Autocrine CSF-1 and CSF-1 Receptor Coexpression Promotes Renal Cell Carcinoma Growth

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Abstract

Renal cell carcinoma is increasing in incidence but the molecular mechanisms regulating its growth remain elusive. Coexpression of the monocytic growth factor colony-stimulating factor (CSF)-1 and its receptor CSF-1R on renal tubular epithelial cells (TEC) will promote proliferation and antiapoptosis during regeneration of renal tubules. Here, we show that a CSF-1-dependent autocrine pathway is also responsible for the growth of renal cell carcinoma (RCC). CSF-1 and CSF-1R were coexpressed in RCCs and TECs proximally adjacent to RCCs. CSF-1 engagement of CSF-1R promoted RCC survival and proliferation and reduced apoptosis, in support of the likelihood that CSF-1R effector signals mediate RCC growth. In vivo CSF-1R blockade using a CSF-1R tyrosine kinase inhibitor decreased RCC proliferation and macrophage infiltration in a manner associated with a dramatic reduction in tumor mass. Further mechanistic investigations linked CSF-1 and epidermal growth factor signaling in RCCs. Taken together, our results suggest that budding RCC stimulates the proximal adjacent microenvironment in the kidney to release mediators of CSF-1, CSF-1R, and epidermal growth factor expression in RCCs. Furthermore, our findings imply that targeting CSF-1/CSF-1R signaling may be therapeutically effective in RCCs.

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Introduction

Renal cell carcinoma has been steadily on the rise for several decades. Renal clear cell carcinoma (RCC) is derived from proximal tubule epithelial cells and is by far the most common (70%–80%) form of kidney carcinomas (1, 2). However, despite the increased incidence of RCCs, the molecular mechanisms that regulate the growth of this tumor remain elusive (3).

Colony-stimulating factor (CSF)-1 and its receptor (CSF-1R) are instrumental during the progression of epithelial tumors of the female reproductive tract and prostate (4–7). For example, CSF-1 and CSF-1R are coexpressed in more than 50% of mammary tumors and elevated circulating CSF-1 levels are an indicator of early metastatic relapse in patients with breast cancer (8, 9). In addition, CSF-1 expression in primary breast carcinoma and RCC correlates with infiltration of inflammatory cells and, in turn, poor prognosis of this tumor (10). In fact, CSF-1 generated by mammary tumors and RCC recruit and activate large numbers of tumor-associated Mø (TAMS) that release trophic cytokines and other growth factors, thereby enhances mammary and renal carcinoma growth and facilitate tumor metastases (10–14). Moreover, an autocrine loop in breast cancer cell lines expressing both CSF-1 and the CSF-1R may contribute to tumor invasion and metastasis (4, 9, 15).

Furthermore, recent findings indicate that CSF-1R is expressed on tubular epithelial cells (TEC) in RCC; however the relevance of numerous tissues (22), we probed for the contribution of inflammatory cells and, in turn, poor prognosis of this tumor (10). In fact, CSF-1 generated by mammary tumors and RCC recruit and activate large numbers of tumor-associated Mø (TAMS) that release trophic cytokines and other growth factors, thereby enhances mammary and renal carcinoma growth and facilitate tumor metastases (10–14). Moreover, an autocrine loop in breast cancer cell lines expressing both CSF-1 and the CSF-1R may contribute to tumor invasion and metastasis (4, 9, 15). Furthermore, recent findings indicate that CSF-1R is expressed on tubular epithelial cells (TEC) in RCC; however the relevance of the development and progression of this tumor was not elucidated (5, 16). Thus, signaling through the CSF-1R may promote the progression of renal epithelial cell tumors.

Inflammation is meant to set the stage for repair. We recently uncovered a CSF-1–dependent mechanism of renal tubular repair (17). After transient renal ischemia, CSF-1 and CSF-1R are coexpressed on TECs, including those in the proximal tubule, in mice and humans. CSF-1 engaging with the CSF-1R induces TECs to proliferate and inhibits further apoptosis leading to the replenishment of injured TECs. CSF-1 is integral in the healing process as CSF-1 injected into mice after ischemia/reperfusion (I/R) hastens tubular healing whereas blocking the CSF-1R prevents renal tubular regeneration (17, 18). However, CSF-1 was originally identified as the principle Mø developmental molecule that stimulates survival, differentiation, proliferation, and activation of Mo (19, 20), and CSF-1 has a sole receptor, the c-fms tyrosine kinase proto-oncogene that is expressed on cells of the monocyte lineage (Mo, dendritic cells; ref. 21) As Mo are implicated in the repair of numerous tissues (22), we probed for the contribution of a CSF-1–dependent Mo along with autocrine TEC renal repair after I/R. We determined that CSF-1–mediated tubular repair is dependent on TEC autocrine Mo-independent and

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Mo-dependent mechanisms following transient injury (17). Thus, signaling via the CSF-1R on TECs and Mo is intended to protect the kidney by mediating tubular repair.

