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NF-κB–Dependent Plasticity of the Epithelial to Mesenchymal Transition Induced by *Von Hippel-Lindau* Inactivation in Renal Cell Carcinomas

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Abstract

The critical downstream signaling consequences contributing to renal cancer as a result of loss of the tumor suppressor gene *von Hippel-Lindau* (*VHL*) have yet to be fully elucidated. Here, we report that *VHL* loss results in an epithelial to mesenchymal transition (EMT). In studies of paired isogenic cell lines, *VHL* silencing increased the levels of N-cadherin and vimentin and reduced the levels of E-cadherin relative to the parental VHL^+ cell line, which displayed the opposite profile. VHL^+ cells grew as clusters of cuboidal and rhomboid cells, whereas VHL-silenced cells took on an elongated, fibroblastoid morphology associated with a more highly invasive character in Matrigel chamber assays. Based on earlier evidence that *VHL* loss can activate NF-κB, a known mediator of EMT, we tested whether NF-κB contributed to VHL-mediated effects on EMT. On pharmacologic or molecular inhibition of NF-κB, VHL-silenced cells regained expression of E-cadherin, lost expression of N-cadherin, and reversed their highly invasive phenotype. Introducing a pVHL-resistant hypoxia-inducible factor 1α (HIF1α) mutant ($HIF\alpha^M$) into VHL^+ cells heightened NF-κB activity, phenocopying EMT effects produced by VHL silencing. Conversely, inhibiting the heightened NF-κB activity in this setting reversed the EMT phenotype. Taken together, these results suggest that *VHL* loss induces an EMT that is largely dependent on HIFα-induced NF-κB. Our findings rationalize targeting the NF-κB pathway as a therapeutic strategy to treat renal tumors characterized by biallelic *VHL* inactivation. *Cancer Res; 70(2); 752–61. ©2010 AACR.*

Introduction

During embryogenesis, in a morphogenetic process known as epithelial to mesenchymal transition (EMT), normal epithelial cells transiently acquire the phenotype of mesenchymal cells, whereby they dislodge from their sites of origin and migrate to distant sites. Increasing evidence supports the notion that epithelial malignancies (i.e., carcinomas), which represent the great majority of human malignancies, subvert their natural tendency to form compact cellular clusters through tight cell-cell adhesion by undergoing EMT and thereby maximize their invasive and metastatic potential (1, 2). During EMT, expression of epithelial markers that promote cellular adhesion, such as E-cadherin and γ-catenin, decreases, whereas expression of proteins that typify motile mesenchymal cells, such as N-cadherin, vimentin, matrix metalloproteinases, integrins, and smooth muscle actin, is acquired (2). A multitude of biochemical signals has been shown to activate the EMT program, including receptor tyrosine kinases, WNT, and transforming growth factor β (TGFβ), to name a few. These signals converge on several transcription factors, including Snail, Slug, Zeb1, Zeb2, E47, and Twist, which transcriptionally induce the EMT program (1, 2). For example, transcriptional downregulation of *CDH1*, the gene that encodes E-cadherin, by Snail, Slug, or Twist, is an essential (but not sufficient) component of EMT.

Renal cell carcinomas (RCC), which comprise several different histologic subtypes, have been increasing in incidence over the last several years. One third of patients present with metastatic disease, and an additional 30% recur after nephrectomy (3). The clear cell variant represents about 80% to 85% of RCCs, and despite the introduction of modestly effective molecularly targeted therapies over the last 2 years, metastatic disease is incurable, although rare patients experience durable complete remissions in response to high-dose interleukin-2 (4). Thus, the genetic and biochemical events that drive the inexorable growth and metastasis of clear cell RCC (CCRCC) must be more fully elucidated to identify appropriate therapeutic targets.

Hereditary CCRCC, which occurs in the setting of the von Hippel-Lindau (VHL) syndrome, arises from germ-line mutations of one of the *VHL* gene alleles. At the molecular level, VHL disease arises from somatic loss or inactivation of the remaining wild-type allele and thus conforms to the Knudson two-hit model. The importance of *VHL* mutations in the pathophysiology of CCRCC is underscored by the fact that

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~90% of sporadic CCRCC cases manifest biallelic loss/inactivation at the *VHL* locus as a consequence of gross genetic loss, nonsense and missense point mutations of *VHL*, as well as hypermethylation of the *VHL* promoter (5).

The protein encoded by the *VHL* gene, pVHL, functions as an E3 ubiquitin ligase that targets various proteins for degradation by the 26S proteasome. A key pVHL target is hypoxia-inducible factor 1α (HIFα), a principal regulator of cellular responses to hypoxia, and HIFα expression is required but not sufficient for renal carcinogenesis mediated by *VHL* loss (6–8). HIFα serves as a transcription factor, and its expression in the context of biallelic inactivation of the *VHL* gene drives the transcription of genes that promote angiogenesis (e.g., *vascular endothelial growth factor*), proliferation (e.g., *TGFα*), anaerobic metabolism (e.g., *glucose transporter 1*), as well as many other cellular functions. However, the precise downstream targets of HIFα and of pVHL for that matter that drive renal oncogenesis are not fully defined.

NF-κB represents a family of transcription factors that modulate expression of genes with diverse functions. The activity of NF-κB is regulated by IκB, the NF-κB inhibitory protein that binds to and sequesters NF-κB family members in the cytoplasm. When the NF-κB pathway is activated, IκB is phosphorylated by IκB kinase (IKK), which phosphorylates IκB at serine residues 32 and 36 (9). Phosphorylated IκB is subjected to ubiquitination and proteasome-mediated degradation, which results in the translocation of NF-κB to the nucleus.

Constitutive NF-κB activity has been implicated in the malignant progression of numerous hematologic and solid malignancies (10). Mounting evidence supports a role for NF-κB in renal oncogenesis. The preclinical evidence for NF-κB activation in RCC is as follows. Constitutive NF-κB activation has been observed in many RCC cell lines (11–13). Inhibition of NF-κB sensitizes RCC cells to tumor necrosis factor α (TNFα) and TNFα-related apoptosis-inducing ligand, and NF-κB blockade retards the growth of murine RCC xenografts (11, 12, 14). The clinical evidence for the role of NF-κB in RCC is highlighted by a study showing that heightened NF-κB activation is associated with the development and progression of RCC in actual patients (15). Moreover, NF-κB activation not only is a frequent observation among RCC patient samples but also correlates with primary tumor size (14).

Recently, we and others reported that biallelic inactivation of *VHL* leads to activation of the NF-κB (12, 16, 17). Activation of NF-κB has been causally linked to an invasive phenotype and can directly or indirectly induce expression of Snail, Slug, Twist, Zeb1, and Zeb2 (18). Thus, we sought evidence that *VHL*-null CCRCCs undergo the transdifferentiation process of EMT in a NF-κB–dependent fashion.

