were prepared as previously reported.²⁵ The differentiated BMDMs from *Ccr2*^{-/-} mice or C57BL/6 mice (male, 8 weeks old) were treated with 100 ng/mL monocyte chemoattractant protein (MCP)-1 (PeproTech, Rocky Hill, NJ) for 1 hour. Fifty nanomolar wortmannin (Santa Cruz Biotechnology), 4 μ M perifosine (Selleck Chemicals, Houston, TX), 500 nmol/L rapamycin (Selleck Chemicals), or 4 μ M/L LB-100 (Selleck Chemicals) was added into the BMDM cultures 1 hour before or 500 nmol/L 2-methoxyestradiol (2-MeOE2) (Selleck Chemicals) 4 hours before MCP-1 stimulation for 1 hour. The cells were next harvested for Western blot analysis of target proteins.

Macrophage Depletion and Adoptive Transfer Studies

Ccr2^{-/-} or WT male mice (8 weeks old) were intraperitoneally injected with clodronate liposomes (200 μ L per mouse) (ClodronateLiposomes, Haarlem, the Netherlands) to deplete the peripheral and resident macrophages or injected with phosphate-buffered saline liposomes (ClodronateLiposomes) to serve as controls. Three days after the injection, three mice were selected from each group to verify the depletion of macrophages, and eight mice from each macrophage-depleted group were subjected to UUO

induction as described. Similarly, another eight mice from each group were adoptively transferred with differentiated WT BMDMs into the left kidneys through the left renal arteries (4×10^5 BMDMs per mouse) and immediately subjected to UUO induction as above. All mice were sacrificed 5 days after UUO induction, and kidneys were collected for experimental purpose.

EMSA

Nuclear proteins from renal tissues were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Lafayette, CO) and then subjected to electrophoretic mobility shift assay (EMSA) using a LightShift Chemiluminescent EMSA Kit (Thermo Scientific, Lafayette, CO) as previously reported.²¹ Biotin-labeled double-stranded oligonucleotides containing the HIF-1 α binding site were used as the probe (forward: Biotin-5'-TCTGTACGTGACCACACTCACCTC-3', reverse: Biotin-5'-AGACATGCACCTGGTGTGAGTGGAG-3'; Biyuntian, Wuhan, China). A mixture of unlabeled probes was used as a negative control.

ChIP Assay and VEGF-C Promoter Reporter Assay

Chromatin immunoprecipitation (ChIP) assays were conducted using a ChIP assay kit (Biyuntian) as previously described.²⁸ The primers used in the ChIP assay were as follows: forward: 5'-GCGAGGAGAGTGGATGGAG-3', and reverse: 5'-CTGCCTGCGCTTATGTGAG-3'. A dual luciferase reporter system (Promega, Madison, WI) was used for the VEGF-C promoter luciferase reporter assays, in which the HIF-1 α binding motif (GCGTG, -498 bp, start codon as +1) within the VEGF-C promoter was mutated to GAGTC as previously reported.²⁸

Rapamycin Intervention Study

For analysis of the involvement of mTOR signaling in UUO-induced renal lymphangiogenesis, male *Ccr2*^{-/-} and WT C57BL/6 mice (8 weeks old) were treated with rapamycin (Selleck Chemicals) by i.p. injection (2 mg/kg per day for 6 days), starting from the day of UUO induction. The mice were sacrificed after day 5 of UUO induction to compare the differences of renal lymphangiogenesis.

Statistical Analysis

Statistical analyses of the data were conducted using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA), and the unpaired *t*-test or the standard one-way analysis of variance when appropriate. Represented data are means $\pm$ SD; all *in vitro* studies were conducted with at least three replications. A *P* value of <0.05 was considered statistically significant.

Results

Loss of *Ccr2* Attenuates Renal Injury and Fibrosis and Manifests Decreased Lymphangiogenesis

We first sought to determine the impact of *Ccr2* deficiency on renal injury and fibrosis after UUO induction. Periodic acid-Schiff staining revealed that the mice deficient in *Ccr2* were protected from UUO-induced renal tubular dilation and loss of tubular brush border integrity compared with WT mice on day 5 or 14 of UUO induction (Figure 1A). Similarly, Masson's trichrome staining indicated less extracellular matrix deposition in the 5- and 14-day UUO-induced *Ccr2*^{-/-} kidneys (Figure 1B). Consistently, Western blot analysis indicated decreased expression of fibrogenic markers fibronectin, collagen I, and α -smooth muscle actin in *Ccr2*^{-/-} mice both in the 5- (Supplemental Figure S1A) and 14-day (Supplemental Figure S1B) UUO-induced kidneys. Together, these results indicated that loss of *Ccr2* attenuates UUO-induced renal injury and fibrosis.

From the above observation, we next sought to demonstrate the effect of *Ccr2* deficiency on UUO-induced renal lymphangiogenesis. To this end, we checked the expression of LYVE-1, a marker specific for LVs in the kidneys of adult mice.²⁹ Interestingly, in the sham-operated kidneys only a few LYVE-1⁺ LVs were detected adjacent to the renal arteries and arterioles, whereas in sharp contrast, significantly higher numbers of LYVE-1⁺ LVs were observed both in the 5- and 14-day UUO-induced kidneys (Figure 1C). To confirm this observation, we costained Ki-67, a marker for cell proliferation; therefore, LECs positive for LYVE-1 and Ki-67 were assumed to undergo proliferation (Figure 1D). Similar as above, the number of LVs (Figure 1C) and proliferating LECs (Figure 1D) were both significantly decreased in the 5- and 14-day UUO-induced *Ccr2*^{-/-} kidneys. Taken together, these results indicated that *Ccr2* deficiency not only attenuated renal injury and fibrosis but also repressed renal lymphangiogenesis in the ureteral obstructed kidneys.

