JAK kinases promote invasiveness in VHL-mediated renal cell carcinoma by a suppressor of cytokine signaling-regulated, HIF-independent mechanism

Karen L. Wu, Hui Miao, and Shenaz Khan

Department of Medicine, Case Western Reserve University, School of Medicine, MetroHealth Campus, Rammelkamp Center for Research, Cleveland, Ohio

Submitted 26 February 2007; accepted in final form 24 September 2007

Wu KL, Miao H, Khan S. JAK kinases promote invasiveness in VHL-mediated renal cell carcinoma by a suppressor of cytokine signaling-regulated, HIF-independent mechanism. Am J Physiol Renal Physiol 293: F1836–F1846, 2007. First published September 26, 2007; doi:10.1152/ajprenal.00096.2007.—von Hippel-Lindau (VHL) disease is a cancer syndrome, which includes renal cell carcinoma (RCC), and is caused by VHL mutations. Most, but not all VHL phenotypes are due to failure of mutant VHL to regulate constitutive proteolysis of hypoxia-inducible factors (HIFs). Janus kinases (JAK1, 2, 3, and TYK2) promote cell survival and proliferation, processes tightly controlled by SOCS proteins, which have sequence and structural homology to VHL. We hypothesized that in VHL disease, RCC pathogenesis results from enhanced SOCS1 degradation, leading to upregulated JAK activity. We find that baseline JAK2, JAK3, and TYK2 activities are increased in RCC cell lines, even after serum deprivation or coinubation with cytokine inhibitors. Furthermore, JAK activity is sustained in RCC stably expressing JAK2, JAK3, and TYK2 activities are increased in RCC cell lines, even after serum deprivation or coinubation with cytokine inhibitors. Additionally, JAK activity is suppressed in RCC stably expressing JAK2, JAK3, and TYK2 activities are increased in RCC cell lines, even after serum deprivation or coinubation with cytokine inhibitors. Additionally, JAK activity is suppressed in RCC stably expressing JAK2, JAK3, and TYK2 activities are increased in RCC cell lines, even after serum deprivation or coinubation with cytokine inhibitors.

Finally, we observe enhanced SOCS2/SOCS1 coprecipitation and reduced SOCS1 expression due to proteasomal degradation in VHL-null RCC compared with wild-type cells. The data suggest that JAK inhibition represents an alternative therapeutic approach.

Janus kinase; suppressor of cytokine signaling; von Hippel-Lindau syndrome

VON HIPPEL-LINDAU (VHL) disease is a heritable multisystem cancer syndrome caused by germline mutations in VHL, which is located on chromosome 3p25 (30). The main causes of death are complications linked to highly angiogenic renal carcinomas and hemangioblastomas within the central nervous system (45, 59). The VHL gene is ubiquitously expressed (60) and the role of the VHL gene product (pVHL) in renal cell carcinoma (RCC) has been extensively studied (31, 49). pVHL contains an ~100-residue NH2 terminus (β-domain) and a smaller COOH-terminal α-helix (α-domain). Under normoxic conditions, the pVHL β-domain interacts with JAK kinases promote invasiveness in VHL-mediated renal cell carcinoma by a suppressor of cytokine signaling-regulated, HIF-independent mechanism.

Address for reprint requests and other correspondence: K. L. Wu, Case Western Reserve Univ., School of Medicine, Dept. of Nutrition, Research Tower, RT600, 2109 Adelbert Rd., Cleveland, OH 44106 (e-mail: lkw@case.edu).

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In this study, we show that VHL mutant RCC cell lines exhibit constitutive, HIF-independent JAK-STAT pathway activation, which regulates RCC invasion. We also find that JAK activity is increased by a mechanism involving SOCS2/SOCS1 association, SOCS1 ubiquitination, and proteasomal degradation, resulting in diminished steady-state SOCS1 expression. The data suggest that RCC therapy directed toward JAK-STAT abrogation may synergize with HIF inhibition strategies.

