Acute Kidney Injury Instigates Malignant Renal Cell Carcinoma via CXCR2 in Mice with Inactivated Trp53 and Pten in Proximal Tubular Kidney Epithelial Cells

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ABSTRACT

Renal cell carcinoma (RCC) is one of the most common urologic malignancies with the highest mortality rates worldwide. However, recent mouse models that recapitulated the genetic alterations found in RCC have been lacking. In this study, we crossed Trp53 and Pten conditional knockout mice with Gt1-Cre mice to generate a Gt1-Cre; Trp53fl/flPtenfl/fl (GPPY) mouse model, which resulted in the formation of dysplastic lesions involving kidney tubular epithelial cells (TEC), with only approximately 25% of mice developing RCC at an advanced age. Combining CRISPR/Cas9-mediated Vhl knockout in these mice increased the frequency of dysplasia, but failed to increase the incidence of RCC. Assessments of whether ischemic injury of TECs in the GPPY kidney without Vhl knockout influences the emergence of RCC revealed that advanced RCC predominantly emerged in the contralateral, noninjured kidney with 100% penetrance at a younger age, but rarely in the injured kidney due to severely damaged ischemic TEC. Injured TEC released CXCL1 into the microenvironment that traveled systemically to activate fibroblasts and recruit neutrophils to enable emergence of RCC in the contralateral kidney. Fibroblasts responded to CXCL1 via CXCR2 and recruited tumor-associated neutrophils, which in turn mediated tumor-promoting inflammation and angiogenesis. Treatment with anti-CXCR2 antibodies abolished the emergence of malignant RCC. Collectively, these results demonstrate a defining functional role of systemic inflammation and microenvironment in the emergence of malignant cancer from preestablished dysplastic precursor lesions.

Significance: These results identify a role for CXCL1/CXCR2 and the tumor microenvironment in the development of RCC.

Graphical Abstract: http://cancerres.aacrjournals.org/content/cancerres/81/10/2690/F1.large.jpg

See related commentary by Kusmartsev, p. 2584

Introduction

Renal cell carcinoma (RCC) accounts for 2%–3% of all malignancies, making it one of the top 10 most common cancers (1). Several histologic RCC subtypes are recognized including clear cell renal cell carcinomas (ccRCC, ~75%), papillary renal cell carcinomas (pRCC, ~10%), chromophobe renal cell carcinomas (chRCC, ~3%), unclassified RCC (uRCC, ~5%), and few extremely rare renal cancers (~5%; ref. 2). Large-scale sequencing studies have identified many frequently mutated tumor suppressor genes, including VHL, TP53, PTEN, BAP1, and MTOR, which implies that there may be many different combinations of genetic alterations cooperating with each other and give rise to the emergence of RCC (3).

VHL is an important tumor suppressor that is commonly inactivated in ccRCC, which encodes an E3 ubiquitin ligase that targets hypoxia-inducible factor α (including HIF1α and HIF2α) for proteasome degradation and acts as a major contributor in cellular adaptation to hypoxia (4, 5). It is well known that loss of VHL via promoter hypermethylation, somatic mutations or gene deletion could affect its ability to capture and ubiquitylate HIFα, and result in constitutive HIF stabilization and increased angiogenesis (6). TP53 and PTEN are recognized as the high penetrance susceptibility genes in many human malignancies, which are also mutated in a subset of renal cancers at high frequency, such as ccRCC, chRCC (7).

Numerous studies had demonstrated that inactivation of Vhl alone in mice is not sufficient for the development RCC, suggesting that the combination of other commonly mutated genes is essential for the emergence of RCC (8). It was also shown that specific codeletion of Vhl and Pten in kidney epithelial cells could give rise to simple and atypical cystic lesions, resembling the proposed precursor lesions of a subset of ccRCC (9). Combined mutation of Vhl and Trp53 in kidney were only able to induce cysts and neoplasms formation, which recapitulated some of the cellular and molecular changes of ccRCC (10). RCC is a heterogeneous disease comprising several distinct histological cell types with different genetics, biology and behavior (1). Different subtypes of RCC harbor unique biology with distinct cells of origin, and the origin has long been controversial (11). Accumulating evidence has indicated that ccRCC, the most frequent histologic subtype...
of RCC, mainly develops from proximal tubule epithelial cells (12). Harlander and colleagues demonstrated that the combination of Vhl, Trp53, and Rbl deletion throughout the nephron can result in ccRCC and shared some molecular markers and mRNA expression profiles with human ccRCC. (12). Although these tumors display expression of proximal tubule epithelial markers, it remains unknown if proximal tubule epithelial cells are the cell of origin in this context (12). This highlights an important limitation in the field: lack of genetically engineered mouse models reflective of the human disease to initiate molecular events that lead to advanced RCC and reproduce genetic events driving tumor progression. Therefore, there is still a need to develop informative mouse models, incorporating clinically relevant tumor microenvironment-derived instigators of malignant progression of RCC.