To probe for the mechanisms that promote RCC growth, we hypothesized that: (i) RCCs coexpress CSF-1 and the CSF-1R and (2) the CSF-1–mediated autocrine feedback loop, intended to promote tubular repair in normal kidneys is “hijacked” by the RCC and instead triggers tumor cell proliferation and inhibits tumor cell apoptosis, thereby promoting tumor growth.

Materials and Methods

Renal biopsy specimens
Renal carcinoma tissues (discarded tissues) with a confirmed pathologic diagnosis were provided by the Department of Pathology, Rush University Medical Center, Chicago, IL, and the Department of Pathology, Johannes-Gutenberg University, Mainz, Germany. RCC samples were analyzed using the morphologic classification of the carcinomas according to World Health Organization (WHO) specifications to evaluate clinical outcome.

CSF-1R and CSF-1 expression

Immunofluorescence. In vitro, we cultured RCC (786-0 and Caki), HK2, and MCF-7 lines according to the manufacturer’s instructions (Cell Line Services). Cells (1 × 10⁴ per well) were stimulated with TPA [12-O-tetradecanoylphorbol 13-acetate] for 48 hours. We fixed cells with methanol for 5 minutes on coverslips and incubated them with rabbit-anti-mouse CSF-1R Ab or rabbit IgG (Santa Cruz) for 1 hour at room temperature. We detected CSF-1R by incubating cells with anti-rabbit Cy3 Ab (Vector) for 30 minutes. We mounted the coverslips with Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector) and analyzed them using a Nikon Eclipse E1000 upright fluorescence microscope.

Immunohistochemistry. Paraffin sections: We determined CSF-1 (CSF-1-Ab, N-16; Santa Cruz), phospho-M-CSFR (rabbit anti-human phospho-M-CSFR Tyr 723 Ab; Cell Signalling), and CSF-1R (CSF-1R-Ab; Santa Cruz) as previously described (23). We determined the number of positive cells in 10 randomly selected high-power fields and evaluated the corresponding areas within serial sections for the correlation analysis.

CSF-1/EGF ELISA
To quantify the levels of CSF-1 in supernatants, we evaluated samples using an ELISA for each as previously described (23). We analyzed epidermal growth factor (EGF) in supernatants using an ELISA (R&D Systems) according to the manufacturer’s instructions.

Human cell lines
The following cell lines were used and cultured as previously described: immortalized RCC lines (786-0 and Caki), immortalized proximal TEC line (HK2; ref. 24), promyelocytic leukemia cell line (HL60; ref. 25), and immortalized T-cell line (JURKAT). Carcinoma cells were purchased from the Cell Line Services.

TECs stimulated with RCC and Mo supernatant
To determine CSF-1R and CSF-1 expression on TECs induced by mediators released by RCC and Mo, we stimulated RCC cell lines and Mo with TNF-α (6 ng/mL), TNF-α/LPS (6 and 12 ng/mL) and phorbol-12-myristate-13-acetate (PMA) for 24 hours. Then, the supernatant was removed and fresh media were added. After an additional 24 hours, the supernatant (undiluted and diluted: 1:1, 1:4, and 1:8 with media) was added to cultured TECs (HK2). After 48 hours, the CSF-1 in the cultured TEC supernatant was evaluated (ELISA assay) and CSF-1 and CSF-1R transcript expression on cultured TEC was evaluated by quantitative real-time PCR (qRT-PCR). We measured CSF-1 concentrations in the supernatant of RCCs and Mo before co inoculation with TECs to determine the baseline expression of CSF-1 in these cells. CSF-1 expression in TECs that were not stimulated served as controls.

CSF-1R and CSF-1 transcript expression
We analyzed the CSF-1R and CSF-1 expression in human cell lines using real-time, 2-step, quantitative PCR as previously described (qRT-PCR; ref. 26). The mRNA levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We used the following PCR primers: GAPDH: sense, 5'-CCC TCA ACG ACC ATG TTA CTA-3'; antisense, 5'-TTC CTC TTG TGC TTC TGC TGG-3'; CSF-1R: sense, 5'-TGA GCA AGA CCT GGA CAA GGA-3'; antisense, 5'-CCA TTG GTC AAT AGC ACG TTA-3'; and CSF-1: sense, 5'-CCC ATG TTA CTG CAC CAC GCA A-3'; antisense, 5'-AAG CAG TAA CTG AGC AAC GGG-3'.