Materials and Methods

Cell lines. RCC cell lines ACHN and SN12C (kind gift of Dr. George Thomas, David Geffen School of Medicine at UCLA, Los Angeles, CA), which endogenously express wild-type pVHL, were transduced with lentivirus that encodes VHL-specific short hairpin RNA (shRNA) or a scrambled control; these cell lines and the VHL shRNA sequences have been described (19). SN12C cells were originally isolated from a radical nephrectomy specimen from a patient with a granular cell type RCC (20), and ACHN cells, which are commercially available (American Type Culture Collection), were derived from a malignant pleural effusion of a young male patient with CCRCC (21). A second isogenic pair of cell lines was generated by transduction of VHL-specific and nonsilencing shRNA expressed from the GIPZ lentiviral construct (Open Biosystems) followed by selection in puromycin. The VHL-specific shRNA sequence was as follows : 5'-*CGGCTAGACTTAGATTCATTAA*TAGTGAAGCCACAGATGTA-3'**TTAATGAATCTAAGTCTAGCCT** (italics, sense; underline, loop; bold, antisense).

In addition, we generated lines of ACHN and SN12C cells that express a pVHL-resistant version of HIF1α ($HIF\alpha^M$) by transducing a retrovirus expressing the $HIF\alpha^M$, in which the proline hydroxylation sites are mutated to alanines by site-directed mutagenesis (a gift of Dr. William Kaelin, Howard Hughes Medical Institute, Dana-Farber Cancer Institute, and Brigham and Women's Hospital, Boston, MA). Transduction of empty retroviral vector (pBabe) served as a negative control. Stable lines were selected in puromycin.

Inhibition of NF-κB activity. A cell-permeable IKKβ inhibitor was purchased from Calbiochem. An adenovirus expressing the IκB "superrepressor" (Ad-IκB-SR) was used as a molecular approach to inhibit NF-κB. The IκB-SR contains mutations at the phosphorylation sites (Ser^{32} to Ala and Ser^{36} to Ala), which thereby renders it resistant to ubiquitination and proteasome-mediated degradation.

Measurement of NF-κB activity. NF-κB activity was measured by electrophoretic mobility shift assay (EMSA), NF-κB–driven reporter gene expression, and an IKKβ *in vitro* kinase assay as we have previously described (22). The sequences of the oligonucleotides used for the EMSAs are as follows: NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′ (wild-type) and 5′-AGTTGAGGCGACTTTCCCAGGC-3′ (mutant); Oct-1, 5′-TGTCGAATGCAAATCACTAGAA-3′ (wild-type) and 5′-TGTCGAATGCAAGCCACTAGAA-3′ (mutant).

Western blotting. Western blotting was performed as previously described (22). The primary antibodies and their final dilutions were as follows: E-cadherin (1:1,000; BD Biosciences), N-cadherin (1:1,500; BD Biosciences), vimentin (1:1,000; Chemicon), HIF1α (1:500; BD Biosciences), actin (1:1,000; Santa Cruz Biotechnology), lamin A/C (1:1,000; Cell Signaling Technology), IKKβ (1:1,000; Cell Signaling Technology), VHL (1:1,000; BD Biosciences), phospho-IκBα (1:1,000; Cell Signaling), Zeb1 (1:1,000; Abcam), Slug (1:500; Abcam), Snail (1:1,000; Abcam), Zeb2 (1:1,000, Abcam), and Twist (1:500; Santa Cruz Biotechnology).

Matrigel invasion assay. The Matrigel invasion assay was performed according to the manufacturer's instructions (BD Biosciences). Briefly, 2.5×10^4 cells in 0.5 mL of medium containing 1% fetal bovine serum (FBS) were added to the Transwell insert, which was seated in 750 μL of complete medium (10% FBS) with or without the IKKβ inhibitor (10 μmol/L). After a 24-h incubation at 37°C in a 5% CO_2 humidified atmosphere, noninvading cells were mechanically removed. Cells that had migrated through the Matrigel were stained with

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the Diff-Quick staining kit (Dade Behring, Inc.) according to the manufacturer's instructions. Cells were counted in five representative microscopic fields (×200 magnification) and photographed.

Anchorage-independent growth assay. This assay was performed as described by us (22).

Results

VHL *loss results in the morphologic, gene expression, and cell biological changes characteristic of EMT.* To recapitulate the effects of *VHL* loss as it occurs during renal oncogenesis, we evaluated the effects of gene silencing of *VHL* by RNA interference in RCC models that endogenously express wild-type pVHL. Thus, we studied EMT in isogenic RCC cell lines (SN12C and ACHN) in which the parental cell line maintains wild-type pVHL expression and therefore lacks HIFα expression, whereas the isogenic partner was transduced with a retrovirus that expresses VHL shRNA, thereby reducing pVHL expression, which results in stabilization and expression of HIFα (Fig. 1*A*). We observed marked differences in the morphology and tendency to form cellular nests or clusters between the VHL^+ parental cells (transduced with a control vector) and their VHL-silenced (VHL^{low}) counterparts (Fig. 1*B*). The VHL^+ cells exhibited a polygonal shape and formed tight clusters of cells, indicative of an epithelial phenotype. In contrast, the VHL^{low} cells took on an elongated, fibroblastic morphology with dendritic processes, consistent with a mesenchymal transition (Fig. 1*B*). VHL^{low} cells also had a greater predilection to break away as single cells and did not form distinct cellular clusters.

We next tested whether the VHL^{low} SN12C and ACHN cells manifested expression of specific EMT markers. As shown in Fig. 1*C*, VHL^{low} cells underwent a "cadherin switch," whereby they manifested reduced E-cadherin expression and increased N-cadherin expression. In further support of an EMT in response to pVHL suppression, vimentin expression increased in the VHL^{low} compared with the VHL^+ cells. Similar results were obtained when we silenced pVHL expression with a second shRNA lentiviral vector targeting a different VHL sequence (Supplementary Fig. S1).

Cells that have undergone EMT tend to exhibit greater migration and invasiveness. As such, we examined these properties in VHL^+ versus VHL^{low} cells in a Matrigel invasion assay. Striking differences were observed. Both ACHN VHL^{low} and SN12C VHL^{low} cells readily migrated through the Matrigel chamber in relatively high numbers, whereas their VHL^+ isogenic partners exhibited a marked reduction in invasion in this assay at 24 hours (Fig. 1*D*). The proliferation of VHL^+ and VHL^{low} did not differ over 24 hours, as shown by cell counting by trypan blue exclusion (data not shown), which excludes differences in cell number as an explanation for the results of the invasion assays. Taken together, our data indicating changes in morphology, growth pattern, protein expression, and invasion support the notion that suppression of pVHL leads to EMT.

VHL^{low} cells exhibit heightened NF-κB activity. We and others have previously shown that inactivation of *VHL* results in activation of NF-κB in multiple RCC models (12, 13, 15). Thus, we sought to determine whether the EMT observed in VHL^{low} RCC cells was attributable to heightened NF-κB activity. First, we documented that NF-κB activity was indeed increased in VHL^{low} compared with VHL^+ ACHN and SN12C cells. As shown in Fig. 2*A* and *B*, NF-κB was demonstrably higher in VHL^{low} cells compared with the VHL^+ counterparts as determined by EMSAs and NF-κB reporter assays. The specificity of gel-shifted bands in the EMSA were documented by cold competition experiments (see Materials and Methods), in which excess cold wild-type but not cold mutant κB probe abrogated the signals from the shifted bands. Moreover, to establish that the effect of pVHL modulation on the NF-κB EMSA was not a generalizable phenomenon, we showed that the Oct-1 EMSA signals were similar in VHL^+ and VHL^{low} cells (Fig. 2*A*). Electrophoretic mobility supershift assays showed that the NF-κB complexes were composed of p65-p50 and p50-p50 dimers (Fig. 2*C*), findings consistent with activation of the classic as opposed to the alternative NF-κB pathway.

