

A Review of VHL Disease and E-Cadherins

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von Hippel-Lindau (VHL) disease is a rare disorder, characterized by a family cancer syndrome, affecting one in 36,000 people (Maher et al., 1991). Individuals affected with von Hippel-Lindau disease are predisposed to different subsets of tumors, including pheochromocytomas (tumors of the adrenal gland), hemangioblastomas (tumors of the retina and central nervous system), and the most lethal, renal cell carcinomas. Hereditary cancers such as VHL are often characterized by the absence of one normal copy of the tumor suppressor gene. Upon a single mutagenic event, the remaining normal copy of the allele can lose its function, which may lead to the initiation of cancer. Although VHL disease is recessive at the cellular level, it is inherited in an autosomal dominant pattern because only one copy of the gene is needed for predisposition to VHL disease. The loss of one copy of the tumor suppressor gene creates this hereditary predisposition as described by Knudson's two hit hypothesis for tumor suppressor genes (Knudson, 1971).

The multi-functional gene product of VHL, pVHL, contains two domains, α and β , that are part of the ubiquitin E3 ligase complex. The α domain of pVHL functions to bind to elongin C, which is part of the complex made with elongin B, Cul2, and Rbx1. The β domain of pVHL is responsible for substrate recognition. One important protein that acts as a substrate to pVHL is Hypoxia Inducible Factor- α (HIF- α). HIF- α is part of the transcription factor HIF, which is composed of a heterodimer of α and β subunits. The α subunits, under normoxic conditions,

become hydroxylated on proline residues at conserved positions. The hydroxylated proline residues create a binding site for the VHL gene product (Cockman et al., 2000). In the presence of oxygen, HIF- α binds to the β domain of pVHL and is ubiquitinated and targeted for proteosomal degradation (Cockman et al., 2000). Under hypoxic conditions, HIF- α does not become hydroxylated, ubiquitinated or targeted for degradation. Instead, the HIF- α binds with HIF- β to activate the transcription of hypoxia inducible genes, such as vascular endothelial growth factor (VEGF), which is involved in angiogenesis, and PHD3, which is a prolyl hydroxylase that regulates protein aggregation (Kaelin, 2002; Rantanen, 2008). When pVHL becomes inactivated under normoxic conditions due to a mutation, it can no longer bind HIF- α nor cause its degradation by the proteasome. VHL inactivation causes an increase in the activity of HIF- α and therefore an increase in the expression of genes it targets.

While the mechanism is still unclear, mutations that cause a loss of VHL function in patients are known to promote tumor development in kidney cells, specifically sporadic clear cell renal cell cancers (CCRCC's), which are epithelial in origin (Kaelin, 2002). A new avenue of VHL research was therefore explored, in order to identify the effects of this type of pVHL inactivation on the glycoprotein E-cadherin. This transmembrane protein serves an important function in cellular adhesion by forming heterodimers between interacting cells (Alberts et al., 2002). By understanding VHL inactivation through E-cadherins and their role in adhesion, we may better understand tumors originating from epithelial cells in the kidney. The following studies have helped elucidate the pathways of E-cadherin downregulation in cells lacking functional VHL through assays of the transcription factor HIF, along with known transcriptional repressors of E-cadherin in various CCRCC cell lines.

In the 2006 research article by Estaban et al., the downregulation of E-cadherin upon VHL inactivation was examined in human kidney tubular cells. As a characteristic of epithelial cancers, loss of E-cadherin expression was examined in vivo through pVHL inactivation, and in vitro through the reintroduction of VHL in pVHL defective sporadic CCRCC tumor cells (Estaban et al., 2006). In order to examine the role of pVHL loss in decreasing E-cadherin expression, Estaban et al. also examined a possible mechanism for the progression of CCRCC in kidney cells. The group questioned if the inactivation of pVHL in the distal tubule may be causing the decrease in E-cadherin expression. It is important to note that E-cadherins are expressed in the distal portion of the renal tubule, and pVHL loss of function first occurs in the renal distal tubule. To test this path of E-cadherin regulation, they first verified the distribution of E-cadherin in the distal portion of the renal tubule. Portions of kidneys from affected patients were then analyzed in order to identify expression of carbonic anhydrase IX (CAIX), which is a target of HIF. Results showed that CAIX was expressed in the distal tubule along with a decreased level of E-cadherin. The purpose of this portion of the study was to associate the loss of pVHL in renal epithelium to the decrease in E-cadherin expression in vivo (Estaban et al., 2006).

The next segment of the study looked at E-cadherin expression in two CCRCC cell lines that had defective pVHL, and a subline with stably restored pVHL. Using immunoblotting and immunofluorescence methods, the results of this assay showed that pVHL defective CCRCC cells had little to no E-cadherins, whereas the VHL restored cells had notably higher levels of E-cadherins. To further analyze these results and the connection between pVHL and E-cadherins, Estaban et al. employed quantitative real time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). First, the mRNA from the VHL negative, and VHL restored cells was isolated

through interactions with its poly-A tail. The mRNA transcripts were then retrotranscribed to DNA using the enzyme reverse transcriptase. PCR was then conducted to amplify this DNA, using E-cadherin primers that contained green fluorescent dye. After each round of replication, the amount of fluorescence was measured, while quantifying the amount of DNA using an automated machine (Esteban et al., 2006). The figure below illustrates the results from the real time RT-PCR assay.