To mechanistically evaluate the role of tissue injury and inflammation in the progression of RCC, we employed mice with deletion of Pten and Trp53 in the proximal tubular epithelial cells that spontaneously develop precursor dysplastic lesions of RCC. These genetically altered mice do not progress beyond dysplasia in the kidney even after 1 year, and therefore an ideal model system to explore microenvironment drivers of malignant transformation. A recent study demonstrated that acute kidney injury (AKI) could drive tumorigenesis and accelerate the development of RCC, which was also a risk factor for RCC recurrence based on different human cohort studies (13). To further explore this mechanism, we subjected GPy mice to ischemic reperfusion injury (IRI) to evaluate whether the resulting regenerative/repair response can instigate the emergence of malignant RCC in the context of Pten and Trp53 deletion. IRI is one of the most frequent types of AKI caused by a temporary impairment of blood flow to kidney followed by subsequent restoration of blood supply (14). It is often associated with maladaptive repair, incomplete resolution, and presence of chronic inflammatory infiltrate within the kidney (15). Renal hypoxia serves as a key player in this pathophysiology. Hypoxia is associated with HIF stabilization, which in turn profoundly damages tubular epithelial cells, activates fibroblasts, and induces inflammatory responses (16). In addition, the recruitment of immune cells (e.g., macrophages and neutrophils) and the production of cytokines and chemokines also play a vital role in mediating inflammation-mediated malignant progression of preexisting neoplastic lesions (17). Here, we explored the functional role of systemic inflammation and tumor microenvironment in the regulation of malignant RCC emergence from preestablished dysplastic precursor lesions.

Materials and Methods

Animal studies

Trp53<sup>fl/fl</sup> mice, Pten<sup>LoxP/LoxP</sup> mice were kindly provided by Dr. Pier Paolo Pandolfi, Beth Israel Deaconess Medical Center, Harvard University (Boston, MA). Rosa26<sup>Cre<sub></sub></sup>LacZ<sup>Stop<sub></sub></sup>YFP mice were purchased from Jackson Laboratories. Ggt1-Cre mice were provided by Eric Neilson, Northwestern University (Chicago, IL), and described previously (18). The Ggt1 promoter is predominantly expressed in the kidney proximal tubules, which was undetectable expression in the liver and pancreas (14, 18). Trp53<sup>fl/fl</sup>Pten<sup>LoxP/LoxP</sup> and Pten<sup>LoxP/LoxP</sup> mice were crossed to Ggt1-Cre<sup>+</sup> mice and the resulting progeny were genotyped. The T7 endonuclease 1 mismatch detection assay was used for PCR validation. The cells were routinely screened and were free of Mycoplasma.

Cell culture and virus transduction

MCT mouse proximal tubular epithelial cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) solution (VWR45000-652) at 37 °C and 5% CO<sub>2</sub>. The cells were short tandem repeat (STR) validated and routinely tested for Mycoplasma. Cells were transfected with lentiviral backbone constructs (described above), a packaging vector (pCMV delta R, Addgene #12263), and an envelope vector (pCMV-YSV-G, Addgene #8454) using Lipofectamine 3000 (L3000015, Life Technologies) based on the manufacturer’s protocol. Supernatant was collected 48 and 72 hours after transfection, followed by centrifuging the media-containing cell culture supernatant at 300 × g for 5 minutes and filtering with a 45-μm filter. Finally, lentivirus was concentrated by PEG-itTM Precipitation (LV810A-1-SBI, System Biosciences) before resuspending in PBS and storing at −80°C. Repeated freeze-thaw cycles were avoided. Scintillation virus titration was determined by the Lenti-X<sup>®</sup>-QRT<sup>®</sup>-PCR Titration Kit (LV900, Applied Biological Materials Inc) according to manufacturer’s instructions.

The T7 endonuclease 1 mismatch detection assay

DNeasy Blood and Tissue Kit (QIAGEN 69506) was utilized for the isolation of genomic DNA isolation from LV/Cas9-Vhl-sgRNA1,2,3-MCT-stable cell line. DNA was quantified via NanoDrop spectrophotometer (Thermo Fisher Scientific). The Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific F530L) was used to amplify target regions during PCR cloning. Purified PCR homocomplex product (250 ng) was denatured under annealing conditions based on a previous study (22). The sequences for the amplification the targeted region are shown in Supplementary Table S2. Briefly, the PCR product was denatured at 95°C for 10 minutes and reannealed at −2°C/second temperature ramp to 85°C, followed by a −0.3°C/second until ramp to 25°C for duplex formation and subsequent heteroduplex formation. For T7 endonuclease 1 (T7E1) mismatch detection assay, T7 endonuclease (NEB #M0302) was used and incubated for 1 hour at 37°C based on the manufacturer’s protocol. TAE agarose gel (6%) was used for the
visualization of PCR products, and the DNA band intensities were quantified and by ImageJ (NIH, Bethesda, MD). Finally, the apparent gene modification percentage of the original cell pool was analyzed using the following equation: indel % = \( 100 \times \left( 1 - \left( \text{density from (i)}/\text{density from (ii)} \right)^{1/2} \right) \), where fcut is the fraction of total cleaved DNA. Fraction cleaved = (density from (i) + density from (ii))/(density density from (i) + density density from (ii) + heteroduplex density) (23).

**In vivo intraparenchymal injection of sgRNA virus**

Briefly, 10–14 weeks old mice were anesthetized with a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) before the left renal pedicle was opened. Three LV/Cas9–Vhl-sgRNA1,2,3 virus solutions were combined and 2.5 × 10^10-11 IU/μL in 20 μL was locally injected with into the renal cortex using a 30G needle.

**Histology**

Five-micron–thick sections of mouse kidney tissue were cut for hematoxylin and eosin (H&E). H&E-stained slides were imaged using Leica Aperio AT2 slide scanner or Leica DM 1000 LED microscope. Dysplasia and tumor were identified by histopathologic analysis and quantified as a percentage of total kidney area using Aperio Image Scope v12.3.2.8013 or ImageJ. The staining of Sirius Red and MTS were visualized with ImageJ (NIH, Bethesda, MD). Finally, the apparent cellular damage of kidney, it was counted by grid intersection method manually via Adobe Photoshop.