MATERIALS AND METHODS

Cell lines. 786-0 RCC cells were transfected with either empty vector (RC3) or wild-type VHL (WT8) (gifts from Dr. B. Kaelin). RCC4 cells (VHLser65Trp) (32) were transfected with wild-type VHL (WT8/WT45) or an inactivating point mutant (L188V) (gifts from Dr. E. Maher). Cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS and 0.5 g/l G418 in humidified incubator (Dr. E. Maher). Cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS and 0.5 g/l G418 in humidified incubator with 5% CO2 at 37°C. Stably transfected 786-0 cells that overexpress VHL (WTVHL) or an inactivating point mutant (L188V) (gifts from Dr. O. Iliopoulos. MCF7 cells were from ATCC and cultured in 10% FBS containing DMEM in humidified incubator with 5% CO2 at 37°C.

Reagents. Anti-phospho-JAK2, anti-phospho-TYK2, and anti-phosphotyrosine-STAT3 antibodies were obtained from Cell Signaling (Danvers, MA). Anti-JAK2, anti-JAK3, anti-TYK2, anti-SOCS1, anti-ubiquitin, and anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-SOCS2 antibody was purchased from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were products from Amersham Biosciences (Piscataway, NJ). Anti-α-tubulin, anti-FLAG M2 IgG, cycloheximide, crystal violet, and IFG-1 were from Sigma (St. Louis, MO). Anti-HIF1α was from NeoMarkers (Fremont, CA). AG490, JAK inhibitor I, JAK3 inhibitor V, AG9, anti-TGF-α, Tranlast, AG1296, PD153035, lactacystin, MG132, and PSI were purchased from BD Biosciences.

Plasmids and transient transfection. Tyrosine kinase domain-deleted dominant-negative TYK2 construct, ΔTK-TYK2, was a kind gift from Dr. S. Pellegrini (69). JAK2 inactivating point mutants, K828E and Y1007F, were kind gifts from Dr. O. Silvennoinen (68). Plasmids were transformed into Top 10 competent bacteria cells according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA), extracted using a Maxiprep kit (Qiagen, Valencia, CA), and amplified by culture in Luria-Bertani-ampicillin broth. cDNAs were transiently transfected into cells according to previously described methods (73). Briefly, cells were plated into six-well plates (0.25 × 10^6 cells/well) and cultured overnight in complete medium. The transient mixture, which contained 1.0 μg of plasmid DNA and 6 μl of Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) in 100 μl of serum-free DMEM medium (Invitrogen), was mixed for 20 min at room temperature and then added to each well with complete medium for 48 h.

Scratch-wound assays. Wounding assays were performed as described previously (15). Briefly, confluent WT8 and RC3 cells plated in 24-well gridded plates were incubated in serum-free DMEM medium 1 h before wounding. Cells’ monolayer was scratch-wounded with a P200 pipette tip. Cells were then incubated with or without JAK inhibitors AG490 (100 μM), P131 (300 μM), JAK3i-V (50 μM), AG9 (100 μM), or JAKi-1 (500 μM). Migration was determined at identical locations by phase-contrast microscopy (×10 magnification) 5 and 15 h after incubation. Representative images were photographed.

Immunoprecipitation and immunoblot analyses. The methods have previously been described in detail (73). Cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5 mM deoxycholate, 0.1% SDS, 1% Triton X-100) containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) for 1 h at 4°C. The lysates were centrifuged at 12,000 rpm for 30 min at 4°C. Proteins in the supernatant were assayed for protein content (Bio-Rad) and 500 μg of protein were immunoprecipitated with appropriate antibodies (1 μg/each, 4°C, overnight). The samples were incubated with γ-bind protein G-Sepharose beads (Amersham; 4°C, 1 h) and then washed in RIPA buffer. Immunoprecipitates were dissolved in 40 μl of 2× SDS sample buffer (125 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 1% β-mercaptoethanol, and 0.003% bromophenol blue) and then evaluated by immunoblotting according to the following methods. Whole cell lysates or immunoprecipitates in 2× SDS sample buffer were denatured by boiling for 5 min, and then samples were fractionated by 6 or 4–20% SDS-PAGE (Invitrogen). The proteins were transferred to polyvinyldiene difluoride membranes, blocked with 5% nonfat milk (5% BSA for anti-phosphotyrosine, -phospho-JAK2, and -phospho-TYK2 antibodies), and incubated with appropriate antibodies (4°C, overnight), followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (1:10,000, 1 h, room temperature). In some experiments, the blots were stripped with Blot Restore Solutions (Chemicon, Temecula, CA) and reprobed with appropriate antibodies. Protein bands were detected by enhanced chemiluminescence (Amersham Biosciences).