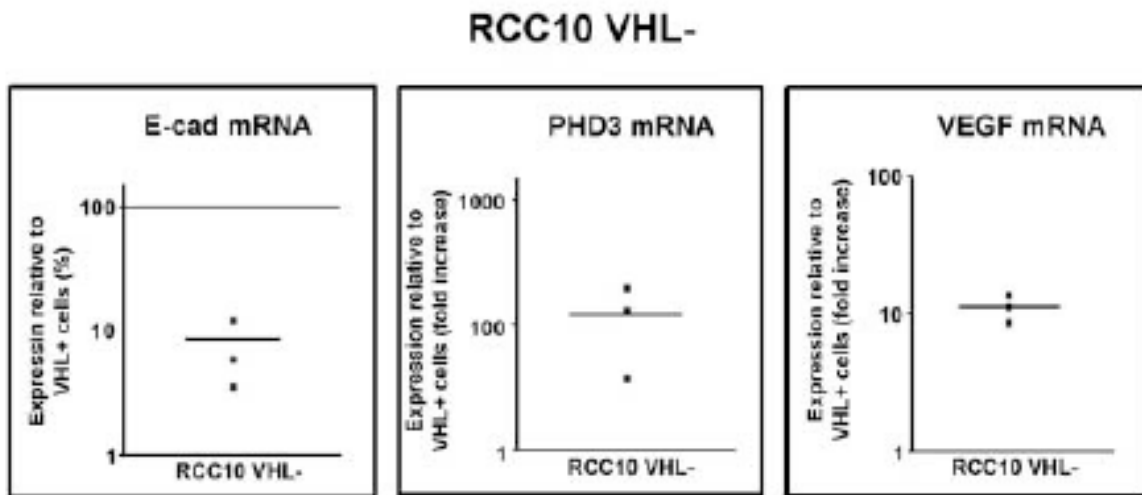


Figure 1: RT-PCR analysis of E-cadherin, and HIF target gene products PHD3, and VEGF mRNA in VHL null and VHL positive CCRCC cells. Note the difference in scales among the three figures on the y-axis (Esteban et al., 2006).

The results from the RT-PCR showed that in VHL restored cells, E-cadherin mRNA levels were significantly higher than in pVHL defective cells. Results also indicated that the mRNA of HIF target gene products vascular endothelial growth factor (VEGF) and prolyl hydroxylase PHD3 was also higher in VHL defective cells, suggesting a relationship between

functional VHL and potential tumor formation. Essentially, the CCRCC cells with stably re-expressed VHL had the ability to restore E-cadherin expression, because of (to some degree) increasing E-cadherin mRNA levels.

In order to examine the suppression of E-cadherin through a possible HIF mediated pathway, Esteban et al. used the genetic approach with VHL mutants. First, they examined missense mutants of VHL that regulated HIF normally in their expression. They then used mutants lacking functional VHL to observe the effects of loss of pVHL function while HIF was being normally expressed. Through immunoblotting, it was determined that the mutants downregulated HIF1 and HIF2 expression while increasing E-cadherin levels. The expression of E-cadherins was confirmed through immunofluorescence microscopy as well as real time RT-PCR. The results of these additional assays all pointed to the suppression of E-cadherin in VHL negative cells by an HIF dependent pathway. The authors had to assume however, that there was no alternate pathway that depended on another target of VHL (besides HIF) which could have regulated growth factors involved in cell proliferation.

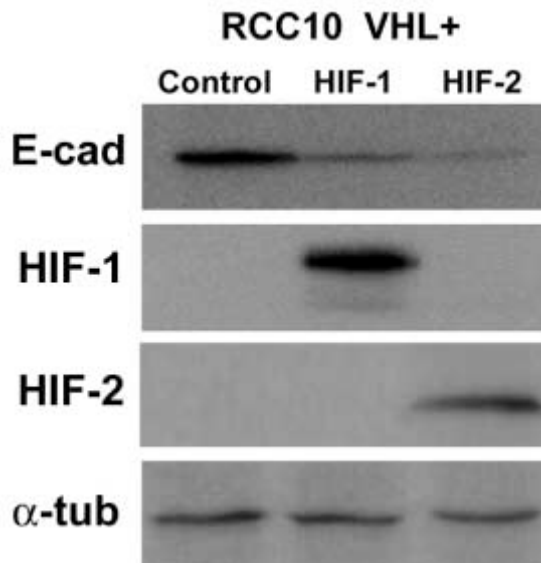


Figure 2: This Western blot shows the activated HIF1 and HIF2 decreasing E-cadherin expression upon re-introduction of VHL to VHL null CCRCC cells, suggesting HIF's involvement in E-cadherin suppression. The RCC10 VHL+ cells were infected with a retrovirus that encoded constitutively active HIF1a (Lane 2), HIF2a (Lane 3), or an empty vector (Control, Lane 1) (Esteban et al., 2006).

To further examine the effects of HIF on E-cadherin expression, a series of experiments was conducted to restore functional VHL into the cells and to activate HIF. Using 1% oxygen (hypoxic conditions), the HIF pathway was triggered, as illustrated through an increase of HIF target gene expression. Through analysis via real time RT-PCR and immunoblotting, it was shown that the hypoxia decreased the levels of E-cadherin mRNA and proteins in cells that had VHL functionally restored.