CSF-1R RNA interference
For transient inhibition of CSF-1R mRNA production, 786-0 and HK2 cell lines were transfected with a commercially available pool of 4 target-specific 20 to 25 nucleotide-long siRNAs designed to "knock down" CSF-1R expression and with a control siRNA (Santa Cruz Biotechnology, Inc.). For stable inhibition of CSF-1R expression, 786-0 and HK2 cell lines were transfected using a short hairpin RNA (shRNA) plasmid [pool of 4 target specific lentiviral vector plasmids each encoding 19–25 nucleotide-long (plus hairpin) shRNAs are designed to target the CSF-1R knockdown] or control shRNA plasmid (Santa Cruz Biotechnology). Each plasmid contains a puromycin resistance gene to select cells stably expressing shRNA. These transfections were conducted with shRNA Plasmid Transfection reagent according to the manufacturer’s instructions (Santa Cruz Biotechnology).

Preparation of von Hippel–Lindau in 786-0⁺ cell line
Using a retroviral gene transfer approach, 293T cells were transfected with pBABe-puro-VHL DNA and a control DNA as previously described (27). 786-0 cells expressing the von Hippel–Lindau (VHL) gene were selected with puromycin (1 μg/mL).

Proliferation

Immunohistochemistry. We stained paraffin sections using a primary Ab against rabbit anti-human/mouse Ki67 (SP6; Lab Vision) to identify proliferating TECs.
**MTT assay.** We cultured RCC [786-0 and Caki-1 (Caki), Caki-2], human TEC (HK2), and human breast carcinoma (MCF-7) lines in 96-well plates (5 x 10^4 cells per well) for 12 hours and stimulated these cells for 72 hours with human recombinant CSF-1 or EGF (Sigma-Aldrich) at various concentrations (5, 20, 40, and 80 ng/mL). To verify specificity by blocking the CSF-1R, we stimulated cells with CSF-1 in combination with CSF-1R Ab (25 ng/mL; Santa Cruz) or CSF-1R tyrosine kinase inhibitor [5-(3-methoxy-4-((4-methoxybenzyl)oxy)benzyl)-pyrimidine-2-4-diamine; Calbiochem] or with EGF and anti-EGFR Ab for 72 hours. We used control antibodies for CSF-1R Ab (rabbit IgG) and EGFR Ab (mouse IgG2B) in all experiments. Furthermore, we stimulated cultured RCC, HK2, and MCF-7 lines with varying concentrations of TNF-α (3, 6, 15, and 30 ng/mL), lipopolysaccharide (LPS; 6, 12, 25, and 50 ng/mL; Sigma-Aldrich) in combination with CSF-1R Ab (25 ng/mL; Santa Cruz) or anti-EGFR Ab. We analyzed proliferation using the MTT colorimetric assay (Roche) according to the manufacturer’s instructions.

**Apoptosis**

We cultured RCC, HK2, and MCF-7 lines in 6-well (apoptosis assay) or 96-well (proliferation assay) plates (5 x 10^4 per well) for 12 hours and stimulated these cells with varying concentrations of human recombinant CSF-1 (5, 20, 39, and 78 ng/mL) in combination with TNF-α (3, 6, 15, and 30 ng/mL), and LPS (6, 12, 25, and 50 ng/mL). After 72 hours, we assessed apoptosis by flow cytometry using an Annexin-V-FITC-PI kit (BD Biosciences) according to the manufacturer’s instructions.

**Mice**

We purchased athymic nu/nu BALB/c mice from The Jackson Laboratory. Mice were housed at Johannes-Gutenberg University. The use of mice in this study was reviewed and approved by the Standing Committee on Animals at the University of Mainz.

**Implanting TECs under the renal capsule**

We implanted RCC (786-0) or HK2 cells under the renal capsule of athymic nu/nu BALB/c mice at 3 months of age as previously described (27).

**Treatment with CSF-1R tyrosine kinase inhibitor**

We injected mice (intraperitoneally) every 24 hours with the CSF-1R tyrosine kinase inhibitor (Calbiochem; 25 mg/kg/body weight) beginning at 1.0 day after surgery and ending at 21 days after surgery. The CSF-1R inhibitor is a cell-permeable diamino- pyrimidine compound that acts as a potent, selective, and ATP-competitive inhibitor of c-fms kinase activity (IC_{50} = 30 nmol/L) with minimal inhibition toward a panel of 26 other kinases (IC_{50} > 5 μmol/L). It is shown to selectively inhibit c-fms-mediated cellular functions in vitro as well as CSF-1-dependent tumor growth in vivo (28).

**Statistical analysis**

The data represent the mean ± SEM and were prepared using GraphPad Prism version 4.0. We used the nonparametric Mann–Whitney U test to evaluate P values. For correlation analysis, we used the Spearman correlation calculation.