Invasion assay. Matrigel (50 μl at 1:8 dilution in DMEM medium) was coated on the top of Transwell filters (6.5-mm diameter, 8.0-μm pore size, Costar, Corning, NY) for 2 h at 37°C. Chemotactic (0.5 μM IGF-1) was added to bottom chambers of transwells. Cells (6.25 × 10^5) were plated on Matrigel in DMEM and allowed to migrate overnight at 37°C through Matrigel. Migrated cells were fixed in methanol (100%, 20 min, room temperature) and stained with crystal violet (0.5 in 20% methanol, 30 min, room temperature). Matrigel and cells, which did not migrate, were gently removed from the upper chamber with Q-tips. Cells migrating through Matrigel to the lower chamber were counted under a dissecting microscope from three to six random fields.

TUNEL assay. TUNEL assay was performed according to the manufacturer’s instruction, as described previously (74). WT8 and RC3 cells were cotransfected with GFP and dominant-negative TYK2 and JAK2 constructs. TUNEL-positive cells within the GFP-expressing population were counted to assess apoptosis.

Protein degradation assay [35S]-labeled pulse chase. Methods have previously been described in detail (74). Cells were cultured to subconfluence, washed in PBS, and incubated with [35S]methionine in methionine-free DMEM (0.1 mCi/ml, 2 h, 37°C). Cells were washed in PBS and chased in methionine-containing DMEM for up to 16 h. Protein lysates (400 μg per sample) were immunoprecipitated with anti-SOCS1 (1 μg, overnight) antibody and resolved by SDS-PAGE. Autoradiograms were developed from dried gels. Individual bands were digitized by phosphoimager (Molecular Dynamics), quantified with Image Quant 5 software (Molecular Dynamics), and normalized to control values.

As an alternative to [35S]-labeled pulse chase, protein degraded was also assessed by cycloheximide-based assay. Cells were plated in six-well dishes (0.25 × 10^6 cells/well) and cultured overnight. The following day, cells were treated with protein synthesis inhibitor, cycloheximide (20 μg/ml in serum-free medium for up to 16 h). Whole cell lysates were resolved by SDS-PAGE and probed with anti-SOCS1 antibodies.

Statistics. Data are representative of three to five experiments per condition, and unless otherwise noted in figure legends, expressed as means ± SE. Statistical significance between two groups was assessed by Student’s t-test and by one-way ANOVA for all other comparisons.

RESULTS

Increased JAK activities in RCC. JAK-STAT pathways have been implicated in carcinogenesis, including in RCC (8). Initial immunoblots demonstrated JAK2, JAK3, and TYK2 expres-
sion in RC3 and RCC4 cell lines, whereas JAK1 expression was not detected in either RCC cell line (data not shown). JAK2 activity was investigated in RCC4, wild-type, and L188V cell lines by immunoblot analysis with anti-phospho-JAK2 antibodies. Figure 1A demonstrates increased JAK2 activity in RCC4 or L188V compared with wild-type cells. To assess JAK3 activity in RCC, cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies and then probed with anti-JAK3 antibodies. The data show robust JAK3 tyrosine phosphorylation in VHL mutant (RCC4, L188V, and RC3) cells compared with wild-type controls (Fig. 1B). Furthermore, TYK2 activation was also assessed in both sets of VHL cell lines by immunoprecipitation and immunoblotting with anti-phosphotyrosine antibodies. As shown in Fig. 1C, significant TYK2 tyrosine phosphorylation was observed in VHL mutant RCC cells compared with wild-type cells.