The next step was to test the exact role of HIF in controlling the expression of E-cadherin mRNA. HIF1 α and HIF2 α mutants that contained mutations of the prolyl residues (which are targets for hydroxylation dependent on oxygen and capture of pVHL) were used in this assay. Cells with functionally reintroduced VHL were made to express these constitutively active HIF1 α and HIF2 α mutants. The results showed that E-cadherin protein and more importantly, mRNA levels were decreased in cells with the HIF missense mutants.

Using small interfering RNA (siRNA), HIF was then inhibited to test its ability to restore E-cadherin expression in cells lacking functional VHL. Results indicated that inhibiting both

HIF1 and HIF2 siRNA increased E-cadherin expression, suggesting that both HIF1 and 2 have a part in controlling E-cadherins in VHL negative CCRCC cells (Esteban et al., 2006).

Since we learned that E-cadherin downregulation can help induce the development and invasiveness of certain cancers, this study demonstrated a possible mechanism whereby loss of pVHL function may potentially control tumor initiation in renal tubular cells at least partly mediated by HIF. The next question to ask would be concerning the pathway of HIF in lowering the expression of E-cadherin. In cancer, the inactivation of E-cadherin may be due to methylation of the gene or transcriptional silencing. Transcriptional silencing can occur by binding transcription repressors to a specific DNA sequence of the promoter of E-cadherin, called the E-box (Esteban et al., 2006). It is possible that HIF could act to induce the repressor, and would therefore decrease the transcription of E-cadherin mRNA. Esteban et al. sought to examine this mechanism and tested the expression of an E-cadherin transcriptional repressor SNAI1 (SNAIL) in VHL negative cells. Using real time RT-PCR techniques, results showed that in cells that lacked functional pVHL, SNAIL mRNA expression increased. This indicated that the increase in SNAIL mRNA expression may be involved in E-cadherin downregulation, as SNAIL may be a repressor of E-cadherin.

In many types of carcinomas, the epithelial-mesenchymal transition of cells plays a key role in pathogenesis. The epithelial-mesenchymal transition (EMT) can be described as a series of morphological changes in epithelial cells moving towards differentiation to mesenchymal cells caused by the epithelial cells' plasticity. As a cell adhesion molecule, E-cadherin is an important factor in this change, causing cells to become more invasive and metastatic. As described previously, the gene for E-cadherin may be regulated on the level of transcription at

the gene's promoter. The expression of transcriptional repressors, including SNAIL and SIP1, are what control the observed changes in the cellular phenotypes of tumor cells. These repressors bind to the E-box of the promoter of E-cadherin to decrease E-cadherin expression, therefore causing the mesenchymal transition (Cancer Development, 2005). Since CCRCC cancer is epithelial in origin, it may be beneficial to look at some studies relating these transcriptional repressors to the EMT and E-cadherin expression.

The most interesting studies of E-cadherin and Snail by Cano et al. concerned the epithelial-mesenchymal transition of epithelial tumor cells. Snail was ectopically expressed in epithelial cell lines, which resulted in the decrease of E-cadherin at the cellular junctions of the transfected cells, as seen below, after 48 hours. Concentrating on figures "b" and "f", there was a visible decrease in expression as compared to the Mock treated cells with an empty vector (figures "a" and "c"). The cells transfected with Snail were significantly different in phenotype than those infected with the empty vector, which paralleled the decrease in expression of E-cadherin in the cellular junctions. These results help to confirm Snail's role in the epithelial-mesenchymal transition of tumor progression. One limitation to this study however was the type of epithelial cell line used. Keratinocytes were assayed in this study, which may not have the same morphological differences than the renal epithelium we are more interested in.

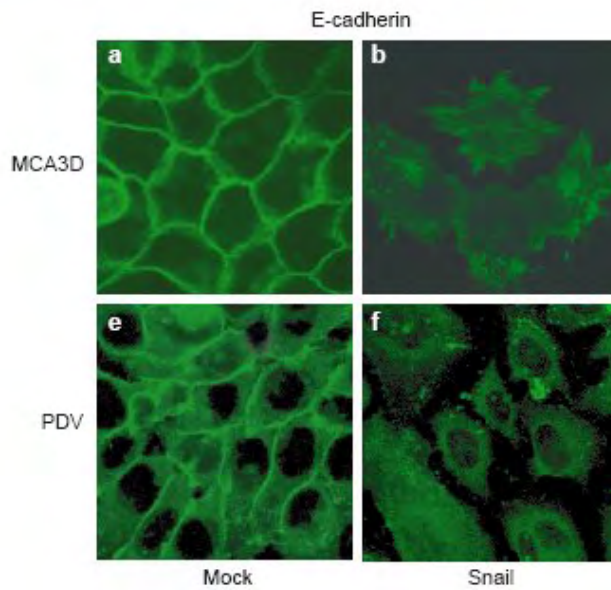


Figure 3: This figure illustrates, via immunofluorescence, expression of E-cadherin after transfection of epithelial cell lines MCA3D and PDV with ectopically expressed Snail. Note the difference in phenotype among the cells (Cano et al., 2000).