Activation of the classic NF-κB pathway is typically mediated by biochemical signaling events that converge on the IKK complex, which consists of the IKKγ (NEMO), IKKα, and IKKβ isoforms (9). The activated IKK complex phosphorylates the inhibitor of NF-κB, IκB, thereby targeting it for ubiquitination and subsequent proteasomal degradation, which allows for the translocation of NF-κB family members (e.g., p65) to the nucleus. Because stimulation of the classic pathway requires recruitment and activation of IKKβ to the IKK complex, we assessed the state of IKKβ activation in VHL^+ versus VHL^{low} cells. Consistent with the EMSA and the NF-κB reporter studies, constitutive IKKβ kinase activity was heightened in VHL^{low} compared with the VHL^+ cells in the SN12C and ACHN isogenic pairs (Fig. 2*D*).

Heightened NF-κB activity in VHL^{low} cells mediates EMT. Having established that suppression of pVHL expression in SN12C and ACHN cells results in elevated NF-κB activity, we next investigated the potential for inhibition of NF-κB to reverse the mesenchymal characteristics of VHL^{low} cells. Toward this end, we used both molecular and pharmacologic means of inhibiting NF-κB in these cells and then compared the resulting phenotype with control-treated cells. Molecular inhibition was accomplished by transduction of an adenovirus that expresses a dominant-active form of IκB (Ad-IκB-SR; see Materials and Methods). Using EMSAs to measure NF-κB activity, we found that the Ad-IκB-SR but not the control virus (Ad-CMV) abolished NF-κB activity when transduced at a multiplicity of infection (MOI) of 10 (Supplementary Fig. S2*A*). The Ad-IκB-SR did not influence an Oct-1 EMSA. We used a commercially available IKKβ inhibitor to pharmacologically block NF-κB activity. The NF-κB inhibitory properties of the IKKβ inhibitor were established by EMSA, NF-κB reporters assays, and IKKβ *in vitro* kinase assays, which all showed a dose-dependent increase in NF-κB inhibitory activity up to a concentration of 10 μmol/L (Supplementary Fig. S2*B–D*).

Inhibition of NF-κB activity by either the Ad-IκB-SR or the IKKβ inhibitor resulted in a change in protein expression characterized by increased E-cadherin and reduced

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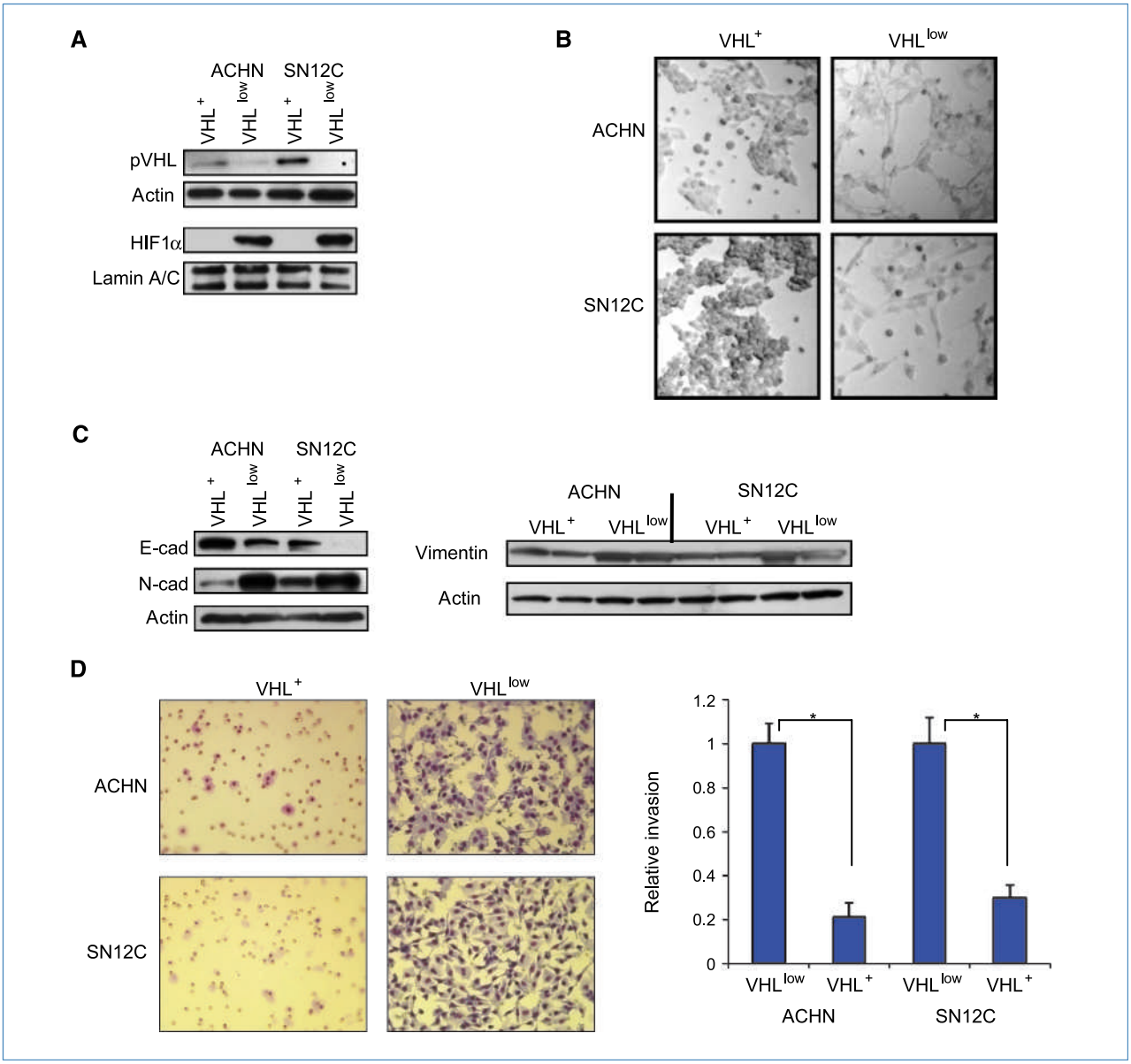

Figure 1. Suppression of pVHL expression results in EMT. *A,* expression of pVHL and HIF1α in VHL$^+$ and VHL^{low} cell lines. Total cellular protein was used to detect pVHL. Actin served as a loading control. Nuclear extracts were the source of protein for detection of HIF1α, so lamin A/C, a nuclear envelope protein, served as the loading control. *B,* phase-contrast photomicrographs of VHL$^+$ and VHL^{low} cells. Original magnification, ×200. *C,* Western blots for E-cadherin, N-cadherin, and vimentin. *D,* Matrigel invasion assay. *Left,* photomicrographs of cells that have passed through Matrigel. Original magnification, ×100. *Right,* quantification of invasion (see Materials and Methods for details). *, $P < 0.05$.