**Results**

**CSF-1R and CSF-1 are expressed in RCC but not papillary carcinoma**

CSF-1 and the CSF-1R are coexpressed on TECs following a transient ischemic injury and via an autocrine/paracrine mechanism–mediated renal tubular repair (17). Moreover, the CSF-1R is expressed on epithelial cells of carcinomas including breast and prostate (6, 29). Thus, we hypothesized that CSF-1 and the CSF-1R are coexpressed on RCC. To test this hypothesis, we probed for CSF-1 and CSF-1R protein expression in human kidney carcinomas and RCC and papillary carcinoma specimens in comparison with normal kidney by immunostaining. We detected robust CSF-1R expression in RCCs as compared with normal kidney that lacked CSF-1R expression (Fig. 1A). Because the CSF-1R is internalized following activation, CSF-1R is expressed on the membrane and in the cytoplasm (30, 31). To determine whether CSF-1R expression is restricted to the RCCs, we examined the TECs adjacent and distant to the RCCs. We detected ubiquitous expression of CSF-1R on TECs adjacent to RCCs, whereas CSF-1R expression was limited to far fewer TECs distant to RCCs (Fig. 1A). In fact, there was a CSF-1R expression gradient emanating from the RCCs and declining in proportion to the distance from the tumor. To determine whether CSF-1 is expressed along with CSF-1R in RCCs, we stained for the presence of CSF-1 in RCCs compared with normal kidney. We detected CSF-1 expression along with CSF-1R expression in RCCs. Moreover, the CSF-1 expression was most pronounced in the TECs contiguous to the tumor, diminished with distance from RCCs, and was not detectable in the areas most distant to the tumor. Furthermore, we detected a correlation in the expression of CSF-1R and CSF-1 in RCCs and the TECs adjacent, but not distant, to RCC (Fig. 1B). In contrast, we rarely detected CSF-1R and CSF-1 expression in papillary carcinoma (Fig. 1A). Taken together, CSF-1 and CSF-1R are ubiquitously coexpressed in RCCs and TECs closest to the RCCs and declines with distance from the tumor.

**CSF-1R and CSF-1 expression in RCCs and TECs adjacent to RCCs correlates with infiltrating Mo but not T cells**

Increased numbers of Mo in human breast cancer is an index of poor prognosis (32, 33). Moreover, mammary tumor metastasis is attenuated in mice with reduced numbers of Mo (34). To determine whether enhanced expression of CSF-1R and CSF-1 on TEC in RCC fosters Mo and T-cell–incited inflammation, we probed for Mo and T cells in RCC and the area adjacent and distant to the tumor. We detected a greater magnitude of Mo in RCCs than in normal kidneys (Fig. 2A). Moreover, Mo were more abundant in the RCC than in the area adjacent and distant to RCC. In fact, the magnitude of CSF-1R and CSF-1 expression correlated with CD68+ Mo in RCCs and TECs adjacent, but not distant, to RCCs (Fig. 2B). Of note, we did not detect an increase in CD3+ T cells in RCCs, nor in TECs adjacent and distant to RCCs (Fig. 2A). And CSF-1R and CSF-1 expression did not correlate with the number of T cells in the...
RCCs and the adjacent TECs (Fig. 2B) and we did not detect an increase in T cells in papillary carcinoma (Fig. 2A). Taken together, our findings suggest that the magnitude of CSF-1R and CSF-1 is an index of the extent of Mø, but not T cells, in RCCs and the adjacent TECs.

**Coexpression of CSF-1 and CSF-1R in RCCs correlates with TEC proliferation**

CSF-1 engaging with CSF-1R on the same or adjacent TEC induces proliferation, thereby replenishing necrotic and apoptotic TEC following transient ischemia (17). To determine whether the CSF-1R and CSF-1 coexpression of TECs in RCCs has an impact on TEC proliferation resulting in an increase in RCCs, we assessed CSF-1, CSF-1R, and proliferation in TECs in sequential kidney sections from patients with RCCs. The magnitude of CSF-1 and CSF-1R expression was greatest in proliferating (Ki67\(^+\)) TEC in RCCs and TECs adjacent to RCCs (Fig. 3). Moreover, we determined that CSF-1R\(^+\) TECs expressed the tyrosine-phosphorylated CSF-1R, indicating that the CSF-1R is signaling on the TEC (Fig. 3). Taken together, this suggests that coexpression of CSF-1 and CSF-1R on RCCs and TECs adjacent to RCCs promotes...
proliferation and is thereby responsible for promoting the expansion of this tumor.

We next investigated whether coexpression of CSF-1R, phospho-CSF-1R, and CSF-1 on proliferating TECs correlated with clinical disease. The criteria for clinical disease included tumor morphology (stage and grade/dedifferentiation) and patient survival. We evaluated 22 patients with RCC (average age of 64 years, 60% male and 40% female). We found that CSF-1R and CSF-1 were associated with enhanced Ki67 expression and with accelerated tumor progression (tumor stage and grade and reduced survival of the patients; Supplementary Fig. S1). Moreover, as the phospho-CSF-1R expression was similarly associated with tumor progression, this indicates that the CSF-1R was functional (Supplementary Fig. S1). This suggests that coexpression of CSF-1 and CSF-1R on RCCs hastens the progression of RCCs by promoting proliferation and differentiation.