To aid in our understanding of tumor progression in epithelial cells, another transcriptional repressor of E-cadherin, SIP1, was analyzed. Its ability to down-regulate E-cadherin expression was examined in the 2001 article by Comijn et al. Since SIP1, like Snail, is a zinc finger protein, this group sought to establish a correlation between SIP1 expression and E-cadherin downregulation, suggesting E-cadherin's significant role in cell invasiveness.

In order to verify the specificity of SIP1's sequence in the transcription of the E-cadherin promoter known to bind SIP1, the authors co-transfected an expression vector for full length SIP1, and reporter genes for the promoter of E-cadherin, in a cell line positive for E-cadherin. As compared to cell lines from other mammals, the human cell line showed an 80% decrease in

E-cadherin expression, due to SIP1's transcriptional repression of E-cadherin's promoter (Comijn et al., 2001). Shown in the graph below is the activity of reporter gene pGL3basic, for the promoter of E-cadherin in the human cell line MCF7/AZ. Measurements of the luciferase depended on the activity of E-cadherin promoters, and were taken after three days. Lanes 3-5 test the specificity of SIP1 to the promoter region by examining mutations in the promoter region.

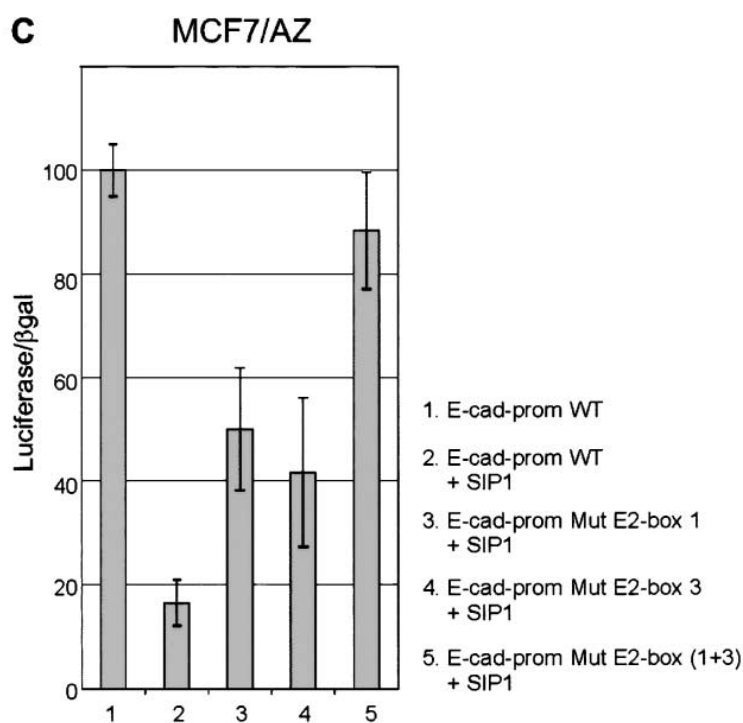


Figure 4: This figure shows the activity of SIP1 cDNA in MCF7/AZ epithelial cells, using a reporter gene to measure activity. E-cadherin promoter activity is impeded by the wild type E-cadherin promoter sequence cotransfected with SIP1. Mutant E-cadherin promoter sequences were also cotransfected with SIP1, which shows that there is a specific sequence of the E-cadherin promoter known to bind to SIP1 and cause E-cadherin's downregulation (Comijn et al., 2001).

The next group to study specific transcriptional repressors of E-cadherin was Krishnimachary et al. The purpose of their 2006 study was to examine whether the dysregulation of HIF1 activity was a factor for the downregulation of E-cadherin, leading to the loss of cellular adhesion and promotion of epithelial-mesenchymal transition. In addition, they examined the HIF1 dependent regulation of E-cadherin, through involvement with transcriptional repressors TCF3, ZFHX1A, and ZFHX1B.

The first part of this study verified the correlation established by Esteban et al. (2006) of loss of VHL function affecting E-cadherin expression through an HIF mediated pathway. By immunoblot assays using VHL negative cells, levels of HIF1 α and HIF2 α were monitored under normoxic conditions. When functional VHL was restored, the levels of HIF decreased, as expected, in addition to the levels of E-cadherin. Immunocytochemistry confirmed VHL negative cells as having low E-cadherin expression, while the restoration of functional VHL increased the expression of E-cadherin. Next, they examined whether the dysregulation of E-cadherin protein also had a decrease in its mRNA levels, though analysis via real time RT-PCR. Results showed that there was statistically significant 27% decrease in E-cadherin mRNA of VHL negative cells in comparison to cells with functionally reintroduced VHL. This preliminary portion of the study was necessary before experiments on HIF's specific role in E-cadherin regulation could be conducted.

The next step of this experiment was to consider whether the dysregulation of HIF1 α and HIF2 α together affected E-cadherin's loss of expression. To do so, they constructed a subclone of the VHL negative cell line whose HIF subunits dimerized and ultimately blocked

transcription. This subclone was called RCC4-DN, for the dominant negative form of HIF1 α it encodes. Since HIF could not transcribe genes, the expression of E-cadherin was increased relative to cells that encoded GFP as a vector. These dominant negative cells had a 9.8 fold increase in the mRNA of E-cadherin when compared to the GFP cells. Using a subclone that mimicked the effects of VHL regulation, the authors suggested that the VHL null cells had lost expression of E-cadherin through an HIF-1 α mediated pathway (Krishnamachary et al., 2006). Upon further evaluation, this pathway may be solidified by immunohistochemical assays for E-cadherin in the RCC4-dominant negative cells compared to cells containing GFP. If the dominant negative cells showed increased E-cadherin expression over that of the GFP cells, it may also suggest that HIF1 α could be necessary to negatively regulate E-cadherin expression. Perhaps an additional assay using small interfering RNA (siRNA) would also be important in providing more evidence that E-cadherin repression depends on HIF1 α only, as Krishnamachary et al. claim.