**IHC**

For kidney IHC staining, 5-μm formalin-fixed paraffin embedded sections were processed consistent with our previous report (24). For the staining of αSMA (Dako, M0851, 1:200), CXCL1 (Santa Cruz Biotechnology, 365870, 1:50), VHL (Santa Cruz Biotechnology, 135657, 1:50), HIF1α (Cayman Chemical Company, 10006421, 1:40), CD163 (Santa Cruz Biotechnology, 33560, 1:50), CD68 (Santa Cruz Biotechnology, 9139, 1:50), CK7 (Abcam, 9021, 1:200), PAX8 (Abcam 53490, 1:20), the M.O.M. Kit (Vector Laboratories, BMK-2202) was applied following the manufacturer’s protocol. For all other staining, sections were incubated with FSP-1 (Dako-A5114, 1:400), anti-type I collagen (SouthernBiotech, 131001, 1:100), Ly6G (Abcam, ab14935, 1:200), Ly6C (Abcam ab25377, 1:100), αSMA–CY3 (Sigma–Aldrich, C6198, 1:200), β-actin (Cell Signaling Technology 9805S, 1:200) over-night at 4°C. Tissue sections were incubated with Alexa Fluor 594 anti-rabbit (Invitrogen, 1:200), Alexa Fluor 647 anti-mouse (Jackson ImmunoResearch Laboratories, 1:200), and Alexa Fluor 647 anti-rat (Invitrogen, 1:200) before DAPI (Vectashield H1200) staining. Representative pictures were acquired with Zeiss Axioskop inverted microscope with Axiocam 506 monochrome camera or LSM800 confocal laser scanning microscope using ZEN software (Zeiss). Images were quantified by counting the number of positive cells from three random visual fields (200×). Representative images at ×200 and ×400 magnification are shown.

**Primary renal dysplastic cancer cell culture and analyses**

Fresh kidney/tumor tissue after IRI was harvested and minced into 0.3–0.5 mm pieces with sterile razor blades prior to the digestion with dispase II (17105041, Gibco, 4 mg/mL) and collagenase IV (17104019, Gibco, 4 mg/mL) in RPMI medium at 37°C for 1 hour, followed by filtration via cell strainer (70 μm) and resuspension in RPMI with 10% FBS medium (25). Primary kidney cells were seeded on Corning BioCoat Cellware, Collagen Type (VWR 62405–636) and maintained in complete RPMI medium supplied with 10% FBS, 1% penicillin–streptomycin–amphotericin B (PSA) antibiotic-antimycotic solution. Dysplastic cancer cells were further isolated by FACs using BD FACsAria II sorter (South Campus Flow Cytometry Core Lab of MD Anderson Cancer Center) based on EpCAM expression (Thermo Fisher Scientific 64–5791–82, 1:100). EpCAM-negative population were collected and denoted as fibroblasts. Fresh kidney tissue without IRI was harvested and processed in the same way. Dysplastic kidney cells without IRI were further isolated by FACs using BD FACsAria II sorter based on YFP expression. The YFP-negative population was collected and denoted as fibroblasts. The sorted cells were subsequently expanded in vitro. For the validation that primary kidney cells were depleted of immune cells, unsorted kidney cells were collected and stained with Pacific Blue anti-mouse CD45 (BioLegend 103126, 1:100) and fixable viability dye eFluor 780 (eBioscience 6–0865–14, 1:1000) in the dark at 4°C for 30 minutes. Mouse splenocytes were used as a positive control. Samples were analyzed using the LSR Fortessa X-20. Testing for Mycoplasma and STR authentication was not performed.

**Quantitative real-time PCR analyses**

RNA was extracted using TRizol reagent (Life Technologies 15596026). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814) after DNase treatment (Promega 9919M610). Primer sequences of relevant target genes are listed in Supplementary Table S3 (F, forward; R, reverse primer). Gapdh was used as an internal control. The relative expression level is presented as fold change 2^ΔΔCt. Statistical comparisons were performed on the basis of ΔΔCt, values in each group.

**Western blot analysis**

Cell lysates from LV/Cas9–Vhl-sgRNA1,2,3–MCT–stable cell line were obtained and resolved on 4%–12% Bis-Tris protein gels (Thermo Fisher Scientific NP0321PK2) as described previously (24). The membrane was blocked with 5% nonfat milk (LabScientific M0841) and incubated with primary antibody; VHL (Santa Cruz Biotechnology, sc-135657, 1:200), β-actin (Cell Signaling Technology 37005S, 1:1,000) overnight at 4°C before the incubation with mouse IgG horseradish peroxidase–conjugated secondary antibody (R&D Systems HAF607, 1:2,000) at room temperature for 1 hour. West-Q Pico ECL Solution (GenDEPOT W3652020) and Amer sham Hyperfilm ECL (GE Healthcare 28906835) were used for
protein detection, and band density was quantified using ImageJ (NIH, Bethesda, MD).

**Cell viability assay**

Fibroblasts (4,000 cells/well) and dysplastic cells (5,000 cells/well) were seeded in a 96-well plate overnight. Cells were treated with different concentration of anti-mouse CXCR2 antibodies (R&D Systems, MAB2164–500) for 24 and 48 hours before incubation with MTT reagent (Abcam ab211091) according to manufacturer’s instructions.

**Ischemic reperfusion injury and CXCR2 antibody treatment**

IRI were performed as described previously (14). Briefly, 24–28 weeks old mice were anesthetized with a mixture of ketamine and xylazine before the left renal pedicle was clamped for 30 minutes. Mice were treated with anti-mouse CXCR2 (R&D Systems, MAB2164–500, 30 μg/mouse) and corresponding IgG2A isotype control (R&D Systems, MAB006, 30 μg/mouse). Antibody administration was started 24 hours after recovery from the surgery by intraperitoneal (i.p.) injection twice per month as described previously (26). All the mice were euthanized after treatment for 20 weeks.