Ccr2 Deficiency Suppresses VEGF-C Expression Independent of TECs

Next, we examined the expression of VEGF-C, an essential growth factor required for lymphangiogenesis.⁸ In sham-operated mice, VEGF-C was almost undetectable in the kidneys; however, UUO induced a significant increase in terms of VEGF-C expression compared with that of sham-operated kidneys (Figure 2, A–C). Nevertheless, *Ccr2* deficiency significantly attenuated VEGF-C expression after day 5 (Figure 2A) and 14 (Figure 2B) of UUO induction, which was in fact consistent with the results of decreased renal lymphangiogenesis (Figure 1, C and D). We then conducted immunohistochemical analysis of serial UUO-induced renal sections by staining VEGF-C and Aquaporin 1, a marker for proximal TECs. VEGF-C was noted to

be predominantly expressed by TECs as evidenced by the Aquaporin 1 staining, and no obvious VEGF-C expression change was noted in TECs between *Ccr2*^{-/-} and WT mice after day 5 or 14 of UUO induction (Figure 2C). Interestingly, a great number of infiltrated inflammatory cells in the renal interstitial area also expressed high levels of VEGF-C (Figure 2C), and immunohistochemical staining of serial UUO-induced renal sections for CD45 (a marker for inflammatory cells) and VEGF-C further confirmed this observation (Supplemental Figure S2A). Importantly, mice deficient in *Ccr2* manifested significantly decreased number for VEGF-C⁺ inflammatory cells after UUO induction (Figure 2, C and D), suggesting that loss of *Ccr2* attenuated UUO-induced VEGF-C expression by repressing the infiltration of VEGF-C-expressing inflammatory cells. To confirm this assumption, we conducted RT-PCR analysis of VEGF-C mRNA and obtained consistent results (Figure 2E). Collectively, these data supported that *Ccr2* deficiency attenuated VEGF-C expression in a manner independent of TECs after UUO induction.

Macrophages Contribute to UUO-Induced Altered VEGF-C Expression

The next key question was to characterize the identity of infiltrated inflammatory cells that express VEGF-C. For this purpose, we stained renal sections with markers specific for different types of inflammatory cells (Supplemental Figure S2B). It was interestingly found that the infiltrated inflammatory cells located in the interstitial space of the kidneys were predominantly F4/80⁺ macrophages after UUO induction, whereas only a few resident macrophages were detected in sham-operated kidneys (Figure 3A). More importantly, the number of infiltrated macrophages was significantly reduced in *Ccr2*^{-/-} mice after day 5 and 14 of UUO induction (Figure 3A). Next, we costained VEGF-C to demonstrate whether those macrophages were responsible for the increased VEGF-C expression after UUO induction. Remarkably, F4/80⁺ macrophages expressed high levels of VEGF-C (Figure 3B). However, fewer VEGF-C-expressing F4/80⁺ macrophages and lower levels of VEGF-C expression in those macrophages were observed in the kidneys derived from *Ccr2*^{-/-} mice after day 5 and 14 of UUO induction (Figure 3, B and C). Taken together, our data indicated that reduced macrophage infiltration along with lower VEGF-C expression in those macrophages accounted for the attenuated VEGF-C levels in UUO-induced *Ccr2*^{-/-} kidneys.

Macrophages Generate a Microenvironment in Favor of Lymphangiogenesis

To further address that the infiltrated VEGF-C⁺ macrophages contributed to altered lymphangiogenesis, we conducted immunohistochemical analysis of serial UUO-induced renal sections. Interestingly, the F4/80⁺/VEGF-C⁺

macrophages were colocalized with the LYVE-1⁺ LVs (Figure 4A), suggesting that the infiltrated macrophages probably generated a microenvironment in favor of lymphangiogenesis. To demonstrate this assumption, we first depleted macrophages by i.p. injection of clodronate liposome into *Ccr2*^{-/-} and WT mice, and injection of phosphate-buffered saline liposome served as a control. As expected, approximately 98% of macrophages were depleted in the kidneys after day 3 of clodronate injection (Figure 4B). Next, we conducted UUO induction in WT mice after clodronate liposome and phosphate-buffered saline liposome injection. In line with our assumption, depletion of macrophages significantly attenuated UUO-induced lymphangiogenesis as evidenced by the detection of LYVE-1 expression (Figure 4C). To address that CCR2 regulates macrophage function to affect UUO-induced lymphangiogenesis, we induced macrophage depletion both in WT and *Ccr2*^{-/-} mice as above, then conducted UUO induction in those mice. Macrophage depletion was confirmed by F4/80 staining as above (Supplemental Figure S3A). Indeed, macrophage-depleted *Ccr2*^{-/-} mice manifested similar levels of renal lymphangiogenesis (Figure 4D) and VEGF-C expression (Figure 4F) as that of WT mice. To further confirm this observation, we adoptively transferred 4×10^5 WT BMDMs into the left kidneys of *Ccr2*^{-/-} and WT mice through the renal artery after macrophage depletion during UUO surgery as above. Adoptive transfer of macrophages restored the capability of WT and *Ccr2*^{-/-} mice for induction of lymphangiogenesis, and, consistently, WT and *Ccr2*^{-/-} mice displayed comparable levels of renal lymphangiogenesis (Figure 4E) and VEGF-C expression (Figure 4G). Immunostaining of F4/80⁺ macrophages indeed confirmed that the transferred macrophages resided in the surrounding area of LYVE-1⁺ LVs (Supplemental Figure S3B). Together, these results supported that CCR2 was required for macrophages to generate a microenvironment (eg, by producing VEGF-C) to mediate renal lymphangiogenesis in our UUO model.