To confirm these results, WT8 and RC3 cells were treated with the specific JAK2 inhibitor AG490 (50 μM, 72 h) and lysed whole cell lysates were immunoblotted for expression of phosphorylated STAT3, the downstream effector for JAK2. Figure 1D shows that RC3 cells contained increased tyrosine phosphorylated STAT3 levels, which was abolished by AG490, indicating that JAK2 is functionally active in RCC cells. Basal phospho-STAT3 was unaffected by AG490 in wild-type cells, suggesting that STAT3 is phosphorylated by a kinase other than JAK2 in the basal state. Taken together, JAK2, JAK3, and TYK2 are activated in two different RCC cell lines.

Increased JAK activity is independent of HIFα. JAK activation in the cell line expressing the L188V VHL mutation was surprising because L188V is associated with VHL syndromes that primarily include pheochromocytoma, rather than RCC. In some instances, L188V overexpression has been shown to suppress RCC growth (27), suggesting that JAKs mediate RCC phenotypes other than proliferation. Since the L188V VHL mutant retains the ability to bind HIFα subunits and Elongin C, and downregulate HIF activity (12, 27), the data suggest that JAK activation may be HIF-independent in RCC. To test the effect of HIF on JAK activity, we examined RC3 cells that stably express short-hairpin siRNA (shRNA) targeting HIF2α. Figure 1E demonstrates that HIF2α shRNA expression did not inhibit JAK2 activation, suggesting that JAK activity in RCC cells is indeed independent of HIFα regulation. To confirm that

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**Fig. 1. Renal cell carcinoma (RCC) JAK activity is increased by a hypoxia-inducible factor (HIF)-independent mechanism.**

**A:** RCC4, wild-type, and L188V von Hippel-Lindau (VHL) mutant cell lines were maintained in serum-free media overnight, to avoid cytokine stimulation. **Top:** whole cell lysates were then probed with anti-phospho-JAK2 antibodies by immunoblot analysis. **Bottom:** blot was stripped and reprobed for JAK2 expression and protein loading with anti-JAK2 antibodies.

**B:** depicted cells were maintained in serum-free media overnight. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibody (α-PY). **Top:** blot was stripped and reprobed for JAK3 expression. **Bottom:** parallel lysates were immunoblotted with anti-JAK3 antibodies.

**C:** cell lines were maintained in serum-free media overnight. **Top:** whole cell lysates were then immunoprecipitated with anti-TYK2 antibodies and probed with anti-α-tubulin antibodies as a loading control. **Bottom:** parallel lysates were immunoblotted with anti-TYK2 antibodies. **Middle:** parallel lysates were immunoblotted with anti-JAK2 antibodies. **Bottom:** blot was stripped and reprobed with anti-α-tubulin antibodies as a protein loading control.

**D:** WT8 and RC3 cells were incubated with JAK2 inhibitor AG490 (50 μM, 72 h). **Top:** cell lysates were immunoblotted with anti-phospho-JAK2 antibodies. **Middle:** parallel lysates were immunoblotted with anti-STAT3 antibodies. **Bottom:** blot was stripped and reprobed for STAT3 expression.

**E:** RC3 cells were stably transfected with either mock vector or shRNA construct targeted to HIF2α. **Top:** whole cell lysates were immunoblotted with anti-phospho-JAK2 antibodies. **Bottom:** blot was stripped and reprobed for HIF2α expression by immunoblot analysis. **Bottom:** blot was stripped and reprobed for anti-α-tubulin antibodies as a loading control.

**F:** top: RC3 and MCF7 cell lysates were immunoblotted with anti-HIF1α antibodies. **Bottom:** blot was stripped and reprobed with anti-tubulin antibodies.
HIF1α isoform is not expressed RC3 cells (48, 77), we examined HIF1α protein expression levels in RC3 cells and a control cell line, MCF7 cells. RC3 cell lysates did not display HIF1α protein expression, whereas MCF7 cells did (Fig. 1F, top). The stripped blot was reprobed with anti-tubulin antibodies as a loading control (Fig. 1F, bottom).