Figure 2. CSF-1R and CSF-1 expression on RCCs and TECs that are adjacent to RCCs correlates with infiltrating Mø, but not CD3⁺ T cells. A, we evaluated the presence of CD68⁺ and CD3⁺ leukocytes in formalin-fixed sections by immunostaining. We confirmed staining specificity using isotype control Abs. Representative photomicrographs are shown (magnification 20×, enlargement 40×). B, correlations of CSF-1R and CSF-1 expression with CD68⁺ and CD3⁺ leukocytes in RCCs and the TECs in areas adjacent and distant to RCCs. Values are the mean ± SEM. hpf, high-power field.
Mediators predominantly released from RCC increase CSF-1R and CSF-1 expression on adjacent TEC

To test the hypothesis that increased expression of CSF-1R and CSF-1 on TECs adjacent to RCCs is induced by mediators released by RCC and/or TAMS, we conducted a series of in vitro experiments. Incubating the TEC cell line (HK2) with RCC (786-0) supernatant led to a concentration (1:1-1:8)-dependent increase in CSF-1 protein (Supplementary Fig. S2) and transcripts (Fig. 4, bottom) as compared with TECs not incubated with RCC supernatant. Following RCC stimulation (TNF-α), the concentration-dependent increase in CSF-1 expression was even higher than in unstimulated RCCs (Fig. 4, top). Baseline expression of RCCs before coincubation with TEC is displayed as a dotted line.

By comparison, incubating TECs with increasing concentrations of Mo (HL60) supernatant modestly increased CSF-1 expression on TECs (Fig. 4, top; Supplementary Fig. S2). Furthermore, we detected a similar pattern of CSF-1R transcript expression on TECs following incubation with supernatant generated by RCCs and Mo (Fig. 4, bottom). This suggests that mediators predominantly released by RCCs induce CSF-1 and CSF-1R expression on TECs adjacent to RCC.

CSF-1 engaging with CSF-1R on RCC promotes survival/proliferation, thereby fostering RCC growth

We hypothesized that CSF-1 engaging with CSF-1R on TEC promotes survival/proliferation in RCCs. To test this...
hypothesis, we first established that human RCC cell lines, 786-0 (Fig. 5) and Caki-1 (Caki wild-type VHL) and Caki-2 (deficient for VHL), express CSF-1R and CSF-1 transcripts and protein. We detected more robust CSF-1R transcript and protein (immunofluorescence) expression on TECs in RCC lines than on a human proximal tubule line (HK2) and a breast carcinoma line (MCF-7). And CSF-1R transcript expression increased when RCC line was stimulated with PMA (Fig. 5; Supplementary Fig. S3). In parallel with CSF-1R expression, we detected an upregulation after stimulation in RCC lines.

As CSF-1 expression increases in injured (TNF-α/LPS, actinomycin D, cisplatin) normal mouse TECs and, in turn, promotes TEC survival/proliferation, (17), we hypothesized that an increase in CSF-1 and CSF-1R displayed on RCCs leads to tumor growth. To test this hypothesis, we blocked the CSF-1R along with TNF-α/LPS stimulation to increase CSF-1 expression and evaluated survival/proliferation (as above). We detected a decrease in RCC mass following blockade of the CSF-1R (by CSF-1R Ab, transient and stable silencing by siRNA and shRNA), enhanced in RCC with prior TNF-α/LPS stimulation (Fig. 6B and C, data not shown for siRNA and shRNA). Thus, RCC generates sufficient CSF-1 to trigger CSF-1R signaling on RCCs resulting in enhanced RCC survival/proliferation, thereby increasing the tumor growth.

Because inactivating mutations in the VHL tumor suppressor gene are associated with RCC (36), we investigated whether CSF-1 and CSF-1R expression in RCCs is dependent on VHL. We did not detect a difference in CSF-1R and CSF-1 expression comparing the Caki (wild-type VHL) with Caki-2 (VHL-deficient) cell line (data not shown) or the 786-0 RCC line with and without VHL expression (786-0-VHL+/−/786-0-VHL−). Furthermore, CSF-1R stimulated a similar increase in proliferation/survival in the Caki/Caki-2 and 786-0-VHL+/−/786-0-VHL− cell lines (Supplementary Fig. S5). Thus, the CSF-1R and CSF-1 expression is not dependent on the VHL mutation.