Loss of *Ccr2* Represses PI3K-AKT-mTOR Signaling along with Reduced HIF-1 α Expression in Macrophages

To further dissect the molecular mechanisms by which loss of *Ccr2* repressed VEGF-C expression in macrophages to attenuate UUO-induced lymphangiogenesis, we checked with the focus on the PI3K-AKT-mTOR pathway.^{30,31} We

first established that the infiltrated macrophages by UUO induction expressed high levels of CCR2 (Supplemental Figure S4). Western blot analysis was then performed to examine the PI3K-AKT-mTOR signaling. Interestingly, the levels of phosphorylated PI3Kp85/AKT/mTOR and even total PI3Kp85 and mTOR were significantly increased in the kidneys after day 5 (Figure 5, A, C, and E) and 14 (Figure 5, B, D, and F) of UUO induction, but they were significantly lower in the *Ccr2*^{-/-} kidneys (Figure 5, A–F). These results prompted us to embark on HIF-1 α , which could be downstream of PI3K/AKT/mTOR signaling. In line with this assumption, a significant increase for HIF-1 α expression was noted after UUO induction, whereas mice deficient in *Ccr2* displayed significantly lower levels of HIF-1 α expression after day 5 (Figure 5G) and 14 (Figure 5H) of UUO induction. Consistently, EMSA results revealed that renal lysates originated from WT mice displayed significantly higher HIF-1 α DNA binding activity than that of lysates from *Ccr2*^{-/-} mice after day 5 of UUO induction (Figure 5I). Co-immunostaining of renal sections further demonstrated that HIF-1 α was expressed by most of the infiltrated macrophages (Figure 5J). Collectively, our data indicated that CCR2 regulated PI3K-AKT-mTOR signaling to modulate HIF-1 α expression in macrophages, which could be relevant to altered VEGF-C production in UUO-induced kidneys.

HIF-1 α Directly Binds to the VEGF-C Promoter for its Transcription

Finally, we sought to address how HIF-1 α , downstream of PI3K-AKT-mTOR signaling, mediated VEGF-C expression in macrophages. To this end, we first conducted bioinformatic analysis of VEGF-C promoter. Interestingly, three putative HIF-1 α binding motifs (ACGTG/GCGTG) were characterized at positions -1571, -716, and -498 bp upstream of transcription start site (start codon as +1) (Figure 6A). MCP-1-stimulated BMDMs were next prepared and then subjected to ChIP assay as described, and the resulting products were used for ChIP-PCR analysis of regions flanking the above indicated putative HIF-1 α binding sites. Indeed, HIF-1 α manifested binding activity to the motif located in the VEGF-C promoter at position -498 bp (Figure 6B). VEGF-C promoter reporter assays were further conducted to confirm the ChIP data. A mutated VEGF-C promoter reporter (MU) was constructed, in which the

Figure 1 *Ccr2* deficiency attenuates unilateral ureteral obstruction (UUO)-induced renal injury and fibrosis and decreases renal lymphangiogenesis. **A:** Periodic acid-Schiff (PAS) staining for the assessment of tubular dilation and tubular brush border integrity. **Insets** show higher magnified images of the representative areas. **B:** Masson's trichrome staining to analyze extracellular matrix deposition. **C:** Immunofluorescence of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1)-positive lymphatic vessels (LVs). Most of the LYVE-1⁺ LVs were adjacent to the renal arteries and arterioles (indicated by asterisks). **D:** Double staining of LYVE-1 (green) and Ki-67 (red) cells for detection of the proliferating lymphatic endothelial cells (LECs) as indicated by arrows. The arteries (asterisks) are highlighted because of the autofluorescence. Data are expressed as means $\pm$ SEM between the *Ccr2*^{-/-} and wild-type (WT) mice 5 days or 14 days after the UUO operation. Bar graphs express tubular damage score for PAS staining (**A**); analysis for extracellular matrix deposition area (**B**); the quantification of the number of LYVE-1⁺ LVs per field in each group (**C**); quantification of the LYVE-1⁺ and Ki-67⁺ proliferating LECs per field (**D**). $n = 3$ (**A–D**, sham); $n = 5$ (**A–D**, 5 and 14 days UUO); $n = 10$ random fields for the number of LYVE-1⁺ LVs in the cortex (**C**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Scale bars = 100 μ m (**A–D**). Original magnification: $\times 400$ (**C**); $\times 1000$ (**A**, insets). SH, sham.

Figure 2 Unilateral ureteral obstruction (UUO)-induced *Ccr2*^{-/-} kidneys display decreased VEGF-C expression independent of tubular epithelial cells (TECs). **A** and **B**: Representative Western blot analysis results of total vascular endothelial growth factor (VEGF)-C expression after the sham operation (SH), 5-day UUO (**A**), and 14-day UUO (**B**). **C**: Immunohistochemical staining of aquaporin 1 and VEGF-C in series sections. Aquaporin 1 and VEGF-C staining for tubular epithelial cells. **Boxed areas** are shown at higher magnification in the **bottom row**. **Arrows** indicate the VEGF-C-expressing inflammatory cells in the interstitial areas. **D**: Quantification of the VEGF-C⁺ inflammatory cells. **E**: Real-time PCR results for the analysis of VEGF-C in the kidneys. *n* = 3 (**A–D**, sham); *n* = 5 (**A–D**, 5 and 14 days UUO); *n* = 2 (**E**, sham); *n* = 8 (**E**, 5 and 14 days UUO). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bars: 100 μ m (**C**, main images); 50 μ m (**C**, bottom row). Gapdh, glyceraldehyde-3-phosphate dehydrogenase; WT, wild-type.

HIF-1 α binding motif (GCGTG) was mutated to GAGTC. Consistently, disruption of the HIF-1 α binding site resulted in a 2.2-fold reduction in MCP-1-induced luciferase reporter activities (Figure 6C). Collectively, our data suggested a novel regulatory network in macrophages, in which CCR2 was required to activate PI3K-AKT-mTOR signaling, which then enhanced its downstream gene HIF-1 α expression, HIF-1 α then directly binds to the VEGF-C promoter to transcribe its expression.