**Profiling of cytokine levels in plasma**

Cytokines secreted from plasma after IRI surgery in WT and GPPY mice were screened using the Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems, ARY006) based on the manufacturer’s instructions. In brief, 400 μL plasma was loaded on to the membranes before incubation with primary biotin-conjugated antibody at 4°C overnight and then incubated with Streptavidin-HRP at room temperature for 1 hour. West-Q Pico ECL Solution (Fisher Scientific W3652020) and Amersham Hyperfilm ECL (GE Healthcare 28906835) were used for protein spots detection. Relative spot density was quantified using ImageJ and compared between each group. The relative fold change was determined by the average value of reference spots in three corners of each array. CXCL1 protein levels in plasma samples from WT and GPPY mice with or without IRI surgery were quantified by ELISA (R&D Systems, MCK008) according to the manufacturer’s protocol.

**Kaplan–Meier survival analyses**

The TCGA data of ccRCC (533 patients), pRCC (290 patients), and chRCC (66 patients) were downloaded from UCSC Xena dataset containing survival data, clinical information, and mRNA expression (https://xenabrowser.net/datapages/?hub=https://tcga.xenahubs.net:443). For each cancer type, we classified the tumor samples into two groups based on the median mRNA expression values of CXCL1. The overall survival time was fit to the univariate Cox proportional hazards model using the R “survival” package (version 3.1–11). Kaplan–Meier survival plots were generated by different cancer types. A log-rank test calculated by the “survdiff” function from the R “survival” package was used to assess the significance ($P < 0.05$).

**Statistical analysis**

Statistical analysis was performed on the means of biological replicates in the indicated groups (GraphPad Software). For comparisons between two groups, unpaired two-tailed Student t test was performed, and Welch correction for unequal variances was applied (determined by an F test). An ordinary one-way ANOVA test with Tukey post hoc analysis was used for the comparison among multiple groups with normal distribution. Kruskal–Wallis test with Dunn post analysis was used when the data were not normally distributed, as stipulated in the figure legends ($* P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ****, $P < 0.0001$; n.s., not significant; n.d., not detected).

**Results**

**Deletion of Trp53 and Pten in kidney results in dysplasia**

To investigate the consequences of combined deletion of Trp53 and Pten in kidney tissues in vivo, we crossed Trp53$^{Lens\text{LoxP}/LoxP}$ and Pten$^{Lens\text{LoxP}/LoxP}$ mice with gamma-glutamine transferase (Ggt1-Cre) transgenic mice to generate Ggt1-Cre; Trp53$^{Lens\text{LoxP}/LoxP}$, Pten$^{Lens\text{LoxP}/LoxP}$ and Trp53$^{Lens\text{LoxP}/LoxP}$ and Pten$^{Lens\text{LoxP}/LoxP}$ (herein referred to as GPPY) mice, which allows for deletion of these genes in proximal kidney tubular epithelial cells (Fig. 1A). GPPY mice were fertile and produced viable offspring at a normal frequency. Cohorts of GPPY mice were analyzed between 8 to 20 weeks ($n = 3$), 24 to 40 weeks ($n = 11$), and 44 to 72 weeks of age ($n = 13$). The size of kidneys in GPPY mice were significantly larger ($P < 0.0001$) compared with control group (Fig. 1B and C). Only 7 of 27 GPPY mice developed RCC tumors at advanced age (48–72 weeks, Fig. 1D). H&E staining of the kidneys of control littermate mice showed that tubules within the cortex and medulla were histologically normal at all ages examined (Fig. 1E). However, the GPPY mice started to display occasional areas of tubular epithelium disorganization after 24 weeks, and ultimately resulted in the emergence of kidney dysplasia (Fig. 1E). The percentage of dysplastic region increased with time (Fig. 1E and F).

**Deletion of Trp53 and Pten in the kidney gives rise to fibrosis**

No overt changes in lung, intestine, liver, and pancreas were observed from histopathologic analyses, despite some reported expression of GGT in those organs (Supplementary Fig. S1A). H&E, Sirius red staining, and Masson Trichrome staining (MTS) analyses of kidneys revealed presence of fibrosis in GPPY mice (Fig. 1G and H). In addition, accumulation of type I collagen and abundance of αSMA+ myofibroblasts were observed in the GPPY mice (Fig. 1G and H), suggesting deletion of Trp53 and Pten gives rise to fibrosis.

**Vhl knockdown in GPPY mice increases the incidence of dysplasia, but is not sufficient to accelerate the emergence of malignant RCC**

We found that GPPY mice developed dysplasia, a precursor to RCC. Because loss of Pten and Trp53 did not result in RCC beyond just the dysplastic lesions in the kidney, we wondered whether priming the dysplastic epithelial cells with a loss of Vhl might increase HIF1α that stimulates pronoecrogenic microenvironmental cues. Therefore, we employed CRISPR/Cas9-mediated loss of Vhl in the GPPY mice. Three gRNAs sequences targeting on murine Vhl were selected and cloned into lentiviral vector (LV) (Supplementary Fig. S2A and S2B). To validate the designed sgRNAs efficiency, mouse kidney epithelial MCT cells were transduced with LV/Cas9-Vhl-sgRNAs and selected for positive cells. After establishment of stable MCT cells with LV/Cas9-Vhl-sgRNAs, genomic DNA were harvested and T7E1 assay was performed for determining indel formation. Indels were identified in samples transduced with LV/Cas9-Vhl-sgRNAs with 44%, 36%, and 28% efficiency, respectively (Supplementary Fig. S2C). To further confirm the effectiveness of Vhl knockdown in stable MCT cells with LV/Cas9-Vhl-sgRNAs, mRNA and protein levels were assessed. Both qPCR and Western blot analysis revealed a significant reduction of the Vhl mRNA and protein expression compared with MCT cells with LV/Cas9-Control sgRNAs (Supplementary Fig. S2D–S2G). Hence, in vitro studies revealed that three sgRNAs delivered by LV/Cas9 vectors were able to induce indel formation efficiently, which directly decreased Vhl mRNA and protein translation.