Looking for evidence of epithelial-mesenchymal transition in VHL negative cells, the authors used transepithelial resistance to measure cell-cell adhesion in VHL null and VHL restored cells. This assay was done to detect the physical effects of VHL expression in relation to levels of E-cadherin. The results, after six days in culture, indicated that loss of VHL was linked to a reduced transepithelial resistance, compared to VHL restored cells. The same assay also compared the dominant negative subclone to the GFP cells, and determined that dominant negative expression of HIF1 α correlated with an increased expression of E-cadherin, as shown through the transepithelial resistance of the cells. Results from both assays are displayed in the figure below.

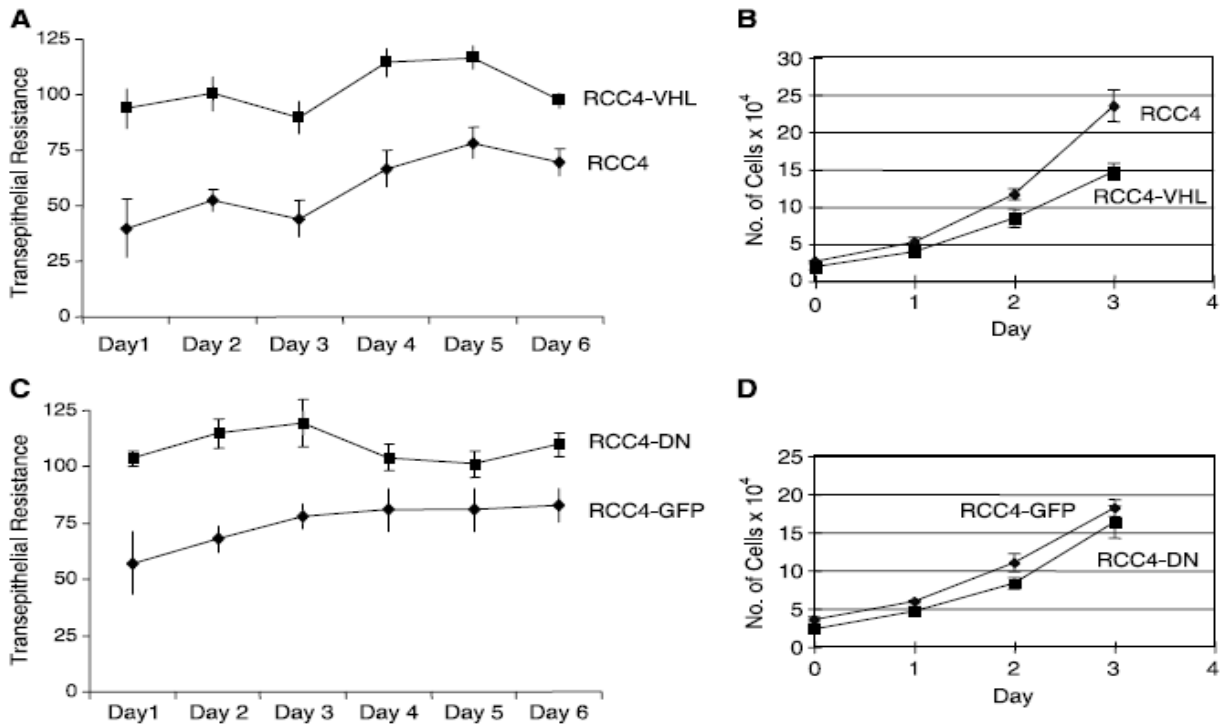


Figure 5: This figure shows the transepithelial resistance of CCRCC cells. The number of cells were determined by staining and counted after six days in culture (DN represents dominant negative cells) (Krishnamachary et al., 2006).

To bring these results into clinical perspective, Krishnamachary et al. investigated the link between VHL, E-cadherin, and HIF1 α by conducting immunohistochemical analyses on sections of normal kidney cells along with biopsies from VHL negative CCRCC tumors. In the normal kidney cells, the E-cadherin expression was located in the distal convoluted tubule, while in VHL null cells, E-cadherin was not detected, as HIF α was being solidly expressed. The

results from this assay, shown below, indicate that E-cadherin expression and HIF α levels are mutually exclusive in samples from CCRCC biopsies.