To further confirm the above assumption, WT and *Ccr2*^{-/-} BMDMs were prepared and then subjected to MCP-1 stimulation. As expected, the levels for the phosphorylated PI3Kp85/AKT/mTOR and HIF-1 α and VEGF-C expression were significantly increased in MCP-

1-stimulated WT BMDMs, whereas no significant change was detected in MCP-1-stimulated *Ccr2*^{-/-} BMDMs (Figure 6D). In line with these results, treatment of WT BMDMs with wortmannin (a PI3K inhibitor), or perifosine (an AKT inhibitor), or rapamycin (an mTOR inhibitor) significantly inhibited MCP-1-stimulated PI3Kp85/AKT/mTOR activity, which then suppressed HIF-1 α and VEGF-C expression (Figure 6E). Similarly, repression of HIF-1 α activity using its inhibitor, 2-MeOE2, attenuated MCP-1-stimulated VEGF-C expression but did not affect PI3Kp85/AKT/mTOR signaling (Figure 6E). Furthermore, treatment of *Ccr2*^{-/-} BMDMs with a PI3Kp85/AKT/mTOR agonist, LB-100 (a protein phosphatase-2A inhibitor), significantly enhanced PI3Kp85/AKT/mTOR signaling

Figure 3 Unilateral ureteral obstruction (UUO)-induced *Ccr2*^{-/-} kidneys manifest decreased macrophage infiltration along with attenuated vascular endothelial growth factor (VEGF)-C expression. **A:** Results for immunohistochemical staining of F4/80. **Boxed areas** are shown at higher magnification in the **insets**. **B:** Double staining of F4/80 (red) and VEGF-C (green) in the sham-operated and 5- and 14-day UUO-induced kidneys. **C:** Bar graph shows the average number of F4/80- and VEGF-C⁺ cells counted per field under magnification. *n* = 3 (A–C, sham); *n* = 5 (A–C, 5 and 14 days UUO). **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Scale bars: 100 μm (A); 30 μm (B). Original magnification: ×400 (A and C). SH, sham operation; WT, wild-type.

along with increased HIF-1α and VEGF-C expression (Supplemental Figure S5). To confirm the involvement of mTOR signaling *in vivo*, we treated *Ccr2*^{-/-} and WT control mice with rapamycin during the course of UUO induction. As expected, rapamycin treatment rendered WT

mice with comparable level of lymphangiogenesis as that of *Ccr2*^{-/-} mice, and WT mice displayed similar expression levels for HIF-1α and VEGF-C as *Ccr2*^{-/-} mice on rapamycin treatment (Supplemental Figure S6). Altogether, these results confirmed that CCR2 mediated VEGF-C

Figure 4 Macrophages express vascular endothelial growth factor (VEGF)-C to generate a microenvironment in favor of lymphangiogenesis.

A: Results for immunohistochemical staining of F4/80, VEGF-C, and lymphatic vascular endothelial hyaluronan receptor 1 (LYVE-1) in serial sections of the sham-operated and 5- and 14-day unilateral ureteral obstruction (UUO)-induced kidneys. The VEGF-C⁺ F4/80 macrophages surround the LYVE-1⁺ LVs. **Arrows** indicate the representative areas. **Asterisks** indicate the location of renal arteries.

B: Clodronate liposomes effectively deplete the resident F4/80⁺ macrophages in the kidneys. **C:** Number of LYVE-1⁺ LVs counted per field in 5-day UUO-induced kidneys pretreated with either PBS liposomes or clodronate liposomes. **D:** Number of LYVE-1⁺ LVs counted per field in 5-day UUO-induced *Ccr2*^{-/-} and wild-type (WT) kidneys pretreated with clodronate liposomes.

E: Number of LYVE-1⁺ LVs counted per field in 5-day UUO-induced *Ccr2*^{-/-} and WT kidneys adoptively transferred with WT bone marrow-derived macrophages (BMDMs). **F** and **G:** Western blot analysis of total VEGF-C expression in 5-day UUO-induced *Ccr2*^{-/-} and WT kidneys pretreated with clodronate liposomes (**F**) or adoptively transferred with WT BMDMs (**G**). Representative Western blot analysis results and the corresponding quantitative results are shown by bar graphs. *n* = 3 (**A** and **B**); *n* = 4 (**C** and **D**); *n* = 8 (**E–G**). ***P* < 0.01, ****P* < 0.001. Scale bars: 100 μm (**A–E**). Original magnification: ×400 (**B–E**). Gapdh, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline.

expression in macrophages by regulating PI3Kp85/AKT/mTOR signaling and HIF-1α expression.

Discussion

Here, we first demonstrated experimental evidence indicating that mice deficient in *Ccr2* were protected from

UUO-induced renal fibrosis as evidenced by the ameliorated renal injury and attenuated expression of fibrogenic markers in the UUO-insulted kidneys. From this observation, we then checked renal lymphangiogenesis and found that *Ccr2* deficiency suppressed UUO-induced lymphangiogenesis as characterized by the decreased number of LVs and proliferating LECs. It was further noted that loss of *Ccr2* significantly reduced the expression of VEGF-C, an