Next, we delivered lentivirus containing all three sgRNAs to the GPPY mice between 10–14 weeks of age. LV harboring Vhl sgRNA1,
sgRNA2, sgRNA3 mixture solution were directly injected into the left renal parenchyma as reported previously (27). At 16 weeks postinfection, we assessed the effects of CRISPR/Cas9-mediated Vhl gene deletion on tumor progression. No overt changes in lung, intestine, liver, and pancreas were observed (Supplementary Fig. S3A). All the mice infected with LV/Cas9-Vhl-sgRNAs did not give rise to RCC (26–30 weeks), but increased the baseline dysplasia (Fig. 2A and B), to a level similar to what was observed at 40–44 weeks in the GPPY mice (Fig. 1F). Loss of Vhl in GPPY mice was also associated with immune infiltrate and formation of cysts (Supplementary Fig. S3B). No difference in kidney weight was observed (Fig. 2C). As expected, VHL expression was observed in the control sgRNA–treated GPPY mice. We found decreased VHL expression in GPPY kidneys with infected with LV/Cas9-Vhl-sgRNAs (Fig. 2D and E). In addition, a concomitant increase in HIF1α levels, the downstream biomarker for the loss of Vhl, was observed (Supplementary Fig. S3C and S3D).

Ischemic reperfusion injury leads to tumor formation

Next, we wondered whether prominent kidney tubular epithelium injury via ischemic damage would prime the microenvironment of the dysplastic lesions to evolve into malignant RCC. AKI is a consequence of ischemia, which has been implicated as an independent risk factor for the development of chronic kidney disease and cancer (13). In this study, GPPY mice at 24–28 weeks were subjected to IRI to induce damage to renal tubular parenchyma. After 20–32 weeks of inducing renal damage to GPPY mice, the kidneys and other organs were examined. The contralateral kidney (nonischemic injured kidney) exhibited increased size compared with the ischemic injured kidney, indicating severe parenchymal damage caused by IRI.
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(Supplementary Fig. S4A and S4B). Less Ki67+ cells were found in the injured kidney compared with contralateral kidney (Supplementary Fig. S4C and S4D), indicating reduced proliferative capacity. In addition, increased fibrosis (evaluated by Sirius red and MTS staining) was noted in GPPY mice, with significant increases (P < 0.001) observed in the contralateral kidneys (Fig. 3F–I). However, no overt changes in lung, intestine, liver, and pancreas were found in GPPY mice with IRI (Supplementary Fig. S4E). To further characterize the molecular subtype of RCC in GPPY mice, we stained for a series of RCC markers. Kidneys with dysplasia were positive for CAIX, CD10, PAX8, and E-cadherin, but negative for CK7, CD117, and vimentin. Tumor tissues were positive for CAIX, CD10, PAX8, and vimentin, but negative for CK7, CD117, E-cadherin (Supplementary Fig. S4F), in agreement with established markers of clear cell RCC (28). IHC analyses revealed more type I collagen deposition (Fig. 3J and K), αSMA+ myofibroblasts activation (Fig. 3L and M), as well increased accumulation of fibrolast specific protein 1 (FSP-1+) positive cells (Fig. 3N and O) and more CD31+ endothelial cells (Fig. 3P and Q). Together, these data suggest a sustained fibrotic response occurs in both injured and contralateral kidneys of GPPY mice.

CXCL1/CXCR2 axis engages stromal fibroblasts associated with the recruitment of protumorigenic neutrophils

Next, we investigated cytokines/chemokines secreted from the ischemic injured kidney into circulation, leading to the emergence of RCC in the contralateral kidney of the GPPY mice. As shown in Fig. 4A, we identified several cytokines and chemokines via cytokine array that were elevated in the circulation of the GPPY mice compared with the WT mice with IRI. We observed that CXCL1, CXCL13, TIMP1, and IL1Rα were upregulated, with CXCL1 having the highest degree of upregulation (Fig. 4B). Quantitative ELISA further confirmed the increased level of CXCL1 in the plasma of GPPY mice with IRI, compared with GPPY and WT mice (Fig. 4C).