JAK activities are constitutive and cytokine-independent in RCC. In most circumstances, JAKs are activated following ligation with cytokine receptors. However, recent studies demonstrate that in some cell lines, JAKs can be constitutively activated (40). To determine cytokine dependence for RCC JAK activities, cytokines were depleted by incubating WT8 and RC3 cells in serum-free media for up to 24 h. WT8 cells displayed progressively decreased phospho-JAK2 levels after 6- and 24-h incubation in serum-free media. RC3 cells demonstrated relatively sustained phospho-JAK2 levels after 6- and 24-h depletion of serum, although some decreased activity has been shown (Fig. 2A). Cytokine depletion resulted in markedly decreased phospho-TYK2 content in WT8 cells after 6 h, which was almost undetectable by 24 h (Fig. 2A). However, RC3 cells displayed sustained TYK2 activity even after 24 h of serum starvation. These results suggest that JAK2 and TYK2 activation in RCC cells is cytokine-independent.

To more directly address this issue, cells were cultured in the presence and absence of PD153035 (EGFR inhibitor), AG1296 (PDGF inhibitor), or Tranilast (VEGF inhibitor) for 48 h, and JAK2 and TYK2 activities were determined by immunoblotting with anti-phospho-JAK2 antibodies. Exposure to cytokine inhibitors diminished phospho-JAK2 and phospho-TYK2 levels in WT8 cells, whereas RC3 cells showed sustained JAK2 and TYK2 phosphorylation (Fig. 2B), indicating that JAK2 and TYK2 are constitutively activated in RCC cells in a cytokine-independent fashion.

Increased JAK activity promotes RCC invasion. To test JAK regulation of pathophysiologically relevant phenotypes, we screened RC3 and RCC4 cells for altered proliferation, apoptosis, and fibronectin secretion. Of these assays, only fibronectin secretion was abnormal, consistent with prior reports (53). However, JAK inhibition had no effect on secretion of fibronectin by RCC or wild-type cell lines (data not shown).

Metastatic potential was measured by assaying RC3 and WT8 invasion through Matrigel (7, 14). To test JAK regulation of invasion, cells were preincubated with pan-JAK inhibitor (JAKI-1), JAK2-specific inhibitor (AG490), JAK3 inhibitor (JAK3I-V), and TYK2 inhibitor (AG9). Figure 3A demonstrates three- to fourfold increases in invasion of RC3 compared with WT8 cells, which were diminished by JAK inhibitors. The greatest inhibition of RC3 invasion was observed with JAKI-1 and AG490, indicating that JAK2-dependent signaling pathways may be important in RCC metastasis in vivo. Suggestive, but statistically insignificant, effects were observed with JAK3I-V and AG9. None of the JAK inhibitors significantly altered invasion in WT8 cells (data not shown). The activity of downstream effector of JAKs, STAT3, was evaluated by testing tyrosine phosphorylation of parallel cell lysates and was diminished by all JAK inhibitors. The greatest inhibition of STAT3 phosphorylation was found with JAKI-1 and AG490, which is consistent with invasion assay data (Fig. 3A, bottom).

Invasion through Matrigel was also assessed in RCC expressing dominant-negative JAK constructs: TYK2 (1-895), also referred to as TYK2 (ΔTK), which has a tyrosine kinase domain deletion, as well as two JAK2-inactivating point mutants (K882E and Y1007F). Figure 3B shows that enhanced RC3 cell invasion was abrogated by expression of dominant-negative TYK2. The parallel cell lysates were immunoblotted

![Figure 2](http://ajprenal.physiology.org/)
for TYK2 tyrosine phosphorylation as an indicator of TYK2 activity. The data show abolished TYK2 activity with TYK2 dominant-negative construct transfection (Fig. 3B, bottom). Figure 3C demonstrates that expression of catalytically inactive JAK2 point mutants also inhibited invasion of RC3, but not WT8 cells. Inhibition was slightly greater in RC3 cells cotransfected with both JAK2 and TYK2 dominant-negative vectors (Fig. 3C), suggesting an additive effect. As expected, JAK2 activity was reduced dramatically in cells overexpressing TYK2 with tyrosine kinase domain deletion (TYK2-ΔTK). The parallel lysates were probed with anti-phospho-TYK2 and anti-α-tubulin antibodies. 