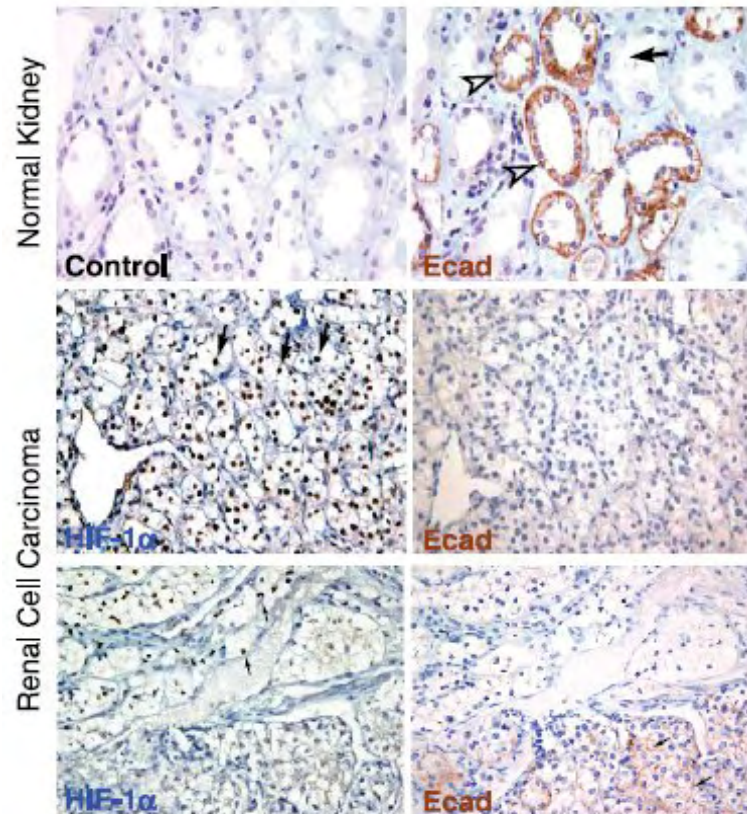


Figure 6: This figure shows the immunohistochemical staining of E-cadherin and HIF-1 α in biopsies of normal kidney cells and renal cell carcinoma cells. The arrows show the lack of E-cadherin in the proximal convoluted tubules, while the arrowheads show the expression of E-cadherin in the distal convoluted tubules (Krishnamachary et al., 2006).

In order to further investigate the mechanism by which E-cadherin mRNA and protein reduction is caused by the increase in HIF1 α expression in VHL null cells, the levels of mRNA from E-cadherin's transcriptional repressors were analyzed via real time RT-PCR. Results demonstrated an increased level of mRNA from transcriptional repressors TCF3, ZFH1A, and ZFH1B (SIP1) in VHL null cells, as opposed to VHL restored cells, and GFP cells. By using the dominant negative cells, the authors were able to conclude that the dysregulation of HIF1 activity caused the expression of E-cadherin's transcriptional repressors to increase, which in turn led to a decrease in E-cadherin protein and mRNA expression, therefore decreasing cell adhesion in the VHL negative CCRCC cells (Krishnamachary et al., 2006). The results from this assay are displayed in the figure below, from real time RT-PCR data.

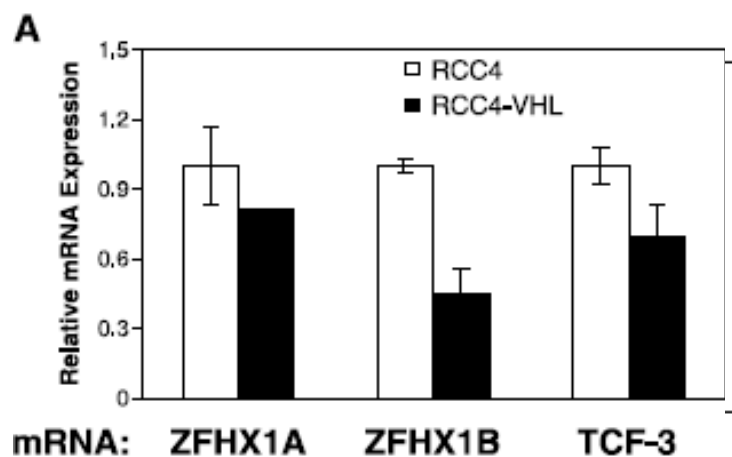


Figure 7: This figure shows the regulation of transcriptional repressors by VHL null and VHL positive CCRCC cells, analyzed through RT-PCR (Krishnamachary et al., 2006).

The results of this study suggest that the loss of E-cadherin expression, mediated in an HIF1 dependent manner, is crucial for the epithelial-mesenchymal transition of tumor cells.

Krishnamachary et al. also indicated that the complex pathway of transcriptional repressors TCF3, ZFHXA, and ZFHXB needed to be further examined in the role of decreasing E-cadherin levels. As seen through various assays of CCRCC cells, the loss of E-cadherin promoted the invasive qualities necessary for metastasis and tumor progression.

The most recent group to examine this pathway of transcriptional silencing involving E-cadherin regulation was Evans et al. (2007). The major goal of these experiments was to reintroduce functional VHL into CCRCC cells and monitor the expression of E-cadherin through expression of its transcriptional repressors, SIP1 and SNAIL. In addition, another experiment in this study was to knockdown HIF α in CCRCC cells to help provide insight into a pathway that links VHL to E-cadherin in its tumor suppression function (Evans et al., 2007).

In the first series of experiments by Evans et al., the technique of oligonucleotide array was employed for over one hundred patients with CCRCC tumors. This procedure used a chip containing an oligonucleotide microarray, hybridized with RNA obtained from the VHL null and VHL positive cells. The chip was then analyzed through a scanner for hybridization and the results were quantified, as shown in the figure "A" below. The results indicated that E-cadherin transcript expression was downregulated in CCRCC cells.