Figure 5 Unilateral ureteral obstruction (UUO)-induced *Ccr2*^{-/-} kidneys show repressed phosphatidylinositol 3-kinase (PI3K)–AKT–mechanistic target of rapamycin (mTOR) signaling along with attenuated hypoxia-inducible factor (HIF)-1α expression. **A**, **C**, and **E**: Renal lysates were prepared from 5-day UUO-induced kidneys and subjected to comparative Western blot analysis of the following proteins in the PI3K–AKT–mTOR signaling pathway: pPI3Kp85 and PI3Kp85 (**A**); pAKT and AKT (**C**); and pmTOR and mTOR (**E**). **B**, **D**, and **F**: Western blot analysis of the following proteins in the PI3K–AKT–mTOR signaling pathway in the 14-day UUO-induced kidneys: pPI3Kp85 and PI3Kp85 (**B**), pAKT and AKT (**D**), pmTOR and mTOR (**F**). **G** and **H**: Western blot analysis of HIF-1α expression in 5- (**G**) and 14-day (**H**) UUO-induced kidneys. Representative Western blot analysis results and their corresponding quantitative results are shown in bar graphs. **I**: Electrophoretic mobility shift assay (EMSA) results for analysis of HIF-1α DNA binding activity in renal lysates from *Ccr2*^{-/-} and wild-type (WT) mice after day 5 of UUO induction. **J**: Results for co-immunostaining of F4/80 and HIF-1α in the 5-day UUO-induced kidneys. *n* = 3 (**A–H**, sham); *n* = 5 (**A–H**, 5 and 14 days of UUO); *n* = 3 (**I** and **J**). **P* < 0.05, ***P* < 0.01. Scale bar = 50 μm. Gapdh, glyceraldehyde-3-phosphate dehydrogenase; p, phosphorylated; SH, sham operation.

Figure 6 Hypoxia-inducible factor (HIF)-1 α is downstream of the phosphatidylinositol 3-kinase (PI3K)/AKT/mechanistic target of rapamycin (mTOR) signaling and transcribes vascular endothelial growth factor (VEGF)-C expression by binding to its promoter. **A:** Putative HIF-1 α binding sites within the VEGF-C promoter region. **B:** Chromatin immunoprecipitation (ChIP)-PCR results for HIF-1 α binding activity to the motif (GCGTG) located in the VEGF-C promoter at position -498 bp. **C:** Luciferase reporter activities in monocyte chemoattractant protein (MCP)-1-stimulated RAW264.7 cells transfected with wild-type (WT) or mutated VEGF-C promoter reporter plasmid. **D:** Loss of *Ccr2* attenuates MCP-1-induced activation of PI3K-AKT-mTOR signaling along with reduced HIF-1 α and VEGF-C expression. Representative Western blot analysis results for the phosphorylated and total PI3Kp85/AKT/mTOR, HIF-1 α , and VEGF-C after 1 hour of MCP-1 stimulation in *Ccr2*^{-/-} and WT bone marrow-derived macrophages (BMDMs) and the corresponding quantitative analysis in bar graphs. **E:** Results for treatment of WT BMDMs with Wort (a PI3K inhibitor), Peri (an AKT inhibitor), Rapa (an mTOR inhibitor), and 2MeOE2 (a HIF-1 α inhibitor) before MCP-1 stimulation. Representative Western blot analysis results for the phosphorylated and total PI3Kp85/AKT/mTOR, HIF-1 α , and VEGF-C expression with different stimulations and the corresponding quantitative results in bar graphs. Data are expressed as means $\pm$ SEM (standard one-way analysis of variance was used in comparisons between each group with the single MCP-1 stimulation group). * P < 0.05, ** P < 0.01, and *** P < 0.001. n = 3 or more independent replications for all *in vitro* studies. ATG, adenine, thymine, guanine, initiation codon; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; 2MeOE2, 2-methoxyestradiol; MU, promoter reporter; p, phosphorylated; Peri, perifosine; Rapa, rapamycin; UTR, untranslated region; Wort, wortmannin.

essential growth factor for lymphangiogenesis, in UVO-induced kidneys. Moreover, no perceptible difference was characterized in terms of VEGF-C expression in TECs between WT and *Ccr2*^{-/-} mice after UVO induction, indicating that the attenuated VEGF-C expression observed in *Ccr2*^{-/-} mice was independent of TECs.

To identify the cells responsible for the discrepancy of VEGF-C expression in the kidneys between UVO-induced WT and *Ccr2*^{-/-} mice, we examined with focus for the infiltrated inflammatory cells. Interestingly, *Ccr2*^{-/-} mice

displayed reduced macrophage infiltration along with lower VEGF-C expression in those infiltrated macrophages after UVO induction, suggesting that macrophages are probably responsible for the reduced VEGF-C expression along with attenuated lymphangiogenesis in UVO-induced kidneys of *Ccr2*^{-/-} mice. Indeed VEGF-C⁺ macrophages showed colocalization with LYVE-1⁺ LVs. More convincingly, WT and *Ccr2*^{-/-} mice displayed comparable lymphangiogenesis once macrophages were depleted by clodronate liposome or WT macrophages were adoptively transferred into

macrophage-depleted *Ccr2*^{-/-} kidneys. Indeed, the adoptive transferred macrophages were located to the surrounding area of LVs. Collectively, our data support that the infiltrated macrophages probably generate a microenvironment (eg, by producing copious amount of VEGF-C) to promote lymphangiogenesis after UUO induction.