Figure 3. Increased JAK activity promotes RCC invasion through Matrigel matrix. Pretreated WT8 or RC3 cells were plated on 8-μm pore transwell filters precoated with Matrigel (0.5-mm thickness, 1:8 dilution), and allowed to migrate from the lower chamber, through the Matrigel and transwell filter in response to chemoattractant stimulation (IGF-1, 0.5 μM, 16 h, 37°C) presented in the bottom chamber. Cells were fixed in 100% methanol, stained with crystal violet, and counted under a dissecting microscope. A: cells were treated with chemical inhibitors of JAKs: JAK1-1 (500 nM), AG490 (100 μM), JAK3I-V (50 μM), and AG9 (100 μM). The parallel lysates were immunoblotted with anti-phospho-STAT3 and anti-α-tubulin antibodies. B: cells were transfected with empty vector or construct encoding TYK2 with tyrosine kinase domain deletion (TYK2-ΔTK). The parallel lysates were immunoblotted with anti-phospho-TYK2 and anti-α-tubulin antibodies. C: cells were transfected with empty vector, inactivating JAK2 point mutations (K882E or Y1007F), TYK2-ΔTK or a combination of JAK mutants. The parallel lysates were immunoblotted with anti-phospho-JAK2 and anti-α-tubulin antibodies. *P < 0.05 compared with WT8 cell invasion.

To determine whether RC3 invasion could be confounded by cytotoxicity from dominant-negative TYK2 and JAK2 expression, transfected cells were assessed for apoptosis by TUNEL assay. These experiments revealed no difference in apoptosis between RC3 cells transfected with dominant-negative JAKs or empty vector (data not shown). In summary, RC3, but not WT8, cells demonstrate an invasive phenotype, which is regulated by JAK2 and TYK2.

As an alternative approach to assessing effects of JAKs on RCC metastatic potential, scratch-wound assays were conducted in confluent WT8 and RC3 cells in the presence and absence of JAK inhibitors. In the absence of JAK inhibitors, WT8 cells failed to fill the wound gap after 15 h (Fig. 4, top). However, RC3 cells closed the gap completely in the same time period, thereby confirming invasion assay results by demonstrating that VHL mutant RCC cell migrate more extensively than wild-type VHL-expressing cells. Moreover, inhibition of JAK2 by AG490 and JAK3 by P131 and JAK3I-V significantly attenuated RC3 cell migration, whereas inhibition of TYK2 by AG9 had a more modest effect on RC3 migration (Fig. 4). Taken together, the data from Figs. 3 and 4 indicate JAKs regulate RCC invasion and migration.

SOCS1 undergoes enhanced proteasomal degradation in RCC. A major mechanism of JAK regulation is through down-regulation by SOCS proteins. To initially investigate the possibility of JAK activation through evasion of SOCS-dependent degradation in RCC, SOCS protein expression was assessed by immunoblot analysis in RCC and wild-type cells. VHL mutant RCC cells (RCC4, RC3, and L188V) demonstrated significantly less steady-state SOCS1 expression compared with wild-type, VHL-transfected control cell lines (Fig. 5A, top). 

To determine whether decreased SOCS1 expression is due to protein degradation, SOCS1 half-life was measured in WT8 and RC3 cells by [35S]pulse chase methods. Figure 5, B and C, demonstrates that SOCS1 decay is enhanced in RCC cells, consistent with enhanced SOCS1 degradation. As an alternative strategy to verify SOCS1 degradation, WT8 and RC3 cells were treated with the protein synthesis inhibitor cycloheximide. The rate of decline of SOCS1 expression following cycloheximide removal was then measured as an index of SOCS1 degradation. Similar to [35S]pulse chase results, these studies demonstrated accelerated degradation of SOCS1 in RC3 cells compared with WT8 cells (Fig. 5D).

It has been suggested that SOCS1 and SOCS3 are degraded through the proteasome pathway (50, 76). To determine whether increased SOCS1 decay is due to proteasomal degra-
SOCS1 protein expression was examined in RC3 and WT8 cells preincubated with the proteasome inhibitors lactacystin, MG132, and PSI. Figure 5 demonstrated once again that SOCS1 expression is relatively decreased in VHL mutant RC3 cells. However, proteasome inhibition restored SOCS1 levels in RC3 (Fig. 5E), consistent with a model, in which SOCS1 undergoes enhanced proteasomal degradation in VHL mutant RCC cells.