E-Cadherin Expression

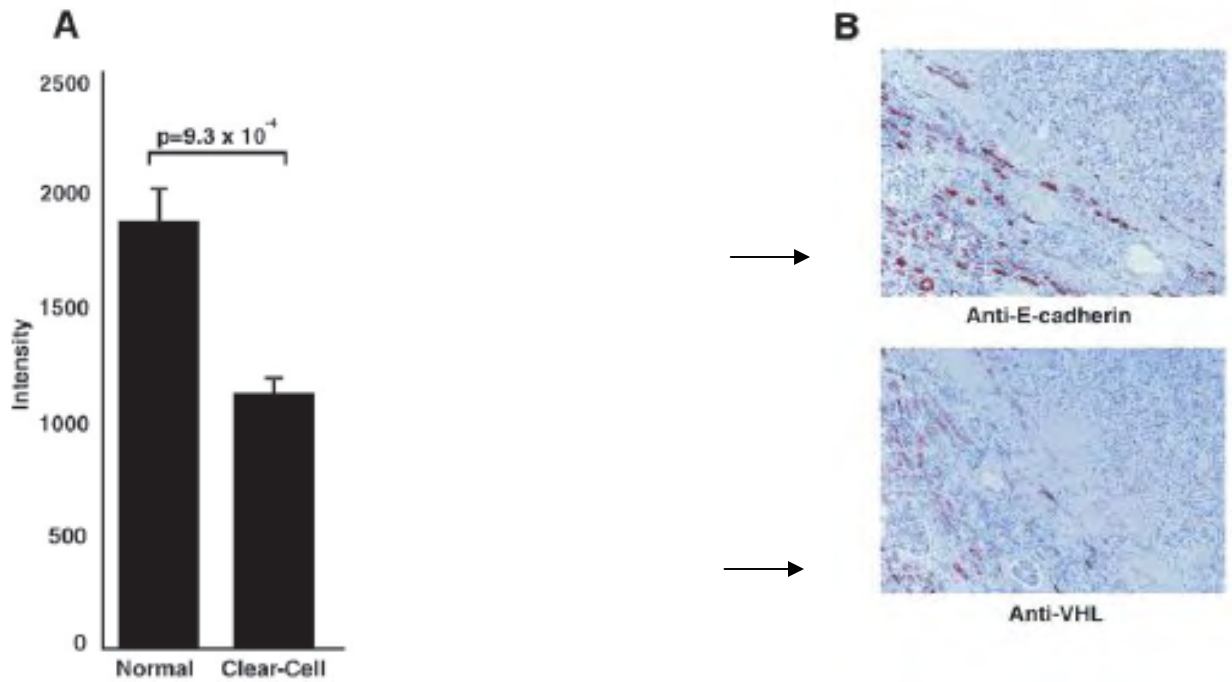


Figure 8: Figure A shows the downregulation of E-cadherin expression in 105 CCRCC tumor samples compared to 12 normal kidney tissue samples, where mean E-cadherin expression was calculated. Figure B shows the staining of CCRCC cells for VHL and E-cadherin. Note the positive staining in the lower left corners of both sections of B, showing the core tubule epithelium (Evans et al., 2007).

To confirm these results using immunohistochemical staining, nephrectomy sections of 13 patients were stained with hematoxylin and eosin. Hematoxylin is a stain that binds to basic amino acids, while eosin binds to acidic residues. These differences in binding allows for better

visualization in staining procedures. The CCRCC tumor sample shown in the figure “B” above has anti-E-Cadherin and anti-VHL staining that correlate positively.

To supplement this evidence of a positive correlation between VHL and E-cadherins, tissue microarrays of 56 CCRCC tumors were used to analyze the link between VHL and E-cadherin expression. Sections of the CCRCC tumor cores were stained with hematoxylin and eosin, and with anti-VHL and anti-cadherin antibodies. The slides were then scanned, as shown in Figure 9 below. The results of the tissue microarray indicated that most of the tumors that had positive staining for VHL also had positive staining for E-cadherin.

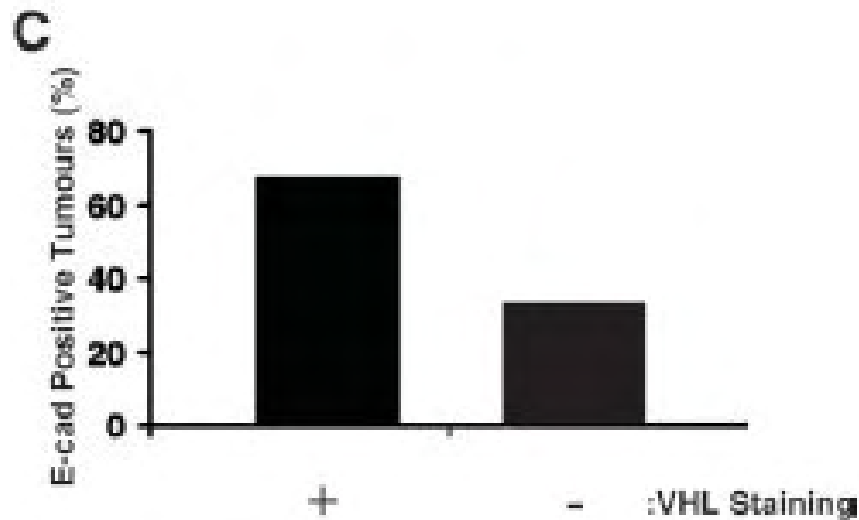


Figure 9: This figure shows the results from the tissue microarray, illustrating the correlation between E-cadherin expression and VHL status in CCRCC cells (Evans et al., 2007).