There is feasible evidence that ligation of MCP-1/CCL2 to CCR2 could be linked to PI3K/AKT/mTOR signaling.^{30,31} We, therefore, first checked their activity by Western blot analysis. Indeed, loss of *Ccr2* resulted in a significant reduction for the phosphorylated PI3K/AKT/mTOR, and even total levels for PI3Kp85 and mTOR were lower compared with that of WT mice after UUO induction. Consistently, MCP-1 induced significantly higher phosphorylated levels of PI3K/AKT/mTOR in WT BMDMs, whereas it failed to stimulate a significant change in *Ccr2*^{-/-} BMDMs. Previous studies suggested that PI3K/AKT/mTOR signaling may modulate the expression of HIF-1 α ,^{32–34} a prominent and well-recognized transcription factor that drives target gene expression involved in angiogenesis,³⁴ glycolysis,³⁵ apoptosis,³⁶ tumor invasion, and metastasis.³⁷ We first noted that UUO induced a significant increase for HIF-1 α expression in the kidneys, whereas much lower levels of HIF-1 α expression were characterized in UUO-induced kidneys of *Ccr2*^{-/-} mice. EMSA analysis further confirmed that renal lysates derived from UUO-induced WT mice were characterized by the significantly higher HIF-1 α DNA binding activity than that of UUO-induced *Ccr2*^{-/-} mice. To confirm that HIF-1 α is downstream of PI3K/AKT/mTOR signaling, we treated WT BMDMs with wortmannin (a PI3K inhibitor), perifosine (an AKT inhibitor), and rapamycin (an mTOR inhibitor) in the presence of MCP-1 stimulation. All of those inhibitors significantly attenuated MCP-1–induced HIF-1 α expression, supporting that HIF-1 α is downstream of PI3K/AKT/mTOR signaling. Of note, two reported studies demonstrated that HIF-1 α expression in macrophages provides protection for mice against UUO-induced renal fibrosis.^{38,39} This conflicting finding suggests that CCR2 may regulate additional pathways other than HIF-1 α in the setting of UUO-induced renal fibrosis, and the phenotype we observed, in fact, resulted from an altered homeostasis of all pathways.

The most critical question is whether HIF-1 α transcribes VEGF-C expression in macrophages. HIF-1 has been known that it could bind to the core DNA sequence 5'-RCGTG-3' (R = A/G) and activate target gene transcription.^{40,41} Bioinformatic analysis of VEGF-C promoter characterized three potential HIF-1 α binding sites located at positions -1571, -716, and -498 bp (Start codon as +1). ChIP assay was then performed and interestingly demonstrated that HIF-1 α only selectively binds to the VEGF-C promoter at position -498 bp. To confirm this observation, promoter reporter assay was then used for analysis whether this particular HIF-1 α binding site affects VEGF-C promoter activity. Indeed, a 2.2-fold decrease in terms of VEGF-C promoter activity was noted once the binding site was mutated. Consistently,

stimulation of BMDMs with MCP-1 in the presence of 2-MeOE2, a HIF-1 α inhibitor, significantly suppressed MCP-1–induced VEGF-C expression in WT BMDMs. Together, these data suggest that HIF-1 α is a transcription factor responsible for VEGF-C expression in macrophages. Interestingly, transforming growth factor- β has also been found to stimulate macrophage expression of VEGF-C,¹⁵ but whether *Ccr2* deficiency also affects transforming growth factor- β –induced VEGF-C expression in macrophages is yet to be investigated.

To further demonstrate that HIF-1 α transcription of VEGF-C expression depends on CCR2-initiated PI3K/AKT/mTOR signaling, we checked VEGF-C expression in MCP-1–stimulated WT BMDMs in the presence of wortmannin (a PI3K inhibitor), perifosine (an AKT inhibitor), and rapamycin (an mTOR inhibitor), respectively. As expected, inhibition of PI3K/AKT/mTOR signaling significantly attenuated MCP-1–induced VEGF-C expression in WT BMDMs. In sharp contrast, treatment of *Ccr2*^{-/-} BMDMs with LB-100, an agonist for PI3K/AKT/mTOR signaling, induced high levels of HIF-1 α and VEGF-C expression. Taken together, these data suggest that HIF-1 α transcription of VEGF-C expression in macrophages requires CCL2 (MCP-1)/CCR2-initiated PI3K/AKT/mTOR signaling. Of note, recombinant MCP-1, which is the main ligand of CCR2, was used to stimulate WT and *Ccr2*^{-/-} BMDMs. Of course, WT BMDMs responded potently to MCP-1 stimulation by CCR2. However, a weak response sometimes was also detected in *Ccr2*^{-/-} BMDMs, which could be caused by the cross-reactions between MCP-1 and other chemokine receptors such as CCR4 expressed on *Ccr2*^{-/-} BMDMs.^{31,42}

Conclusions

We demonstrated experimental evidence indicating that macrophages play a critical role in UUO-induced lymphangiogenesis, whereas the expression of CCR2 on macrophages is required for UUO-induced lymphangiogenic processes. Our studies characterized a novel regulatory network in macrophages, in which CCR2 is required to activate PI3K-AKT-mTOR signaling, which then enhances its downstream gene HIF-1 α expression, and by which HIF-1 α directly binds to the VEGF-C promoter to transcribe its expression, thereby enhancing UUO-induced lymphangiogenesis.

Acknowledgments

Y.-C.G. is the major contributor for most of the experiments; M.Z. and F.-X.W. supervised animal model studies; F.S. provided help for bone marrow–derived macrophage studies; Y.Z. analyzed immunohistochemistry and immunofluorescence data; X.H. performed electrophoretic mobility shift assay experiments; Y.W. and J.S. contributed

to chromatin immunoprecipitation and reporter assays; F.-M.Z. jointly performed some of the experiments; J.L., P.Y., F.X., S.Z., and Q.Y. oversaw animal housing and breeding; G.C.P., Y.Y., and C.-Y.W. designed the experiments; K.H. revised the manuscript; Y.-C.G., N.S.P., and C.-Y.W. wrote the manuscript. C.-Y.W. takes full responsibility for the data and manuscript submission.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.04.007>.