SOCS1/SOCS2 interaction leads to SOCS1 degradation in RCC. To investigate whether SOCS1 degradation is driven by Elongin B/C E3 ligase complex-directed ubiquitination, WT8 and RC3 cell lysates were immunoprecipitated with anti-SOCS1 antibodies and then immunoblotted with anti-ubiquitin antibodies. In Fig. 6A, increased SOCS1 ubiquitination was observed in RCC cells compared with wild-type, VHL-expressing cells (top), as demonstrated by the upper molecular weight polyubiquitin smear. Although the data are consistent with SOCS1 degradation by direct binding to the Elongin B/C complex, we were unable to coprecipitate SOCS1 with Elongin C (data not shown), suggesting that ubiquitinated SOCS1 is bound to the Elongin B/C through an intermediary protein. Based on a recent report demonstrating that SOCS2 can accelerate SOCS3 proteasomal degradation in Ba/F3 cells (67), we queried whether SOCS2 could play a similar role in RCC cells, by interacting with SOCS1. Figure 6B shows enhanced coprecipitation of SOCS1 with SOCS2 in RC3 vs. WT8 cells. However, no difference in SOCS2/Elongin C interaction was observed between RCC and WT8 cells (data not shown).

DISCUSSION
Our findings provide clear evidence that JAK2, JAK3, and TYK2 tyrosine kinase activities were enhanced in VHL mutation-induced RCC. The JAK/STAT signaling pathway has been extensively characterized in many systems, including hematopoietic, gastrointestinal, prostate, and vascular endothelial cells (2, 4, 5, 17). In the context of cancers, JAK kinases can activate STATs, as well as many other signals, to promote cell survival and proliferation. Constitutive activation of STATs, particularly STAT3, has been reported in a number of tumors, including RCC (70). Several recent reports indicate that gain-of-function JAK2 mutations may account for the majority of cases of polycythemia vera (37, 39, 65). To the best of our knowledge, this is the first report regarding the role of JAK/STAT signaling in VHL mutation-induced RCC pathogenesis.

Most previous studies implicated cytokine- and growth factor- induced JAK/STAT activation in disease pathogenesis (4,
However, recent evidence in transformed pre-B cells demonstrates that cytokine-independent phosphorylation of SOCS1 on Ser/Thr residues by v-Abl allowed JAKs to bypass SOCS regulation, resulting in constitutive JAK activation (40). Consistent with these results, our data illustrate that increased JAK activity in VHL mutation-induced RCC is independent of cytokines and growth factors. The mechanism of constitutive JAK activation was not established, but we speculate it may be related to posttranscriptional SOCS1 modification, which promotes both SOCS1 degradation and JAK release.

It has been repeatedly reported that enhanced JAK activity promotes cell proliferation and survival in both malignant and benign tumors (16, 18, 29, 38, 42, 56). Neither RCC cell line demonstrated significant differences in proliferation or apoptosis, so the effect of JAK inhibition could not be determined for these phenotypes. JAKs were not required for diminished extracellular fibronectin secretion, which is a pathologic feature unique to RCC. However, JAK activity was linked to cell migration and invasion through Matrigel, suggesting that JAKs may regulate RCC cell metastasis in vivo.
The mechanism of JAK regulation in RCC is complex, including interaction with SOCS proteins, which are endogenous inhibitors of JAKs and are upregulated by JAK/STAT activation (26). There are at least three families of proteins that inhibit JAK/STAT signaling: SOCS, protein inhibitors of activated STATs (PIAS), and the SH2-containing phosphatase (SHP-1). SOCS proteins are of particular interest in this study due to structural similarity with VHL. All SOCS gene products contain a COOH-terminal SOCS box, which is homologous to BC box in the α-domain of VHL, which confers ability to bind the Elongin B/Elongin C/Cullin 2 or Cullin 5 ubiquitin ligase complex, resulting in proteasome-mediated protein degradation (21, 32, 33, 64). Furthermore, SOCS1 and SOCS3 possess an NH2-terminal kinase inhibitory region, which directly regulates JAK activity (71).