CXCL1 is a CXCL chemokine family member, which was reported to induce angiogenesis and promote tumor progression via the activation of CXCR2 signaling (29). We hypothesized that the CXCL1/CXCR2 axis might be activated as an important determinant for the emergence of RCC upon IRI in GPPY mice. IHC staining revealed that CXCL1 and CXCR2 were increased in IRI kidneys of GPPY mice (Fig. 4D and E). CXCL1 was predominantly associated with dysplastic epithelial cells in the ischemic injured kidney but not the dysplastic cells from
Ischemic reperfusion injury leads to tumor formation. A, The size and external morphology of injured and contralateral kidney from GPPY mice. B, Quantification of kidney weight to body weight between two groups. WT, *n* = 8; GPPY, *n* = 8. C, Representative IRI and contralateral histological sections of kidney from WT and GPPY mice after IRI injury. Examples of dysplasia (D) and tumor (T) are circled. D and E, Quantification of dysplasia region (D) and tumor region (T) as a percentage of total kidney area. Data are represented as mean ± SEM. IRI, injured ischemic kidney with IRI surgery. Contralateral, contralateral kidney without IRI injury. WT, *n* = 8; GPPY, *n* = 8. F–P, Representative images of Sirius Red (F), Masson's trichrome staining (MTS; H), and IHC staining for collagen I (J), cSMA (L), FSP-1 (N), and CD31 (P). G–Q, Quantification of positive area/visual field (200×). Data are represented as mean ± SEM. WT, *n* = 5; GPPY, *n* = 5. For the statistical analysis of B, an unpaired one-tailed *t* test with Welch correction was used. The significance of *D* and *E* was determined by Kruskal-Wallis test with Dunn post hoc analysis. For *G*, *I*, *K*, *M*, *O*, and *Q*, one-way ANOVA with Tukey post hoc analysis was used. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. Scale bar, 100 μm; inset scale, 40 μm.
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Figure 4.
CXCL1/CXCR2 activation in epithelial and stromal cells after kidney injury. A, Analysis of cytokines present in the plasma of IRI mice. Plasma samples from each group after IRI were screened by cytokine array. The cytokines profiled are indicated. B, Heatmap of relative cytokine levels. Data are represented as mean ± SEM. WT, n = 2; GPPY, n = 2. C, Quantification of CXCL1 levels in plasma by ELISA from the indicated groups. Data are represented as mean ± SEM. WT, n = 4; GPPY, n = 4. D and E, Representative images of IHC staining for CXCL1 (D) and CXCR2 (E) from the indicated experimental groups. IRI, injured ischemic kidney with IRI surgery. Contralateral, contralateral kidney without IRI injury. F, Quantification of CXCL1-positive area/visual field (200×). Data are represented as mean ± SEM. WT, n = 5; GPPY, n = 8. G, Quantification of CXCR2-positive area/visual field (200×). Data are represented as mean ± SEM. WT, n = 5; GPPY, n = 5. H, Relative Cxcl1 mRNA levels in dysplastic cells derived from injured kidney and contralateral kidney were determined by qPCR. Expression value is normalized to dysplastic IRI cells and is presented as 2⁻¹⁴Dt. Data are presented as mean ± SEM, n = 3. J and K, Proliferation of dysplastic cells purified from injured kidney (J) and contralateral kidney (K) was treated with different concentrations of CXCR2 antibody (12.5, 25, 50 µg/mL) analyzed by MTT assay at specified timepoints. Data are normalized to untreated cells and is represented as mean ± SEM, n = 3. For the statistical analysis of C, one-way ANOVA with Dunnett T3 post hoc test was used. The significance of F, G, J, and K was determined by one-way ANOVA with Tukey post hoc analysis. For H and I, unpaired two-tailed t test with Welch correction was used. *, P < 0.05; **, P < 0.01; ***, P < 0.001; n.s., not significant. Scale bar, 100 µm; inset scale, 40 µm.
contralateral kidney (Fig. 4D and F). In contrast, CXCL1 was found in stromal fibroblasts in the contralateral kidney in the GPPY mice with IRI (Fig. 4D). In addition, CXCL1 expression was minimal in the kidneys of the GPPY mice without IRI and WT mice (Supplementary Fig. S4G and S4H). These results suggested that CXCL1 was systemically secreted from ischemic injured tubular epithelial cells, which may interact with CXCR2 expressed on fibroblasts in the contralateral kidney after IRI.

Next, IHC staining for CXCR2 (the receptor for CXCL1) demonstrated that CXCR2 was mainly identified on stromal fibroblasts in GPPY mice but not WT mice (Fig. 4E and G). To further address the interaction between dysplastic tubular epithelial cell–derived CXCL1 and adjacent fibroblasts, we isolated dysplastic tubular epithelial cells and EpCAM–negative cells enriched for fibroblasts from both ischemic and contralateral kidneys based on EpCAM expression (Supplementary Fig. S5A and S5B). Although the morphology of EpCAM–negative cells is spindle-shaped characteristic of fibroblasts, it is possible that EpCAM–negative cells could be a mixture of fibroblasts and EpCAM–negative tumor cells that have undergone epithelial-to-mesenchymal transition (EMT). Recombination PCR was performed with GPPY kidney isolated cells to confirm the deletion of Trp53 and Pten in dysplastic cells (Supplementary Fig. S5C). To validate that epithelial cells and fibroblasts were isolated, flow cytometry analysis for CD45 was performed. CD45− cells were not detected in primary kidney cells, indicating lack of immune cell contamination (Supplementary Fig. S5D). qPCR analysis was performed to determine the source of CXCL1 and CXCR2 in these cells. Our results show that the Cxcl1 mRNA expression level was significantly higher in the dysplastic cells derived from injured kidneys when compared with dysplastic cells derived from contralateral kidneys (Fig. 4H). In contrast, Cxcr2 mRNA was lower in dysplastic cells when compared with fibroblasts isolated from the contralateral kidney (Fig. 4I). In addition, Cxcr2 mRNA was also minimal in fibroblasts isolated from the kidney without IRI (Supplementary Fig. S5E and S5F). To further rule out potential autocrine influence of CXCL1 on the proliferation of dysplastic cells, both fibroblasts and dysplastic cells were treated with different concentrations of the CXCR2 neutralizing antibodies, but inhibition of cell viability was not observed (Fig. 4J and K).