References

- Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K: Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 2004, 5: 74–80
- Koltowska K, Lagendijk AK, Pichol-Thievend C, Fischer JC, Francois M, Ober EA, Yap AS, Hogan BM: Vegfc regulates bipotential precursor division and Prox1 expression to promote lymphatic identity in Zebrafish. *Cell Rep* 2015, 13:1828–1841
- Alitalo K: The lymphatic vasculature in disease. *Nat Med* 2011, 17: 1371–1380
- Dieterich LC, Detmar M: Tumor lymphangiogenesis and new drug development. *Adv Drug Deliv Rev* 2016, 99:148–160
- Henri O, Poueche C, Houssari M, Galas L, Nicol L, Edwards-Levy F, Henry JP, Dumesnil A, Boukhalfa I, Banquet S, Schapman D, Thuillez C, Richard V, Mulder P, Brakenhielm E: Selective stimulation of cardiac lymphangiogenesis reduces myocardial edema and fibrosis leading to improved cardiac function following myocardial infarction. *Circulation* 2016, 133:1484–1497; discussion 1497
- Cui Y, Liu K, Monzon-Medina ME, Padera RF, Wang H, George G, Toprak D, Abdelnour E, D'Agostino E, Goldberg HJ, Perrella MA, Forteza RM, Rosas IO, Visner G, El-Chemaly S: Therapeutic lymphangiogenesis ameliorates established acute lung allograft rejection. *J Clin Invest* 2015, 125:4255–4268
- Kerjaschki D, Regele HM, Moosberger I, Nagy-Bojarski K, Watschinger B, Soleiman A, Birner P, Krieger S, Hovorka A, Silberhumer G, Laakkonen P, Petrova T, Langer B, Raab I: Lymphatic neoangiogenesis in human kidney transplants is associated with immunologically active lymphocytic infiltrates. *J Am Soc Nephrol* 2004, 15:603–612
- Zheng W, Aspelund A, Alitalo K: Lymphangiogenic factors, mechanisms, and applications. *J Clin Invest* 2014, 124:878–887
- Ji H, Cao R, Yang Y, Zhang Y, Iwamoto H, Lim S, Nakamura M, Andersson P, Wang J, Sun Y, Dissing S, He X, Yang X, Cao Y: TNFR1 mediates TNF-alpha-induced tumour lymphangiogenesis and metastasis by modulating VEGF-C-VEGFR3 signalling. *Nat Commun* 2014, 5:4944
- Kamezis T, Shayan R, Caesar C, Roufai S, Harris NC, Ardipradja K, Zhang YF, Williams SP, Farnsworth RH, Chai MG, Rupasinghe TW, Tull DL, Baldwin ME, Sloan EK, Fox SB, Achen MG, Stacker SA: VEGF-D promotes tumor metastasis by regulating prostaglandins produced by the collecting lymphatic endothelium. *Cancer Cell* 2012, 21:181–195
- Maruyama K, Li M, Cursiefen C, Jackson DG, Keino H, Tomita M, Van Rooijen N, Takenaka H, D'Amore PA, Stein-Streilein J, Losordo DW, Streilein JW: Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest* 2005, 115:2363–2372
- El-Chemaly S, Malide D, Zudaire E, Ikeda Y, Weinberg BA, Pacheco-Rodriguez G, Rosas IO, Aparicio M, Ren P, MacDonald SD, Wu HP, Nathan SD, Cuttitta F, McCoy JP, Gochuico BR, Moss J: Abnormal lymphangiogenesis in idiopathic pulmonary fibrosis with insights into cellular and molecular mechanisms. *Proc Natl Acad Sci U S A* 2009, 106:3958–3963
- Kataru RP, Jung K, Jang C, Yang H, Schwendener RA, Baik JE, Han SH, Alitalo K, Koh GY: Critical role of CD11b+ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood* 2009, 113:5650–5659
- Sakamoto I, Ito Y, Mizuno M, Suzuki Y, Sawai A, Tanaka A, Maruyama S, Takei Y, Yuzawa Y, Matsuo S: Lymphatic vessels develop during tubulointerstitial fibrosis. *Kidney Int* 2009, 75:828–838
- Suzuki Y, Ito Y, Mizuno M, Kinashi H, Sawai A, Noda Y, Mizuno T, Shimizu H, Fujita Y, Matsui K, Maruyama S, Imai E, Matsuo S, Takei Y: Transforming growth factor-beta induces vascular endothelial growth factor-C expression leading to lymphangiogenesis in rat unilateral ureteral obstruction. *Kidney Int* 2012, 81:865–879
- Lee AS, Lee JE, Jung YJ, Kim DH, Kang KP, Lee S, Park SK, Lee SY, Kang MJ, Moon WS, Kim HJ, Jeong YB, Sung MJ, Kim W: Vascular endothelial growth factor-C and -D are involved in lymphangiogenesis in mouse unilateral ureteral obstruction. *Kidney Int* 2013, 83:50–62
- Naert G, Rivest S: A deficiency in CCR2+ monocytes: the hidden side of Alzheimer's disease. *J Mol Cell Biol* 2013, 5:284–293
- Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA: International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* 2000, 52:145–176
- Willenborg S, Lucas T, van Loo G, Knipper JA, Krieg T, Haase I, Brachvogel B, Hammerschmidt M, Nagy A, Ferrara N, Pasparakis M, Eming SA: CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. *Blood* 2012, 120:613–625
- Izhak L, Wildbaum G, Jung S, Stein A, Shaked Y, Karin N: Dissecting the autocrine and paracrine roles of the CCR2-CCL2 axis in tumor survival and angiogenesis. *PLoS One* 2012, 7:e28305
- Zhang M, Guo Y, Fu H, Hu S, Pan J, Wang Y, Cheng J, Song J, Yu Q, Zhang S, Xu JF, Pei G, Xiang X, Yang P, Wang CY: Chop deficiency prevents UUO-induced renal fibrosis by attenuating fibrotic signals originated from Hmgb1/TLR4/NFkappaB/IL-1beta signaling. *Cell Death Dis* 2015, 6:e1847
- Committee for the Update of the Guide for the Care and Use of Laboratory Animals; National Research Council: Guide for the Care and Use of Laboratory Animals: Eighth Edition. Washington, DC, National Academies Press, 2011
- Yang J, Zhu F, Wang X, Yao W, Wang M, Pei G, Hu Z, Guo Y, Zhao Z, Wang P, Mou J, Sun J, Zeng R, Xu G, Liao W, Yao Y: Continuous AMD3100 treatment worsens renal fibrosis through regulation of bone marrow derived pro-angiogenic cells homing and T-cell-related inflammation. *PLoS One* 2016, 11:e0149926
- Hu S, Zhang Y, Zhang M, Guo Y, Yang P, Zhang S, Simsekylmaz S, Xu JF, Li J, Xiang X, Yu Q, Wang CY: Alopine protects mice against ischemia reperfusion (IR)-induced renal injury by regulating PI3K/AKT/mTOR signaling and AP-1 activity. *Mol Med* 2015, 21: 912–923
- Yao Y, Wang Y, Zhang Z, He L, Zhu J, Zhang M, He X, Cheng Z, Ao Q, Cao Y, Yang P, Su Y, Zhao J, Zhang S, Yu Q, Ning Q, Xiang X, Xiong W, Wang CY, Xu Y: Chop deficiency protects mice against bleomycin-induced pulmonary fibrosis by attenuating M2 macrophage production. *Mol Ther* 2016, 24:915–925
- Yang J, Ning Y, Qiu J, He JS, Li W, Ma ZF, Shao JF, Li YQ, Zeng R, Zhang M, Cheng J, Chen SF, Xu G, Wang CY, Yao Y: TJ0711, a novel vasodilatory beta-blocker, protects SHR rats against hypertension induced renal injury. *Am J Transl Res* 2013, 5: 279–290
- Zou R, He Y, Li YQ, Han M, Ma ZF, Liu XC, Zeng R, Shao JF, Guo YC, He XY, Yang P, Xu G, Wang CY, Yao Y: Telmisartan protects 5/6 Nx rats against renal injury by enhancing nNOS-derived