After establishing the correlation between E-cadherin expression and VHL status, Evans et al. sought to understand the mechanisms by which E-cadherin expression was controlled by VHL in an HIF-dependent pathway. Wild type VHL cells were kept under hypoxic and normoxic conditions and were then analyzed for E-cadherin expression by Western blotting. The results of this experiment showed that E-cadherin expression decreased under hypoxic conditions while VHL expression remained constant. By analyzing VHL mutations that varied in their ability to regulate HIF, Evans et al. determined that E-cadherin expression was inversely related to levels of HIF2 α , suggesting that HIF negatively regulates E-cadherin levels.

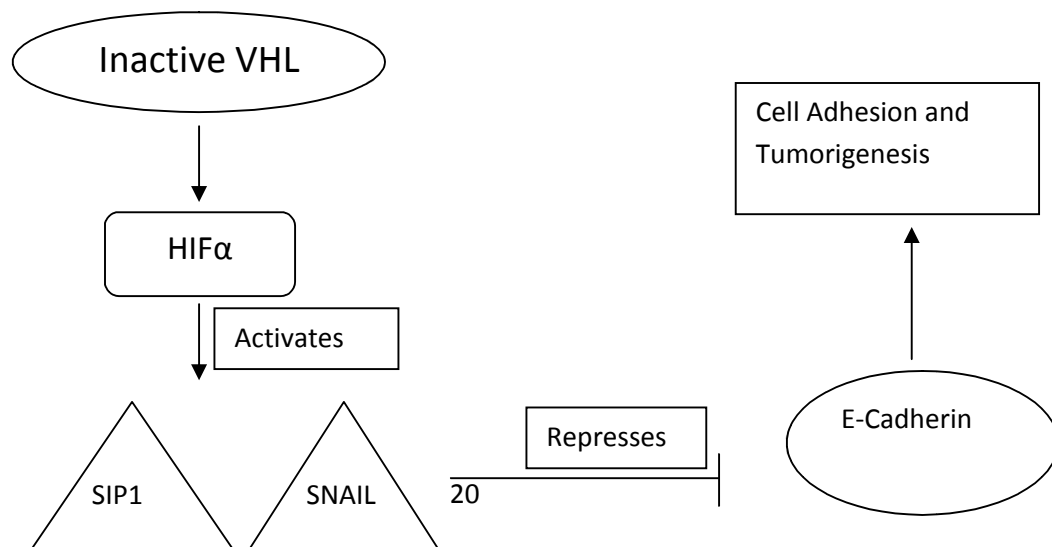
Evans et al. went on to look at specific transcriptional repressors of E-cadherin, Snail and SIP1. These regulators of E-cadherin transcription bind to conserved E2 boxes of the E-cadherin promoter to inhibit the transcription of E-cadherin. Using real time RT-PCR it was shown that the cells that had functional VHL restored decreased their Snail and SIP1 expression. These results were expected, as the restoration of functional VHL would lead to the increase in E-cadherin expression, correlating with the decrease of its repressors. By downregulating E-cadherin's transcriptional repressors, VHL may perhaps increase the expression of E-cadherin itself.

Since the transcriptional repressors Snail and SIP1 were known to decrease transcription by blocking RNA Polymerase II's (Pol II) connection to DNA, Evans et al. examined VHL's role in Pol II's engagement on the promoter of the E-cadherin gene. Using the technique of chromatin immunoprecipitation (ChIP), chromatin preparations were made from the wild type VHL cells, and from mutant VHL cells that were defective in HIF regulation, and were probed with anti-RNA Polymerase II antibody. The amount of Pol II at the promoter of E-cadherin

decreased in the mutant VHL cells, indicating that VHL negatively controlled HIF, which was needed for E-cadherin's transcriptional activity. The authors therefore proposed that HIF controlled the activity of the transcriptional repressors Snail and SIP1, which prevented RNA Polymerase II's attachment to the promoter of E-cadherin, which in turn decreased E-cadherin expression (Evans et al., 2007). Upon confirmation that VHL loss led to a significant decrease in the expression of E-cadherin in patient derived CCRCC tumor cells, the results from the study by Evans et al. showed that the repressors Snail and SIP1 regulated E-cadherin transcription in an HIF-dependent manner, elucidating this pathway between VHL and E-cadherin.

Through these experiments and others, the association between VHL disease and the pathways of tumorigenesis can be further understood. E-cadherin is only one protein that is affected by VHL and HIF, but proved its importance in the areas of cell invasiveness. By studying these proteins and others in patients with VHL, we can develop a better understanding of the mechanisms by which tumors form in the epithelium of renal cells. Figure 10 below summarizes the proposed mechanism by which VHL inactivation may cause tumor proliferation in CCRCC tumors.

Figure 10:



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