N-cadherin expression, consistent with reversion to an epithelial phenotype (Fig. 3*A* and *B*). The effects of the IKKβ inhibitor were dose dependent (Fig. 3*B*). In time course experiments, we showed that IKKβ inhibition induces a rapid (i.e., within 1–4 hours) and sustained cadherin switch (Fig. 3*C*). Importantly, inhibition of NF-κB by either the Ad-IκB-SR or the IKKβ inhibitor led to a marked decrease in the invasiveness of VHL^{low} cells compared with control virus–treated and vehicle-treated cells, respectively (Fig. 3*D*). Despite these changes in protein expression and invasiveness attributable to NF-κB blockade, we did not observe any profound changes in the morphology or growth patterns of VHL^{low} ACHN or SN12C cells in two-dimensional culture (data not shown), an observation that implicates NF-κB–independent biochemical events that contribute to the EMT phenotype in VHL^{low} cells.

VHL^{low} cells exhibit NF-κB–dependent upregulation of Slug and Twist, transcriptional regulators of the EMT program. The gene expression program that mediates EMT is regulated by one or more transcription factors, including Snail, Slug, Zeb1, Zeb2, and Twist (2, 18). These transcription factors influence the expression of cadherins and metallopro-

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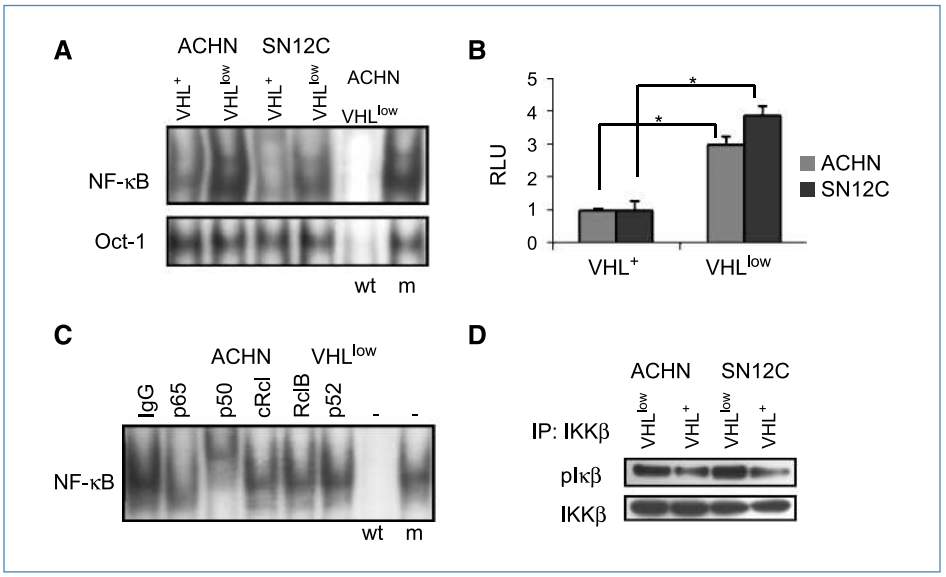

Figure 2. Heightened state of the classic NF-κB pathway in VHL^{low} cells. *A,* EMSAs for NF-κB (*top*) and Oct-1 (*bottom*). *Far right two lanes,* cold competition experiments. *wt,* wild-type; *m,* mutant. *B,* NF-κB reporter assays. Cells were cotransfected with a NF-κB–driven *firefly* luciferase reporter and a *Renilla* luciferase reporter for normalization of transfections efficiency. *Columns,* mean of triplicates obtained at 48 h; *bars,* SD. *, $P < 0.05$ *C,* electrophoretic mobility supershift assay in ACHN VHL^{low} cells. Similar results were obtained for SN12C VHL^{low} cells. *D,* IKKβ *in vitro* kinase assays in VHL^{+} and VHL^{low} cell lines.

teinases, among other proteins involved in EMT. That these transcription factors are transcriptionally induced by upstream signaling pathways, including NF-κB (18), prompted us to study their differential expression in VHL^{low} versus VHL^{+} SN12C and ACHN cells. Similar levels of expression were observed for Snail, Zeb1, and Zeb2 in the VHL^{low} and VHL^{+} SN12C and ACHN cells (Fig. 4*A*). However, VHL^{low} ACHN and SN12C cells exhibited substantial upregulation of Slug and Twist as compared with the levels in VHL^{+} cells (Fig. 4*A*). Pharmacologic inhibition of IKKβ in VHL^{low} cells resulted in a dose-dependent decrease in Slug and Twist expression, a finding that indicts the heightened state of NF-κB as an etiologic biochemical force underlying Slug and Twist overexpression in VHL^{low} cells (Fig. 4*B*). The effects of IKKβ inhibition on Slug and Twist were shown in time course experiments as shown in Fig. 4*C*. These findings indicate that the augmented expression of Twist and Slug that occurs in the setting of inactivation/loss of pVHL is mediated by heightened NF-κB activity.

Expression of a pVHL-resistant HIF1α in VHL^{+} cells induces EMT in a NF-κB–dependent manner. Recent reports have established an etiologic link between HIFα expression in *VHL*-null CCRCC cells to suppression of E-cadherin and acquisition of a mesenchymal phenotype through a mechanism that involves upregulation of the transcription factors, such as Twist, Zeb1, Zeb2, and Snail, which are known to regulate the EMT program by transcriptionally repressing *CDH1*, the gene that encodes for E-cadherin (23–25). We have previously reported that the NF-κB activation that develops in the setting of *VHL* loss/inactivation occurs through a HIFα-dependent mechanism (16). Thus, we hypothesized that the EMT that RCC cells undergo in response to HIFα expression occurs, at least in part, due to the HIFα-mediated activation of the NF-κB pathway.

Using a retroviral delivery system, we stably introduced a pVHL-resistant HIF1α mutant (HIFα^{M}; see Materials and Methods) into VHL^{+} ACHN cells. We isolated a subclone that manifests HIF1α expression that is equivalent to that of VHL^{low} cells (Fig. 5*A*) so that any potential results derived from studies involving HIFα^{M} expression would not be attributable to an artifact of overexpression. Whereas HIF1α was undetectable in the parental VHL^{+} cells transduced with control retroviral particles (pBabe), the HIF1α levels in VHL^{+}/HIFα^{M} cells were comparable with those in the VHL^{low} cells. Next, we confirmed that NF-κB activity did in fact increase in these VHL^{+}/HIFα^{M} model systems. Indeed, VHL^{+}/HIFα^{M} exhibited heightened NF-κB activity as determined by IKKβ *in vitro* kinase assays and EMSAs (Fig. 5*B* and *C*).