- NO generation via regulation of PPARgamma signaling. *Am J Transl Res* 2014, 6:517–527
28. Cheng J, Song J, He X, Zhang M, Hu S, Zhang S, Yu Q, Yang P, Xiong F, Wang DW, Zhou J, Ning Q, Chen Z, Eizirik DL, Zhou Z, Zhao C, Wang CY: Loss of Mbd2 protects mice against high-fat diet-induced obesity and insulin resistance by regulating the homeostasis of energy storage and expenditure. *Diabetes* 2016, 65:3384–3395
 29. Lee HW, Qin YX, Kim YM, Park EY, Hwang JS, Huo GH, Yang CW, Kim WY, Kim J: Expression of lymphatic endothelium-specific hyaluronan receptor LYVE-1 in the developing mouse kidney. *Cell Tissue Res* 2011, 343:429–444
 30. Roca H, Varsos ZS, Mizutani K, Pienta KJ: CCL2, survivin and autophagy: new links with implications in human cancer. *Autophagy* 2008, 4:969–971
 31. Rivero M, Kortenkamp A, Silva E: Non-tumorigenic epithelial cells secrete MCP-1 and other cytokines that promote cell division in breast cancer cells by activating ERalpha via PI3K/Akt/mTOR signaling. *Int J Biochem Cell Biol* 2014, 53:281–294
 32. Bardos JJ, Ashcroft M: Negative and positive regulation of HIF-1: a complex network. *Biochim Biophys Acta* 2005, 1755:107–120
 33. Kim CH, Cho YS, Chun YS, Park JW, Kim MS: Early expression of myocardial HIF-1alpha in response to mechanical stresses: regulation by stretch-activated channels and the phosphatidylinositol 3-kinase signaling pathway. *Circ Res* 2002, 90:E25–E33
 34. Guru SK, Pathania AS, Kumar S, Ramesh D, Kumar M, Rana S, Kumar A, Malik F, Sharma PR, Chandan BK, Jaglan S, Sharma JP, Shah BA, Tasduq SA, Lattoo SK, Faruk A, Saxena AK, Vishwakarma RA, Bhushan S: Secalonic acid-D represses HIF1alpha/VEGF-mediated angiogenesis by regulating the Akt/mTOR/p70S6K signaling cascade. *Cancer Res* 2015, 75:2886–2896
 35. Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR, Chi H: HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med* 2011, 208:1367–1376
 36. Mecklenburgh KI, Walmsley SR, Cowburn AS, Wiesener M, Reed BJ, Upton PD, Deighton J, Greening AP, Chilvers ER: Involvement of a ferroprotein sensor in hypoxia-mediated inhibition of neutrophil apoptosis. *Blood* 2002, 100:3008–3016
 37. Wong CC, Gilkes DM, Zhang H, Chen J, Wei H, Chaturvedi P, Fraley SI, Wong CM, Khoo US, Ng IO, Wirtz D, Semenza GL: Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. *Proc Natl Acad Sci U S A* 2011, 108:16369–16374
 38. Kobayashi H, Gilbert V, Liu Q, Kapitsinou PP, Unger TL, Rha J, Rivella S, Schlondorff D, Haase VH: Myeloid cell-derived hypoxia-inducible factor attenuates inflammation in unilateral ureteral obstruction-induced kidney injury. *J Immunol* 2012, 188:5106–5115
 39. Tateishi Y, Osada-Oka M, Tanaka M, Shiota M, Izumi Y, Ishimura E, Motoyama K, Inaba M, Miura K: Myeloid HIF-1 attenuates the progression of renal fibrosis in murine obstructive nephropathy. *J Pharmacol Sci* 2015, 127:181–189
 40. Semenza GL: Hypoxia-inducible factor 1 and cardiovascular disease. *Annu Rev Physiol* 2014, 76:39–56
 41. Tan M, Gu Q, He H, Pamarthy D, Semenza GL, Sun Y: SAG/ROC2/RBX2 is a HIF-1 target gene that promotes HIF-1 alpha ubiquitination and degradation. *Oncogene* 2008, 27:1404–1411
 42. Ness TL, Ewing JL, Hogaboam CM, Kunkel SL: CCR4 is a key modulator of innate immune responses. *J Immunol* 2006, 177:7531–7539