Transcriptional regulation of SOCS has been investigated in many diseases. For example, inactivation of the SOCS gene by methylation has been previously described in hepatocellular, pancreatic, lung, ovarian, and breast carcinomas (25, 35, 51, 66, 75). Since CpG island methylation generally results in complete absence of transcription, and increased baseline SOCS1 synthesis was observed in RC3 relative to WT8 cells (see Fig. 5B), we reason that hypermethylation of SOCS1 is not a mechanism in RCC. However, our results provide strong evidence that SOCS1 undergoes rapid proteasomal degradation in VHL mutant RCC compared with wild-type VHL-expressing cells. Although a specific mechanism was not determined, we speculate that posttranslationally modified SOCS1 may be more susceptible to degradation. Recent reports revealed that phosphorylation of non tyrosine SOCS1 residues, possibly by Pim1 kinase, disrupts SOCS1 interaction with the proteasome, thereby preventing SOCS1 from targeting activated JAKs for degradation (10, 40, 41). Tyrosine-phosphorylated SOCS3 was also found to interact with and activate Nck and Crk-L (61). Finally, Haan et al. (24) reported that JAK-mediated tyrosine phosphorylation of SOCS3 disrupted interaction with Elongin C and accelerated SOCS3 proteasomal degradation. Tyrosine phosphorylation of SOCS1 in VHL mutation-induced RCC was not observed (data not shown).

Among eight SOCS family molecules, SOCS1 and SOCS3 have been most extensively investigated as regulators of kinase activities, particularly in JAK/STAT signaling pathways. In addition, recent studies demonstrated regulatory cross-talk between SOCS family members. For example, Tannahill et al. (67) found that SOCS2 can accelerate SOCS3 proteasomal degradation in Ba/F3 cells by binding with both Elongin C and SOCS3. Our data are consistent with a similar role for SOCS2 in RCC in this study. Based on these data, we speculate that SOCS2 constitutively binds Elongin B/C to form an E3 ligase complex in wild-type and RCC cells, but SOCS2 preferentially binds SOCS1 in RCC, leading to enhanced SOCS1 ubiquitination and proteasomal degradation.

The mecanism of JAK regulation in RCC is complex, including interaction with SOCS proteins, which are endogenous inhibitors of JAKs and are upregulated by JAK/STAT activation (26). There are at least three families of proteins that inhibit JAK/STAT signaling: SOCS, protein inhibitors of activated STATs (PIAS), and the SH2-containing phosphatase (SHP-1). SOCS proteins are of particular interest in this study due to structural similarity with VHL. All SOCS gene products contain a COOH-terminal SOCS box, which is homologous to BC box in the α-domain of VHL, which confers ability to bind the Elongin B/Elongin C/Cullin 2 or Cullin 5 ubiquitin ligase complex, resulting in proteasome-mediated protein degradation (21, 32, 33, 64). Furthermore, SOCS1 and SOCS3 possess an NH2-terminal kinase inhibitory region, which directly regulates JAK activity (71).

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was also independent of HIF. We now provide additional evidence for HIF-independent regulation of RCC phenotype, namely JAK-dependent cell migration and invasiveness.

In summary, in VHL mutant RCC cells, SOCS1-SOCS2 interaction led to enhanced SOCS1 ubiquitination, SOCS1 instability and degradation, resulting in constitutive JAK activity and accelerated RCC invasiveness. These observations suggest that impaired SOCS-dependent JAK degradation may represent a biochemical mechanism of RCC metastasis in vivo. There are currently no established, successful therapies, e.g., radiation or chemotherapy, for RCC other than nephrectomy, which applies only to patients with no evidence of metastases. We maintain that a better understanding of pathophysiological mechanisms involving JAK-STAT pathway activation may provide new therapeutic approaches for RCC.

ACKNOWLEDGMENTS

We thank Drs. J. Schellang and J. Sedor for assistance with study design, data interpretation, and editing of the manuscript. We appreciate Dr. M. Zhou for anti-HIF-1α antibodies.

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