Having established that VHL^{+}/HIFα^{M} cells manifest heightened NF-κB activity, we next assessed these cells for evidence of EMT. Compared with VHL^{+} cells, VHL^{+}/HIFα^{M} cells exhibited an increase in N-cadherin expression and suppression of E-cadherin (Fig. 6*A*). This cadherin switch was associated with increased Slug and Twist expression but no notable changes in Snail, Zeb1, or Zeb2 expression (Fig. 6*A*), findings that are reminiscent of those observed in VHL^{low} cells (see Fig. 4*A*). The role of NF-κB in modulating these protein expression changes is illustrated by the effects of inhibition of NF-κB in VHL^{+}/HIFα^{M} cells by exposure to the IKKβ inhibitor, which reversed the cadherin switching phenomenon as well as reduced the expression of Slug and Twist (Fig. 6*B*).

Growth of VHL^{+}/HIFα^{M} cells and its dependence on heightened NF-κB activity were evaluated in three-dimensional models. For example, invasiveness of VHL^{+}/HIFα^{M} cells through a Matrigel chamber was significantly greater than that of VHL^{+} cells (Fig. 6*C*). Similarly, anchorage-independent growth in soft agar was enhanced in VHL^{+}/HIFα^{M} and VHL^{low} cells compared with their VHL^{+} counterparts (Fig. 6*D*). The enhanced invasion and anchorage-independent growth of VHL^{+}/HIFα^{M} cells was abrogated by NF-κB blockade by means of ectopic expression of the IκB-SR by adenoviral

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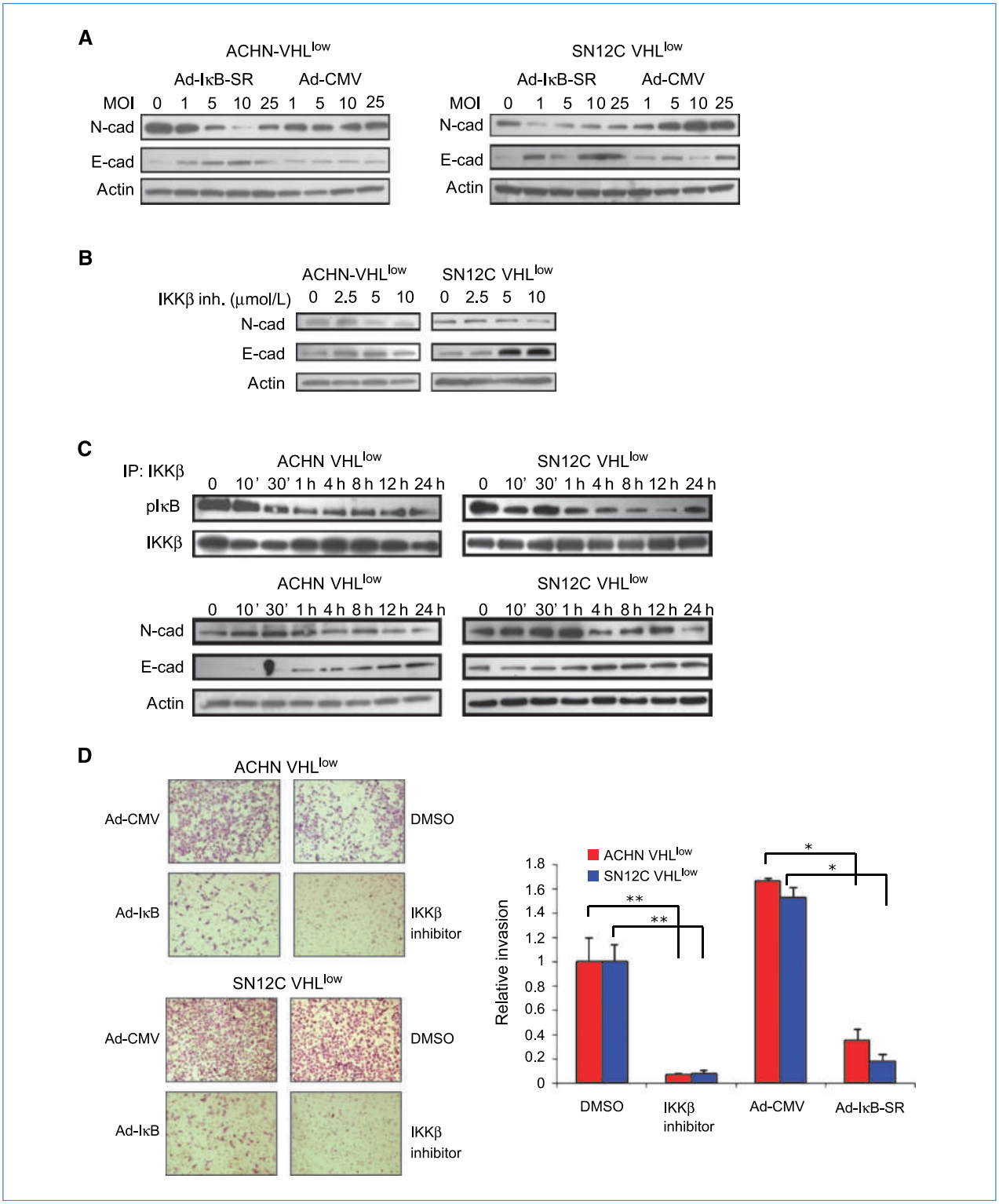

Figure 3. Inhibition of NF-κB reverses EMT characteristics of VHL^{low} cells. *A,* effects of expression of the Ad-IκB-SR on N-cadherin and E-cadherin expression in ACHN and SN12C VHL^{low} cells. *B,* dose-dependent effects of a 24-h exposure to IKKβ inhibitor on N-cadherin and E-cadherin expression in VHL^{low} cells. *C,* time course experiments of IKKβ inhibitor (10 μmol/L) on E-cadherin and N-cadherin expression. *Top,* efficacy of the IKKβ inhibitor. *D,* Matrigel invasion assays. *Left,* photomicrographs after cells were treated with Ad-IκB-SR (MOI, 10; see Supplementary Fig. S2) or IKKβ inhibitor (10 μmol/L) or appropriate respective controls. Original magnification, ×100. *Right,* quantification of invasion assay (see Materials and Methods for details). *, $P < 0.05$; **, $P < 0.01$.

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transduction and exposure to the IKKβ inhibitor (Fig. 6*C* and *D*). Thus, the augmentation in invasiveness and anchorage-independent growth in RCC cells that are typified by biallelic inactivation of the *VHL* gene occurs as a result of HIFα-dependent activation of the NF-κB pathway.

NF-κB has been recently shown to induce transcription of *HIFα* (26). Indeed, when we exposed VHL^{low} to the IKKβ inhibitor and thereby blocked NF-κB activation, HIFα expression was reduced (Supplementary Fig. S3). These results imply that in RCC cells typified by biallelic inactivation of *VHL*, a "vicious cycle" exists whereby HIFα expression induces NF-κB activity, which in turn promotes HIFα expression.

Discussion

A connection between biallelic inactivation of *VHL* and suppression of E-cadherin expression has been previously reported in both preclinical models and patient specimens, and HIFα has been indicted as mediating this phenomenon through induction of Twist, Slug, Snail, Zeb1, and Zeb2 depending on the cellular context (23–25, 27). However, the molecular underpinnings of HIFα-induced upregulation of these EMT-inducing transcription factors have been largely undefined, although evidence in support of the ability of HIF1α to directly induce *Twist* transcription through binding to a hypoxia response element within the *Twist* proximal promoter has been recently described in human embryonic kidney cells (28).