**CSF-1 engaging with CSF-1R on RCC suppresses apoptosis, thereby increasing RCC growth**

CSF-1-dependent dampened apoptosis contributes to renal tubular repair, following transient ischemic injury (17). Therefore, we hypothesized that CSF-1 mediates an increase in RCC mass via CSF-1–dependent suppression of apoptosis in RCC. To test this hypothesis, we exposed RCC lines to increasing concentrations of TNF-α/LPS to increase CSF-1 and CSF-1R and evaluated the magnitude of RCC apoptosis. We detected an increase in the magnitude of apoptotic RCC cells with increasing concentrations of TNF-α/LPS (Fig. 6C). Moreover, blocking signaling through the CSF-1R on RCC lines using anti-CSF-1R Ab led to an increase in apoptotic cells indicating that eliminating the CSF-1R signaling suppresses apoptosis (Fig. 6C). Taken together, CSF-1–dependent RCC autocrine/paracrine mechanisms promote survival/proliferation and dampen apoptosis, thereby fostering tumor growth.

**Blocking CSF-1R signaling in vivo inhibits RCC growth**

We hypothesized that blocking the CSF-1R suppresses RCC growth. To test this hypothesis, we evaluated the impact of a CSF-1R tyrosine kinase inhibitor on human RCC in vitro. We stimulated an RCC line with increasing concentrations of CSF-1 and added the CSF-1R tyrosine kinase inhibitor. The CSF-1R...
tyrosine kinase inhibitor suppressed CSF-1–dependent proliferation and survival (data not shown) of the RCC line. Thus, the CSF-1R tyrosine kinase inhibitor effectively dampens renal tumor cell growth.

To determine whether blocking the CSF-1R on RCC inhibits RCC growth in vivo, we constructed an RCC model in BALB/c mice. We implanted human RCC (786-0) under the renal capsule in athymic nu/nu BALB/c mice, as these cells will not be rejected. To block the CSF-1R, we compared mice injected with a CSF-1R tyrosine kinase inhibitor (28, 37) and PBS for 21 days (Fig. 7). We implanted human TECs (HK2) under the renal capsule to serve as a negative control. We detected a dramatic decrease in local tumor growth following CSF-1R tyrosine kinase inhibitor compared with PBS treatment (Fig. 7). However, provision of the CSF-1R tyrosine kinase inhibitor did not reduce the RCC mass to baseline (level of implanted normal human TECs; Fig. 7). Thus, CSF-1R tyrosine kinase blockade suppresses human RCC growth. To determine whether the reduced tumor cell expansion is a result of reduced RCC proliferation, we evaluated the number of proliferating (Ki67+) RCCs in CSF-1R tyrosine kinase–treated mice compared with the mice implanted with HK2 cells (Fig. 7). In fact, the magnitude of Mo in CSF-1R tyrosine kinase–inhibited mice was reduced to the level of those implanted with nonmalignant HK2 cells. Taken together, blocking the CSF-1R effectively suppresses the growth of RCCs.

EGF mediates CSF-1–dependent RCC increased proliferation and suppressed apoptosis

Breast carcinoma cells generate CSF-1 that, in turn, stimulates Mo to express EGF and EGF stimulates breast carcinoma cells to express CSF-1 (4, 15). Thus, we hypothesized that EGF stimulates RCCs to express CSF-1, thereby promoting proliferation. To test this hypothesis, we incubated RCCs with EGF and measured CSF-1 in the supernatant and cell mass. We detected a progressive increase in the level of CSF-1 released in the supernatant (Fig. 8A), accompanied by increased proliferation of RCCs in response to EGF (Fig. 8B). As expected, blocking the EGF receptor (EGFR) suppressed EGF-mediated CSF-1–stimulated TEC proliferation. Moreover, inhibiting EGF along with CSF-1R additively suppressed RCC proliferation. In conclusion, the interaction of CSF-1 and EGF enhances the expansion of RCC by promoting RCC proliferation (Supplementary Fig. S6).

To determine whether EGF-stimulated CSF-1 suppresses apoptosis of RCC cells, we stimulated RCCs with TNF-α/LPS. Adding EGF dampened apoptosis of RCCs.
apoptosis is specific for EGF as blocking with anti-EGFR Ab restored apoptosis and replacing the EGFR Ab with a control (mouse IgG2b) did not restore apoptosis. To determine whether EGF stimulates CSF-1 and thereby promotes proliferation and dampens apoptosis in RCCs, we repeated the studies in Fig. 8B and C and blocked the CSF-1R. Adding anti-CSF-1R Ab to the EGF-stimulated TECs decreased proliferation (Fig. 8B) and restored apoptosis (Fig. 8C), thereby supporting the concept that EGF mediates proliferation and apoptosis via a CSF-1R-dependent mechanism. Thus, EGF induces CSF-1–dependent increased RCC proliferation and reduced apoptosis.