**CXCR2-dependent accumulation of fibroblasts and neutrophils contribute to the emergence of RCC**

CXCL1 mediates neutrophil recruitment by binding to and activating CXCR2, and this process has been implicated in various inflammatory diseases and cancer (26, 30–32). We observed significantly higher number of Ly6G− (biomarker for neutrophils) in contralateral kidney of GPPY mice compared with ischemic injured kidney ($P < 0.001$, Fig. 5A and B). CXCR2 is expressed on macrophages and contributes to the initiation and disease progression after AKI (33). CD68 and CD163 are used to identify pan-macrophage and M2 macrophage populations, respectively, and increased accumulation of macrophages is associated with poor prognosis in RCC (34). We found both CD68− and CD163− macrophages were recruited into both kidneys of the GPPY mice with IRI at equal levels (Supplementary Fig. S6A–S6D). Tumor-associated neutrophils (TAN) were reported to suppress tumor-infiltrating T lymphocytes in the tumor microenvironment (35). Reduced CD3+ T cells were also observed at the same level in both the kidneys of the GPPY mice with IRI (Fig. 5C and D).

IHC staining and immunofluorescence revealed that CXCR2 predominantly expressed on FSP1− cells (Fig. 5E and F) and Ly6G− neutrophils (Fig. 5G and H). However, CXCR2 was not expressed in αSMA− myofibroblasts or vimentin+ cells (Supplementary Fig. S6E–S6H). These results support previous findings that show that CXCR2 is involved in the recruitment of neutrophils and FSP1− stromal cells (29, 36). In addition, MAPK/ERK signaling is involved in the regulation of CXCL1/CXCR2 pathways (37). CXCL1 was shown to locally recruit TANs via binding to the chemokine receptor CXCR2 (32). In this study, higher expression of phospho-p44/42 MAPK (ERK1/2) was observed in the contralateral kidney compared with injured kidney (Supplementary Fig. S6I and S6J), which may act to stimulate TAN recruitment.

**CXCR2 blockade inhibits the emergence of RCC from dysplastic lesions**

To determine the functional role of CXCR2+ neutrophils and FSP1− stromal cells in the emergence of RCC in the GPPY mice with IRI, we treated with an anti-CXCR2 neutralizing antibody. Interestingly, tumors were not detected in the GPPY mice with IRI after anti-CXCR2 treatment but dysplasia was still present, as observed in the isotype antibody–treated GPPY mice after treatment for 20 weeks (Fig. 6A–C). While anti-CXCR2 treatment reduced tumors in IRI-treated mice, it had no impact on dysplasia (Fig. 6A and C). Histologic examination of lung, intestine, liver showed no overt histologic changes (Supplementary Fig. S7A). IHC staining further revealed that CXCR2 inhibition significantly reduced fibrosis (Supplementary Fig. S7B–S7E), decreased the number of FSP1− cells (Fig. 6D and E), and inhibited angiogenesis (Supplementary Fig. S7F and S7G). The infiltration of TANs was also impaired by CXCR2 blockade (Fig. 6F and G), and more CD3+ (Fig. 6H and I) and CD8+ T cells (Supplementary Fig. S7H and S7I) were observed in the ischemic injured and the contralateral kidneys after anti-CXCR2 treatment when compared with the isotype treatment group.

Additionally, in order to correlate our findings with the human cancer data related to the CXCL1/CXCR2 signaling axis, we mined the TCGA dataset to develop Kaplan-Meier plots (3). Survival analysis showed that high CXCL1 levels were associated with poor overall survival of 533 ccRCC patients (Fig. 6J), but no difference in 290 pRCC (Fig. 6K) and 66 chRCC patients (Fig. 6L). However, the role of CXCL1/CXCR2 axis in cancer initiation needs to be further elucidated.

**Discussion**

We show that the conditional combined deletion of Trp53 together with Pten in proximal renal tubular cells in GPPY mice results in the formation of dysplastic lesions without progression to RCC except in rare cases. Combination of CRISPR/Cas9–mediated Vhl knockdown in the GPPY mice contributed to more dysplastic lesions but again failed to generate RCC, despite increased HIF1α accumulation and nuclear translocation. It appears likely that the additional inactivation of Vhl acts on Trp53− and Pten−mutant cells to enhance proliferation and generate more precursor lesions in kidney. It is possible that Trp53 and Pten genetic alterations act cooperatively and further sensitize Vhl-deficient renal tubular epithelial cells to aberrant cell growth.

Previous studies have also shown that the cooperation between loss of Vhl with the deletion of other tumor suppressor genes such as Pten (9) Trp53 (10), Bap1 (38), Notch1 (39), Kif3a (40) results only in uncontrolled renal epithelial proliferation, loss of primary cilia, kidney cyst formation and early neoplastic events, but without aggressive tumor growth. In this study, we also observed the cyst formation inside...
of the dysplasia region in GPPY mice when treated with Vhl-sgRNA. In this regard, it was demonstrated that inactivation of the Vhl is associated and leading to the development of both hereditary and sporadic renal cysts (41). Combined renal epithelium-specific deletion of Vhl, Trp53 and Rb1 under inducible renal epithelial cell–specific (Ksp1.3-CreERT2) was able to generate ccRCC (12). An RCC model using Pax8-Cre, which encodes a transcription factor expressed in the mesonephros, metanephros, nephric duct, and ureteric bud, to delete Vhl together with either Bap1 or Pbrm1 resulted in tumor growth with different grades (1). But in our study, the deletion of Pten, Trp53, and