Here, we have shown that the EMT program attributable to *VHL* loss is driven by activation of the classic NF-κB pathway. This EMT program is characterized by N-cadherin and vimentin expression and E-cadherin suppression, striking morphologic changes, and a highly invasive mesenchymal phenotype. Importantly, this mesenchymal transformation can in large part be reversed by inhibiting NF-κB through either molecular or pharmacologic approaches, although NF-κB blockade does not promote a complete reversion to an epithelial phenotype, as evidenced by maintenance of the fibroblastic morphology in the face of IKKβ pharmacologic inhibition or ectopic expression of the IκB-SR. This latter finding suggests the existence of VHL-dependent, NF-κB–independent EMT regulatory pathways that remain to be defined.

We have also found that the two principal transcription factors that are differentially regulated in *VHL* wild-type compared with their pVHL-suppressed isogenic partners are Twist and Slug. The expression of these transcription factors can be induced in *VHL* wild-type cells by the ectopic expression of a pVHL-resistant HIF1α. These HIF1α mutant cells exhibit heightened NF-κB activity, the inhibition of which results in the downregulation of Twist and Slug. Moreover, NF-κB blockade in these cells results in reversion of the cadherin switch to that of an epithelial phenotype typified by reduced invasion and anchorage-independent growth,

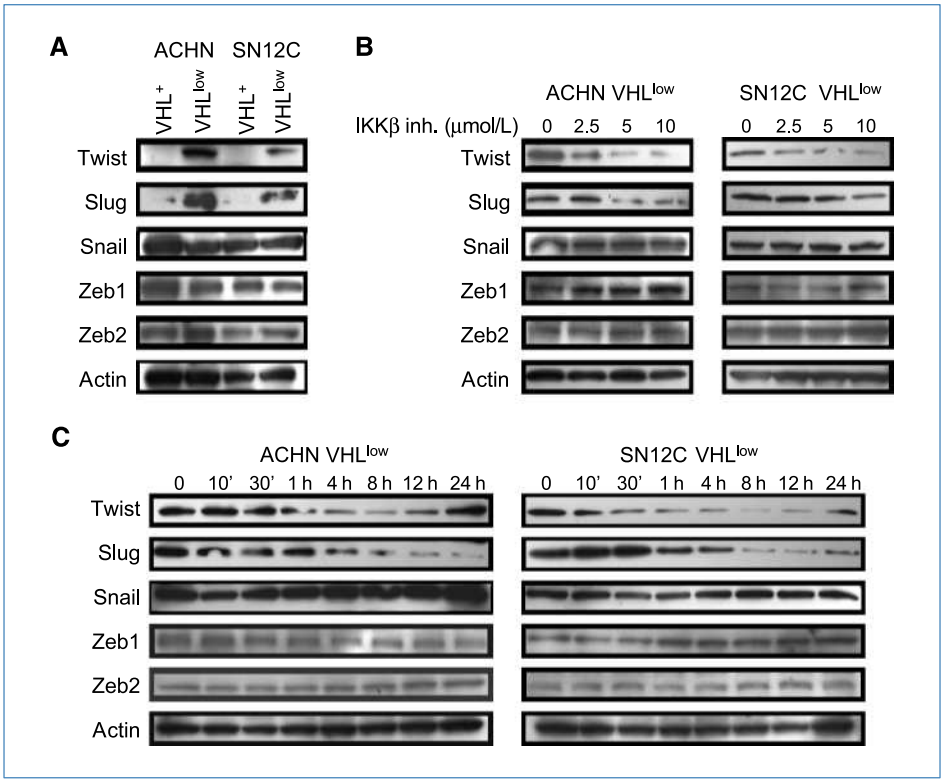

Figure 4. Heightened expression of Twist and Slug in VHL^{low} cells is attributable to increased NF-κB activity. *A,* baseline expression of EMT-mediating transcription factors: Twist, Slug, Snail, Zeb1, and Zeb2 in VHL^{+} versus VHL^{low} cells. *B,* dose-dependent effects of a 24-h exposure of the IKKβ inhibitor on expression of Twist, Slug, Snail, Zeb1, and Zeb2. *C,* expression of Twist, Slug, Snail, Zeb1, and Zeb2 after exposure to the IKKβ inhibitor at 10 μmol/L for the indicated times.

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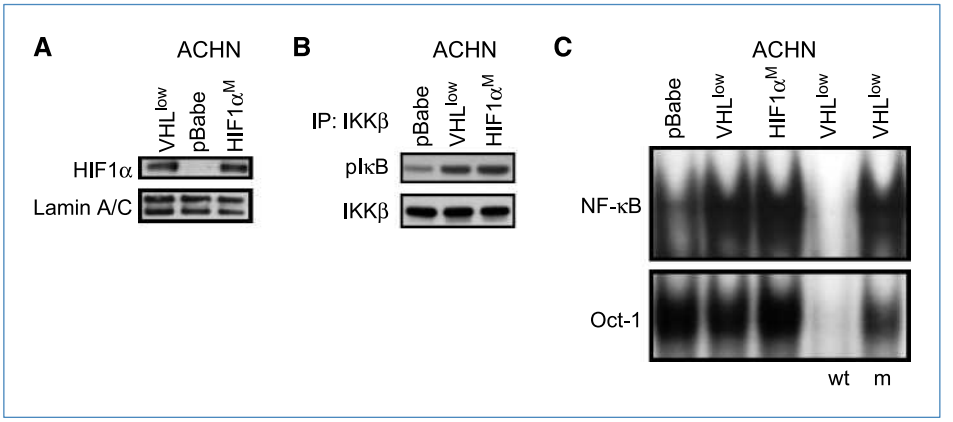

Figure 5. Ectopic expression of a pVHL-resistant mutant version of HIF1α in VHL$^+$ cells induces NF-κB activation. *A,* HIF1α expression in VHL^{low} cells, VHL$^+$ cells transduced with pBabe, and VHL$^+$ cells transduced with a mutant HIF1α that is resistant to pVHL-mediated ubiquitination and subsequent degradation. *B,* IKKβ *in vitro* kinase assays in VHL^{low}, VHL$^+$/pBabe, and VHL$^+$/HIFα^M. *C,* same as *B* but NF-κB EMSAs. *Right two lanes,* cold competition EMSA; *bottom,* Oct-1 EMSA.

findings that implicate NF-κB as a principal mediator of the HIFα-induced EMT program in *VHL*-null cells. Thus, because Twist and Slug transcriptionally repress the gene encoding E-cadherin (*CDH1*) and are regulated by NF-κB (18), the correlation between *VHL* loss and E-cadherin is apt to result from heightened NF-κB activity. In addition, our findings that the EMT program is induced by expression of the HIFα^M is consistent with a previous report showing that HIFα contributes to EMT by reducing expression of the tight junction and adherens junction proteins occludin and claudin 1, respectively (29). One potential connection between HIFα and suppression of occludins and claudins may relate to the Slug expression observed in HIFα-expressing cells because Slug has been shown to transcriptionally repress expression of *claudins* and *occludins* (30).

