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

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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 proteolytic 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. Finally, we observe enhanced SOCS2/SOCS1 coprecipitation and reduced SOCS1 expression due to proteasomal degradation in VHL-null RCC compared with wild-type cells, and blocked by dominant-negative JAK expression or JAK 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 support a new VHL-independent mechanism of RCC metastasis, whereby SOCS2 recruits SOCS1 for ubiquitination and proteasome degradation, which lead to unrestricted JAK-dependent RCC invasion. In addition to commonly proposed RCC treatment strategies that target HIFs, our 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). There are currently no established, successful therapies, e.g., radiation or chemotherapy, for RCC other than nephrectomy in situations with no evidence of metastases.

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 α-subunits of hypoxia-inducible factors (HIF)-1 and -2 transcription factors (48, 60), while the VHL α-domain and a small portion of the β-domain interact with Elongin C, a component of an E3 ubiquitin ligase complex that targets HIF-α subunits for proteasomal degradation (31, 43, 64) and thereby prevents HIF transcription factor binding to target genes. Mutations in either VHL α- or β-domains disrupt HIF degradation, which contributes to tumorigenesis by permitting transactivation of HIF target genes, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-α, erythropoietin, epidermal growth factor (EGF) receptor, Glut1 glucose transporter, and carbonic anhydrase IX (CAIX), with the net effect of upregulated angiogenesis and/or cell proliferation (22, 23, 44, 62). In addition to HIFs, pVHL also targets other proteins for degradation (3, 6, 20, 53–55, 57, 58), suggesting that the RCC phenotype may not be regulated entirely by HIF-dependent pathways.

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway contributes to tumorigenesis through regulation of growth factor activity, apoptosis, and angiogenesis. Constitutive activation of STAT1, STAT3, and STAT5 has been implicated in many primary cancers, including RCC (8). Stimulation of cytokine and growth factor receptors leads to direct receptor-JAK interaction, and receptor tyrosine kinase phosphorylation of JAKs. Phosphorylated/activated JAKs then recruit and phosphorylate STAT proteins, which promote dimerization. STAT homo- or heterodimers are rapidly transported from the cytoplasm to the nucleus and are competent for DNA binding (1).

JAK activity is modulated by a negative feedback loop, wherein STATs transcriptionally upregulate suppressor of cytokine signaling (SOCS) expression (11). SOCS then modulates JAK activity by multiple mechanisms. All SOCS isoforms (SOCS 1-7 and CIS) contain a central SH2 domain, which binds and inhibits the tyrosine-phosphorylated JAK catalytic domain (11). In addition, SOCS1 has an NH2-terminal JAK pseudosubstrate domain, which inhibits JAK activity by blocking JAK access to true substrates (50). Finally, SOCS proteins bind the Elongin C component of the ubiquitin ligase complex, which stabilizes SOCS, and leads to proteasomal degradation of SOCS-bound proteins, including JAKs. A recent report from Tannahill et al. (67) demonstrates that SOCS3 and SOCS2 heterodimerize, and binding of the complex to Elongin C led to SOCS2 stabilization and SOCS3 degradation.

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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 vector (RC3) or wild-type VHL (WT8) (gifts from Dr. B. Kaelin). Cells were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS and 0.5 μg/l G418 in humidified incubator (Dr. E. Maher). Cells were cultured in 10% FBS containing DMEM in humidified incubator.

RCC4 cells (VHLSer65Trp) (32) were transfected with wild-type vector (RC3) or wild-type VHL (WT8) (gifts from Dr. B. Kaelin). Cells were transfected with a P200 pipette tip. Cells were then incubated with or without JAK inhibitors AG490 (100 μM), P131 (300 μM), JAK31-V (50 μM), AG9 (100 μM), or JAK1-1 (500 μM). Migration was determined at 10 magnification.

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, K88E and Y1007F, were kind gifts from Dr. O. Silverstein (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 using a Maxiprep kit (Qiagen, Valencia, CA), and amplified by culture according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA), extracted with a P200 pipette tip. Cells were then incubated with or without JAK inhibitors AG490 (100 μM), P131 (300 μM), JAK31-V (50 μM), AG9 (100 μM), or JAK1-1 (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 polyvinylidene 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 Restorations 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 assay (0.5 μM IGF-1) was added to bottom chambers of transwells. Cells (6.25 × 10^4) 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 RCC3 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 confluence, washed in PBS, and incubated with [35S]methionine in methionine-free DMEM (0.1 μCi/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 degradation 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 phospho-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) and probed with anti-JAK3 antibodies. 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 α-PY. Bottom: stripped blot was reprobed for anti-TYK2 antibody as a 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-STAT3 antibodies. Middle: parallel lysates were immunoblotted with anti-STAT3 antibodies. Bottom: blot was stripped and reprobed with anti-α-tubulin antibodies as a protein loading control. E: RC3 cells were stably transfected with either mock vector or shRNA construct targeted to HIF2α. Both groups were maintained in serum-free media overnight. Top: whole cell lysates were immunoblotted with anti-phospho-JAK2 antibodies. Panel 2: blots were stripped and reprobed for JAK2 expression. Panel 3: to verify efficacy of shRNA knockdown, lysates were probed for HIF2α expression by immunoblot analysis. Bottom: blot was stripped and reprobed with 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 (JAK1-I), 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 JAK1-I 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 JAK1-I 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...
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. C: 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-STAT3 and anti-α-tubulin antibodies. *P < 0.05 compared with WT8 cell invasion.

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.
B), 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-express-
ing cells. Although a specific mechanism was not determined,
we speculate that postranslationally modified SOCS1 may be
more susceptible to degradation. Recent reports revealed that
phosphorylation of nontyrosine 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 be-
tween 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 ubiquiti-
nation and proteasomal degradation.

The net effect of diminished SOCS1 expression was
decreased JAK inhibition, thereby promoting RCC migra-
tion through activation of JAK-dependent pathways. Be-
cause in vitro invasion and migration are surrogate assays
for metastasis in vivo, we suggest that JAKs may regulate
RCC metastasis through a SOCS-dependent mechanism. To
assess JAK regulation of RCC invasion and migration, we used
pharmacological inhibitors and dominant-negative JAK mu-
tants in two different assay systems. In some cases, there was
slight discrepancy in magnitude of the effect on invasion/migra-
tion between different inhibitors of the same kinase,
which could be due to variable intracellular access to JAKs or
overlapping specificities. However, when viewed in aggregate,
we conclude that JAK2, JAK3, and TYK2 each contribute to
regulation of RCC motility.

The VHL literature is replete with studies highlighting the
significance of HIF in RCC pathogenesis (36, 46, 48, 52).
HIF1α interaction with wild-type VHL is required for con-
stitutive HIF1 ubiquitination, proteasomal degradation, and as
a consequence, inhibition of HIF1 substrates activities (13, 34).
HIF1α activation due to impaired binding with mutant VHL
results in the angiogenic phenotype of VHL-associated tumors
in VHL kidneys (46, 48); reconstitution of VHL restores
HIF1α ubiquitination and reverses the angiogenesis phenotype
(34). However, HIF1α activation may not be sufficient to
explain all aspects of VHL mutation-dependent RCC (47). A
recent study showed that VHL inactivation disrupts intercellu-
lar junctions and cell shape through HIF-independent events in
RCC cells, supporting the concept that VHL has additional
functions beside its role in the regulation of HIF (9). Hsu et al.
(28) demonstrated that in VHL null RCC cell lines, deficient
FGF receptor cycling, resulting in enhanced cell migration,
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.

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