Figure 5.
CXCR2-dependent accumulation of neutrophils and fibroblasts promotes tumor progression. A, Representative images of Ly6G distribution in the indicated groups. B, Quantification of Ly6G-positive cells/visual field (200 ×). Data are represented as mean ± SEM. n = 4 per group. IRI, injured ischemic kidney with IRI surgery. Contralateral, contralateral kidney without IRI injury. C, Representative images of CD3 in the indicated groups. D, Quantification of CD3-positive area/visual field (200 ×). Data are represented as mean ± SEM. WT, n = 5; GPPY, n = 5. E and G, Representative images of coimmunolabeling for CXCR2 (green) with FSP-1 (red; E) and CXCR2 (red) with Ly6G (green; G). F and H, Quantification of CXCR2 and FSP-1 double positive cells/visual field (F) and CXCR2 and Ly6G double-positive cells/visual field (200 ×; H). Data are represented as mean ± SEM. WT, n = 5; GPPY, n = 5. The significance of D was determined by one-way ANOVA with Tukey post hoc analysis. For B, F, and H, unpaired two-tailed t test was used. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Scale bar, 100 μm; inset scale, 40 μm.
CXCR2 blockade prevents tumor formation. A, Representative IRI and contralateral histologic sections of kidney from WT and GPPY mice after IRI injury with isotype and anti-CXCR2 antibody treatment. Examples of dysplasia (D) and tumor (T) are circled. IRI, injured kidney with IRI surgery. Contralateral, contralateral kidney without IRI injury. B and C, Quantification of relative tumor (B) and dysplasia (C) region as a percentage of total kidney area. Data are represented as mean ± SEM. CXCR2 isotype treatment group: WT, n = 5; GPPY, n = 5. Anti-CXCR2 antibody treatment group: WT, n = 5; GPPY, n = 5. D, F, and H, Representative images of IHC staining for FSP-1 (D), Ly6G (F), and CD3 staining (H). E, Quantification of FSP-1-positive area/visual field (200x). Data are represented as mean ± SEM. WT, n = 5; GPPY, n = 5. G, Quantification of Ly6G-positive cells/visual field (200x). Data are represented as mean ± SEM. WT, n = 4; GPPY, n = 4. I, CD3-positive area/visual field (200x). Data are represented as mean ± SEM. WT, n = 5; GPPY, n = 5. J–L, Kaplan-Meier survival curves of CXCL1 in ccRCC (530 patients; J), pRCC (290 patients; K), and chRCC (66 patients; L). The significance of B and C was determined by Kruskal–Wallis test with Dunn post hoc analysis. For E, G, and I, one-way ANOVA with Tukey post hoc analysis was used. *, P < 0.05; **, P < 0.01; ***, P < 0.001. n.s, nonsignificant. Scale bar, 100 μm; inset scale, 40 μm.
Vhl in Ggt1-expressing proximal kidney tubular cells did not result in malignant RCC.

We tested whether ischemic epithelial injury could potentially prime the emergence of RCC in the GPPY mice. In this regard, AKI is a frequent and serious health issue with increasingly common complications in hospitalized patients (42). Patients with AKI are more likely to have reduced life expectancy that was result from multiple comorbidities, such as diabetes mellitus, cardiovascular disease, chronic liver disease, and cancers, in particular RCC (43). A previous study from a large Danish population-based cohort revealed that the 1-year incidence was 17.5% while the 5-year risk was 27% of AKI in patients with cancer (44). Among all the patients with cancer, almost 75% were more than 60 years old, and relative younger people had less comorbidity (44). A recent study demonstrated that AKI could drive tumorigenesis and accelerate the development of pRCC in a Notch 1-dependent manner, and decreased levels of PTEN, β-catenin, VHL, mTOR, SMAD4, and increased NOTCH1 expression were related to the late stage of pRCC among 161 patients with pRCC (13).

There are some key differences between our study and the one mentioned above (13). In our study, specific deletion in the Tpr53 and Pten in proximal tubular epithelial cells with ischemic injury resulted in more RCC in the nonischemic contralateral kidney mediated by CXCL1/CXCR2 signaling axis but not injured kidney. This suggests that injury to the epithelial cells with deletion of Tpr53 and Pten may generate oncogenically unfit epithelial cells that are too damaged to generate malignant RCC, as we saw lot of necrosis in injured kidney. Moreover, less cell proliferation was observed in the damaged to generate malignant RCC, as we saw lot of necrosis in injured kidney. Taken together, our findings suggest that individuals with TP53 and PTEN deletion in proximal tubular kidney epithelial cells may develop dysplasia and opportunistic AKI in this setting could lead to malignant RCC, and blocking CXCL1/CXCR2 signaling axis could be viable strategy for the control of RCC.

Authors’ Disclosures

No disclosures were reported.

Authors’ Contributions

X. Zhou: Conceptualization, validation, investigation, methodology, writing—original draft. F. Xiao: Conceptualization, formal analysis, validation, investigation. H. Sugimoto: Formal analysis, investigation, methodology. B. Li: Investigation, methodology, writing—review and editing. K.M. McAndrews: Investigation, writing—review and editing. R. Kalluri: Conceptualization, supervision, project administration, writing—review and editing.

Acknowledgments

The authors thank Valerie LeBleu for guidance on the project. The authors gratefully acknowledge Patricia Phillips for technical support with histological staining. They thank Sujuan Yang, Janine Hensel, Jianli Dai, and Juliennne Carriens for help with in vivo experiments. The authors wish to thank the South Campus Flow Cytometry Core Lab of MD Anderson Cancer Center for FACS sorting and analysis (partly supported by NCI grant P30CA16672). This work was supported by research funds from University of Texas MD Anderson Cancer Center.

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Received August 26, 2020; revised December 14, 2020; accepted February 1, 2021; published first February 8, 2021.

References


AKI Provokes the Development of RCC via CXCL1/CXCR2


Acute Kidney Injury Instigates Malignant Renal Cell Carcinoma via CXCR2 in Mice with Inactivated Trp53 and Pten in Proximal Tubular Kidney Epithelial Cells

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Cancer Res 2021;81:2690-2702. Published OnlineFirst February 8, 2021.

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doi:10.1158/0008-5472.CAN-20-2930

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