CSF-1 and CSF-1R expression on TEC adjacent to RCC is mediated, in part, by EGF

CSF-1 and the CSF-1R are upregulated on TECs adjacent, not distant, by mediators released primarily from RCCs (Fig. 4). Therefore, we tested the hypothesis that EGF is released by RCCs and is responsible for inducing CSF-1 and CSF-1R on TECs adjacent to RCCs. Stimulating RCC and TEC lines with EGF increases CSF-1 expression (Fig. 8A). Thus, we hypothesized that the TECs adjacent to the RCCs are stimulated by RCCs and TAMs to release EGF. Incubating the TEC cell line (HK2) with supernatant of TNF-α/LPS–stimulated RCC (786-0) or Mø (HL60) led to a concentration-dependent increase in EGF expression (Fig. 8D; Supplementary Fig. S2, bottom) as compared with TECs that were not incubated with RCC or Mø supernatant. This suggests that RCCs and TAMs release mediators that induce EGF expression on TECs adjacent to RCCs that, in turn, upregulate CSF-1/CSF-1R expression and lead to an increase in TEC proliferation and a reduction in TEC apoptosis.

**Discussion**

We now report the novel finding that autocrine CSF-1–dependent RCC mechanisms are central to the growth of RCCs. While recent findings indicate that the CSF-1R is expressed on RCCs (5, 16), our study clarifies the CSF-1–mediated
mechanisms central to promoting RCCs. In summary, CSF-1R and CSF-1 are abundantly coexpressed in RCC and in intra-renal TECs adjacent, but not distant, to RCCs. The engagement of CSF-1 with its sole cognate receptor, CSF-1R, promotes RCC survival and proliferation and reduces apoptosis. This suggested that signaling via the CSF-1R promotes RCC growth. Moreover, blocking the CSF-1R with a CSF-1R tyrosine kinase inhibitor in human RCC xenografts in mice decreases the survival and proliferation of the RCC and the infiltration of Mø, thereby leading to a dramatic reduction in tumor mass. Furthermore, EGF induces CSF-1 and the CSF-1R on RCCs, promoting tumor cell proliferation and dampening tumor cell apoptosis. RCC has an impact on the kidney microenvironment adjacent to the tumor, as it releases mediators that induce CSF-1, CSF-1R, and EGF expression on these TECs. Taken together, we suggest that targeting the CSF-1R is a potential therapeutic approach for human RCCs.

Our findings highlight the CSF-1-dependent autocrine mechanism that fosters RCC growth. A wealth of evidence has detailed the involvement of CSF-1R–bearing TAMS in tumor progression and metastasis (11, 38). The paracrine interactions between tumor cells and TAMS facilitate the spread of tumor in the host by promoting tumor cell migration, invasion, and metastasis (4, 15). As CSF-1 generated by the tumor is essential in regulating TAMS, CSF-1- and CSF-1R–expressing Mø are key components in the tumor microenvironment regulating the fate of the host. To identify CSF-1–dependent TAMS-mediated mechanisms central to tumor progression, investigators studied tumors lacking the CSF-1R, thereby eliminating CSF-1–dependent tumor autocrine mechanisms (34, 39). For example, CSF-1 blockade in human colon, devoid of CSF-1R, indicates that CSF-1–dependent Mø-mediated molecular mechanisms regulate tumor growth (40). However, malignant tumors such as breast and ovary coexpress CSF-1 and the CSF-1R (5, 7, 9). Thus, CSF-1 and CSF-1R coexpression on breast and ovarian carcinoma prompted investigations to uncover a CSF-1–dependent autocrine loop that contributes to tumor invasiveness and metastasis.
Moreover, heightened proliferation is evident in mammary glands of CSF-1 transgenic mice that result in mammary tumors. Our findings uniquely highlight the pivotal position of a CSF-1–dependent autocrine mechanism in the most common kidney tumor in human RCCs. While a recent report indicates that the CSF-1R is expressed in RCC (5, 16), using sequential sections from patients with RCC, our findings illustrate CSF-1 and CSF-1R.
coexpression on proliferating TECs and establish that the CSF-1R is phosphorylated. Also, we detect enhanced tumor cell proliferation and dampened tumor cell apoptosis, proportional to CSF-1 expression in RCC lines, thereby indicating that RCC growth is mediated by CSF-1. Moreover, an increase in TAMS in the RCC and kidney adjacent to RCC suggests that, as in other cancers (38), CSF-1R-expressing Mø may contribute to the progression of RCCs. Taken together, CSF-1-dependent mechanisms are central to RCCs.