Other groups have identified Zeb1, Zeb2, and Snail (as opposed to Twist and Slug) as central regulators of E-cadherin suppression and EMT in CCRCC models (24, 25), which suggests that the specific transcription factors that are modulated by *VHL* loss are context dependent. Importantly, all of these EMT-modulating transcription factors, including Twist, Snail, Slug, Zeb1, and Zeb2, are regulated by NF-κB (18) so that it is plausible that the NF-κB activation resulting from *VHL* loss represents a unifying biochemical event that accounts for the EMT observed in CCRCCs, the vast majority of which are characterized by biallelic inactivation of *VHL*.

Two mechanisms to explain the augmentation of NF-κB activity as a result of *VHL* loss have been reported. We originally reported that inactivation of *VHL* leads to NF-κB activation in a HIFα-dependent fashion, whereby growth factors elaborated by HIFα (e.g., TGFα) engage the epidermal growth factor receptor in an autocrine fashion, which subsequently leads to a phosphoinositide 3-kinase/AKT–dependent activation of the IKK complex (16). In this scenario, IKKα, as a component of the IKK complex that also includes IKKβ and IKKγ, is directly activated by AKT. More recently, another group identified a HIFα-independent mechanism of NF-κB activation, in which the inhibitory phosphorylation of Card9, an activator of the NF-κB pathway, is prevented in the absence of pVHL (17). Our data presented herein, in which ectopic expression of pVHL-resistant HIF1α to a degree that approximated the expression of wild-type HIF1α in pVHL^{low} cells, provide additional evidence in support of the existence of a HIFα-dependent mechanism. Thus, the biochemical mechanism that links *VHL* inactivation to NF-κB activation may operate in a fashion that is dependent on the cellular context. Nonetheless, both of the proposed biochemical pathways leading to NF-κB activation converge on the IKK complex.

Recently, it was reported that NF-κB transcriptionally induces HIFα expression in murine macrophages, liver, and brain (26). We found that pharmacologic inhibition of NF-κB resulted in reduced HIFα expression in both ACHN VHL^{low} and SN12C^{low} cells (Supplementary Fig. S3). These findings suggest the existence of a biochemical loop, whereby HIFα activates NF-κB and vice versa. Interrupting this loop in VHL-deficient RCC cells at one or more of the many biochemical steps that link HIFα to NF-κB is apt to have a significant effect on RCC cellular growth.

The finding that EMT induced by *VHL* loss is reversed by inhibition of NF-κB is a demonstration of a mesenchymal to epithelial transition (MET). This EMT-MET reversibility is an expression of the plasticity of RCC cells. This plasticity is a critical observation with respect to therapeutic intervention especially given that the initiating EMT event is a genetic and/or epigenetic event (i.e., biallelic activation of *VHL*). Given that gene therapy to restore the expression of an inactivated tumor suppressor gene such as *VHL* is not a feasible therapeutic approach with currently existing technologies, the understanding of the biochemistry downstream of *VHL* inactivation that drives the EMT process offers the potential for targeted therapeutic intervention.

Therapeutic targeting of hyperactivated kinases has proven to be a successful approach to the treatment of several malignancies, both in preclinical models and clinical trials (31). For instance, the development of multitargeting tyrosine kinase inhibitors, such as sunitinib and imatinib, has favorably influenced the clinical outcomes of several human malignancies, including CCRCC, gastrointestinal stromal cell tumors, and chronic myelogenous leukemia. Consequently, therapeutic targeting of kinases downstream of *VHL* loss, most notably IKK isoforms such as IKKβ, represents a potentially viable approach to the management of CCRCC. Indeed, small-molecule inhibitors of IKKβ are making

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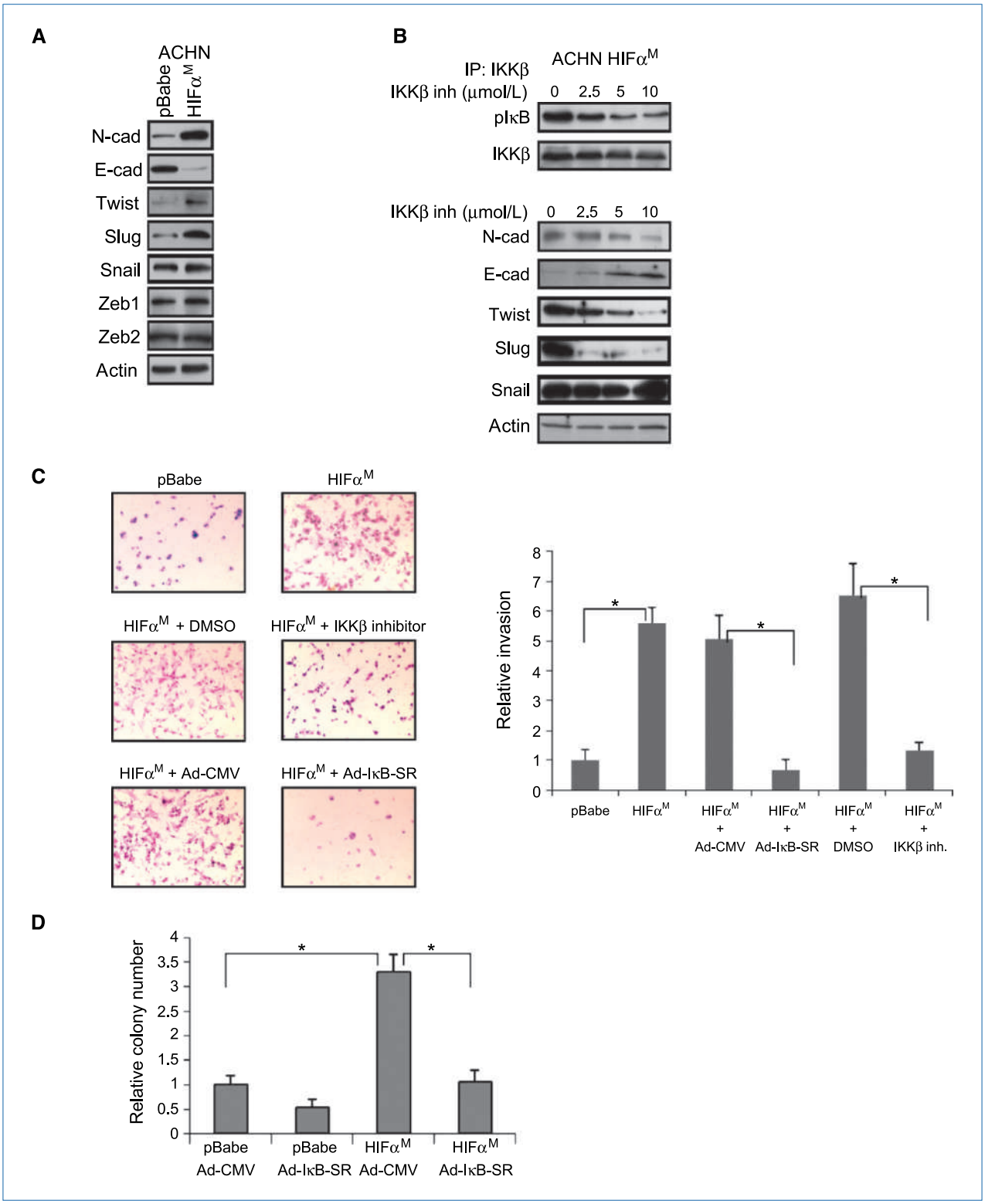