Mediators released by the RCC maybe instrumental in altering the TECs adjacent to the RCCs. We determined that CSF-1 and the CSF-1R are upregulated on the TECs adjacent, but not distant, to RCCs. As TAMS are more abundant in the kidney adjacent to RCCs, this suggests that mediators generated by the RCCs and/or TAMS induce CSF-1 and CSF-1R on the TECs closest to the tumor. We determined that the RCCs, and to a far lesser extent Mø, release mediators, including EGF, that induce CSF-1 and the CSF-1R on TECs. There are several possible consequences of upregulating CSF-1 and the CSF-1R coexpression on TECs adjacent to RCCs. It is possible that RCC injures the TECs closest to the tumor and as we previously reported, CSF-1 and CSF-1R coexpression triggers TEC proliferation and dampens TEC apoptosis, thus replenishing damaged tubules (17). On the other hand, upregulated CSF-1 and the CSF-1R on TECs may be harmful. For example, overexpression of EGF promotes proliferation and in mutated EGFR leads to oncogenesis (43, 44). Thus, upregulated CSF-1 and CSF-1R coexpression on TECs adjacent to RCCs enhances autocrine TEC proliferation, and along with exposure to additional triggers, may lead to malignant transformation of TECs. Alternatively, our findings indicate that CSF-1, generated by TECs adjacent to RCCs, contributes to Mø recruitment. Thus, it is possible that these Mø are instrumental in promoting tumor growth. Despite the consequence of upregulating these molecules in TECs adjacent to RCCs, it is clear that RCC releases mediators that trigger CSF-1 and CSF-1R expression in the kidney microenvironment.

Is the CSF-1R a therapeutic target for human RCC? We now report that blocking the CSF-1R on RCCs dramatically suppresses RCC growth. CSF-1R tyrosine kinase inhibitor, known to block the CSF-1R in vivo (28, 37) delivered into a human RCC xenograft mouse (human RCC cells implanted under the kidney capsule of athymic nude mice), dramatically reduced RCC mass. Our findings are consistent with other therapeutic approaches (antisense, interfering RNA, antibodies) that target the CSF-1R in other forms of cancer (colon, mammary) and lead to tumor suppression (40, 45, 46). However, our findings are in contrast to the failure of a mouse anti-CSF-1R Ab to inhibit tumor growth in subcutaneous mesothelioma and Lewis lung carcinoma models (47). Recalling that Mø, and some malignant tumors, express the CSF-1R, blocking the CSF-1R may suppress tumor expansion via CSF-1-dependent autocrine and paracrine mechanisms. While these tumors (mesothelioma and lung) are similar to RCC as they originate from cells of epithelial origin, CSF-1R expression on these tumors was not explored. And despite decreasing TAMS using this anti-CSF-1R Ab, the CSF-1–dependent mechanisms that may regulate these particular tumors have not been detailed. Thus, CSF-1R blockade may be a promising therapeutic approach for some, but not all, malignant tumors. It is worth noting that sunitinib, a multityrosine receptor tyrosine kinase inhibitor, that targets the CSF-1R along with many other receptor tyrosine kinases, is a first-line therapy for advanced RCCs (1, 48, 49). Taken together, our findings clearly indicate that targeting the CSF-1R is a potential therapeutic for human RCCs. Detailing the relative contribution of autocrine CSF-1–dependent and the unexplored potential of Mø-mediated paracrine mechanisms will be central to tailoring therapeutic strategies to halt human RCCs.

CSF-1 and EGF expression and functions are closely linked in the kidney during inflammation. Following acute renal injury, heparin-binding EGF is upregulated on TECs and promotes TEC proliferation, findings that parallel CSF-1 expression and function (50–52). Moreover, an increase in CSF-1R and EGF expression correlates with poor prognosis of human tumors (43, 44). And in mammary tumors, blocking EGF and CSF-1R inhibits carcinoma invasiveness (53). Finally, CSF-1 and EGF are components of a positive feedback loop. EGF generated by Mø induces CSF-1 expression in breast carcinoma cells, and in turn, CSF-1 generated by breast carcinoma cells induces EGF expression in Mo (15). Thus, we hypothesized that EGF induces CSF-1 in RCCs. We determined that EGF stimulates CSF-1 expression in RCC lines and promotes RCC cell proliferation and dampens RCC cell apoptosis. As blocking EGF and CSF-1R reverses these EGF-incited findings, we suggest that EGF induces CSF-1 in RCC. In addition, we determined that RCCs, and to a lesser degree Mø, release mediators that induce EGF expression and, in turn, CSF-1/CSF-1R expression on TECs and thereby modify the kidney microenvironment.

In conclusion, we have identified CSF-1–dependent autocrine mechanisms that are instrumental in fostering RCC growth. Taken together, an autocrine CSF-1–dependent mechanism, intended to promote tubular repair following renal injury, has been subverted and now drives tumor expansion. Our findings suggest that CSF-1R is a potential therapeutic target for human RCCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


Autocrine CSF-1 and CSF-1 Receptor Coexpression Promotes Renal Cell Carcinoma Growth

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