Figure 6. NF-κB blockade reverses the EMT driven by the HIFα^M. *A,* baseline levels of indicated proteins in VHL$^+$/pBabe and VHL$^+$/HIFα^M ACHN cells. *B,* dose-dependent effects of a 24-h exposure to the IKKβ inhibitor on the indicated proteins. *C,* effects of NF-κB inhibition in ACHN VHL$^+$/HIFα^M cells by either the Ad-IκB-SR or the IKKβ inhibitor or the respective controls on invasion (Matrigel assay). *Left,* photomicrographs at original magnification of ×100; *right,* histogram to illustrate invasion assay results. *D,* effects of Ad-IκB-SR inhibition of NF-κB on anchorage-independent growth of ACHN VHL$^+$/HIFα^M cells. *, $P < 0.05$.

their way to the clinic and may serve as alternative strategies for the treatment of CCRCC and offer the potential of reversing the mesenchymal phenotype that typifies CCRCCs that are predicted to result in the most adverse clinical outcomes.

Disclosure of Potential Conflicts of Interest

A.J. Pantuck: commercial research grant, Pom Wonderful. The other authors disclosed no potential conflicts of interest.

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References

1. Weinberg RA. Twisted epithelial-mesenchymal transition blocks senescence. Nat Cell Biol 2008;10:1021–3.
2. Berx G, Raspe E, Christofori G, Thiery JP, Sleeman JP. Pre-EMTing metastasis? Recapitulation of morphogenetic processes in cancer. Clin Exp Metastasis 2007;24:587–97.
3. Waters WB, Richie JP. Aggressive surgical approach to renal cell carcinoma: review of 130 cases. J Urol 1979;122:306–9.
4. Glaspy JA. Therapeutic options in the management of renal cell carcinoma. Semin Oncol 2002;29:41–6.
5. Nickerson ML, Jaeger E, Shi Y, et al. Improved identification of von Hippel-Lindau gene alterations in clear cell renal tumors. Clin Cancer Res 2008;14:4726–34.
6. Iliopoulos O, Kibel A, Gray S, Kaelin WG, Jr. Tumour suppression by the human von Hippel-Lindau gene product. Nat Med 1995;1:822–6.
7. Kondo K, Kaelin WG, Jr. The von Hippel-Lindau tumor suppressor gene. Exp Cell Res 2001;264:117–25.
8. Kondo K, Klco J, Nakamura E, Lechpammer M, Kaelin WG, Jr. Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell 2002;1:237–46.
9. Baldwin AS, Jr. Series introduction: the transcription factor NF-κB and human disease. J Clin Invest 2001;107:3–6.
10. Basseres DS, Baldwin AS. Nuclear factor-κB and inhibitor of κB kinase pathways in oncogenic initiation and progression. Oncogene 2006;25:6817–30.
11. Oya M, Ohtsubo M, Takayanagi A, Tachibana M, Shimizu N, Murai M. Constitutive activation of nuclear factor-κB prevents TRAIL-induced apoptosis in renal cancer cells. Oncogene 2001;20:3888–96.
12. Qi H, Ohh M. The von Hippel-Lindau tumor suppressor protein sensitizes renal cell carcinoma cells to tumor necrosis factor-induced cytotoxicity by suppressing the nuclear factor-κB-dependent antiapoptotic pathway. Cancer Res 2003;63:7076–80.
13. An J, Fisher M, Rettig MB. VHL expression in renal cell carcinoma sensitizes to bortezomib (PS-341) through an NF-κB-dependent mechanism. Oncogene 2005;24:1563–70.
14. Sourbier C, Danilin S, Lindner V, et al. Targeting the nuclear factor-κB rescue pathway has promising future in human renal cell carcinoma therapy. Cancer Res 2007;67:11668–76.
15. Oya M, Takayanagi A, Horiguchi A, et al. Increased nuclear factor-κB activation is related to the tumor development of renal cell carcinoma. Carcinogenesis 2003;24:377–84.
16. An J, Rettig MB. Mechanism of von Hippel-Lindau protein-mediated suppression of nuclear factor κB (NF-κB) activity. Mol Cell Biol 2005;25:7546–56.
17. Yang H, Minamishima YA, Yan Q, et al. pVHL acts as an adaptor to promote the inhibitory phosphorylation of the NF-κB agonist Card9 by CK2. Mol Cell 2007;28:15–27.
18. Min C, Eddy SF, Sherr DH, Sonenshein GE. NF-κB and epithelial to mesenchymal transition of cancer. J Cell Biochem 2008;104:733–44.
19. Thomas GV, Tran C, Mellinghoff IK, et al. Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. Nat Med 2006;12:122–7.
20. Naito S, von Eschenbach AC, Fidler IJ. Different growth pattern and biologic behavior of human renal cell carcinoma implanted into different organs of nude mice. J Natl Cancer Inst 1987;78:377–85.
21. Kochevar J. A renal cell carcinoma neoplastic antigen detectable by immunohistochemistry is defined by a murine monoclonal antibody. Cancer 1987;59:2031–6.
22. An J, Mo D, Liu H, et al. Inactivation of the CYLD deubiquitinase by HPV E6 mediates hypoxia-induced NF-κB activation. Cancer Cell 2008;14:394–407.
23. Esteban MA, Tran MG, Harten SK, et al. Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. Cancer Res 2006;66:3567–75.
24. Evans AJ, Russell RC, Roche O, et al. VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail. Mol Cell Biol 2007;27:157–69.
25. Krishnamachary B, Zagzag D, Nagasawa H, et al. Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. Cancer Res 2006;66:2725–31.
26. Rius J, Guma M, Schachtrup C, et al. NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. Nature 2008;453:807–11.
27. Gervais ML, Henry PC, Saravanan A, et al. Nuclear E-cadherin and VHL immunoreactivity are prognostic indicators of clear-cell renal cell carcinoma. Lab Invest 2007;87:1252–64.
28. Yang MH, Wu MZ, Chiou SH, et al. Direct regulation of TWIST by HIF-1α promotes metastasis. Nat Cell Biol 2008;10:295–305.
29. Harten SK, Shukla D, Barod R, et al. Regulation of renal epithelial tight junctions by the von Hippel-Lindau tumor suppressor gene involves occludin and claudin 1 and is independent of E-cadherin. Mol Biol Cell 2009;20:1089–101.
30. Kajita M, McClinic KN, Wade PA. Aberrant expression of the transcription factors snail and slug alters the response to genotoxic stress. Mol Cell Biol 2004;24:7559–66.
31. Hunter T. Tyrosine phosphorylation: thirty years and counting. Curr Opin Cell Biol 2009;21:140–6.

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