Knockdown of von Hippel-Lindau Protein decreases lung cancer cell proliferation and colonization

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Abstract

Although von Hippel-Lindau protein (pVHL) is known as a tumor suppressor in kidney and other organs, it remains unclear whether pVHL plays a role in lung cancer development. We investigated the role of pVHL in lung cancer cell proliferation, migration, and colonization using stable A549 cells with knockdown of pVHL. We found that knockdown of pVHL promotes epithelial-mesenchymal transition (EMT) in lung cancer cells. Knockdown of pVHL decreased tumor colonization in a tail-vein injection model and decreased cell proliferation, whereas overexpression of constitutive active HIF increased tumor colonization, suggesting a HIF-independent function of pVHL in lung. Knockdown of pVHL decreased phosphorylation of FAK and expression of integrin, suggesting that pVHL regulates lung cancer development via integrin/FAK signaling pathway.

Keywords: Focal adhesion protein, integrin, von Hippel-Lindau protein

Abbreviations:

von Hippel-Lindau protein (pVHL)

epithelial-mesenchymal transition (EMT)

non-small cell lung cancer (NSCLC)

hypoxia inducible factor (HIF)

focal adhesion kinase (FAK)

fetal bovine serum (FBS)

small hairpin RNAs (shRNAs),

α-mooth muscle actin (α-SMA)

1. **Introduction**

Lung cancer is the most common cause of cancer-related death in the United States [1]. There are two main types of lung cancer, small cell lung cancer and non-small cell lung cancer (NSCLC) [1], which accounts for 80% of lung cancer cases. Patients with metastatic cancer have poorer survival rates [2-6]. Tumor metastasis includes many steps: loss of cellular adhesion, increased motility and invasiveness, entry and survival in the circulation, exit into new tissue and colonization at a distant site [7]. The rate-limiting step is the acquisition of motility and invasive ability, which are characteristics of cells that have undergone epithelial-mesenchymal transition (EMT) [5,8]. EMT is a molecular and cellular process, during which epithelial cells lose epithelial markers such as E-cadherin and gain mesenchymal markers such as α smooth muscle actin (α-SMA) and vimentin [9-11]. The significance of EMT in tumor metastasis has recently been reported in a few tumor models [12,13].

Von Hippel-Lindau protein (pVHL) was first identified as a tumor suppressor because the mutation of pVHL gene is associated with tumors in kidney [14,15]. The most studied function of pVHL is acting as a component of ubiquitin protein ligase (E3) for the proteasomal degradation of hypoxia inducible factor (HIF)-1/2α [16-18]. In renal carcinoma and other cancers, mutation of pVHL leads to stabilization of HIF, which drives angiogenesis and cancer development. Although lungs express levels of pVHL comparable to kidneys [19,20] and loss of *VHL* allele frequently occurs in patients with non small cell lung cancer [21-23], it remains unclear whether pVHL plays a role in lung cancer development.

We investigated the role of pVHL in the proliferation, migration, and colonization of lung cancer cells. Our results suggest that although knockdown of pVHL in lung cancer cells induces EMT phenotypes *in vitro*, it decreases lung cancer cell colonization in lungs of nude mice, possibly through decreased cell proliferation in a HIF-independent but integrin/FAK signaling dependent manner.

2. Methods

2.1 Cell culture

A549 (Human lung adenocarcinoma cells) were purchased from the American Type Tissue Collection and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2-95% air at 37°C. A549-sh-VHL is a stable cell line with suppression of pVHL by small hairpin RNAs (shRNAs), as previously described [24]. Since A549 cells expressing shRNA of *Drosophila melanogaster* HIF (A549-sh-dHIF) were used as control and dHIF shRNA construct does not target human HIF and has no effect on endogenous HIF [24]. These cell lines were maintained in media containing 1 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO). A549-Lxin (retroviral vector infected cells) and A549-HIF-DPA (express constitutive active HIF-1α) were described previously [25] and were maintained in media containing geneticin G418 (400 µg/ml; Invitrogen, Carlsbad, CA). Cell cultures were routinely passaged when they are 85–90% confluent. Cell numbers were counted with Bio-Rad TC10 automated cell counter (Bio-Rad, Hercules, CA).

2.2 Western blotting

Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in mRIPA buffer (50 mM Tris pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, and protease inhibitors). The cell lysates were cleared by centrifugation at 13,000 *g* for 5 min and protein concentrations of the supernatants were determined using a DC protein assay (Bio-Rad, Hercules, CA). Typically, 25–50 µg of protein were then separated by SDS-polyacrylamide gel electrophoresis. The gel was transferred using a Semi-Dry transfer cell (Bio-Rad) to BA-S 85 nitrocellulose membrane (OPTITRAN, Middlesex, UK). Proteins were detected with SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, Rockford, IL). The following

antibodies were used in this study: α-tubulin, actin, vimentin (Sigma-Aldrich, St. Louis, MO), α-smooth muscle actin (α-SMA) (R&D Systems, Minneapolis, MN), E-cadherin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Focal adhesion kinase (FAK) and pFAK (Invitrogen, Carlsbad, CA), integrin α4 (Chemicon,Temecula, CA), integrin β1 (Santa Cruz Biotechnology, Santa Cruz, CA), HIF-2α (Novus Biologicals, Littleton, CO), HIF-1α (BD transduction Laboratories, Franklin Lakes, NJ).

2.3 Scratch assay

Cells were cultured at the density of 0.8 million cells per 35 mm dish and incubated overnight. Wounds were created by scratching a straight line with a 250 ul tip vertically in the center of the dish. Dishes were washed with complete media once to remove detached cells and the first image of the wound was taken under microscope. The width of the wounds was measured with AxioVision LE software (Zeiss, Oberkochen, Germany). Dishes were then put back in an incubator and incubated for 24 hours and the width of the wounds was measured as described above. The difference between the widths is taken as the migration distance.

2.4 Cell invasion assay

Cell invasion assay was performed with BD Matrigel invasion chambers (BD BioCoat, Bedford, MA, USA). Briefly, after transwells and inserts were warmed up, 0.5 ml cell suspension ($5x10^4$/ml) and 0.75 ml media were added into inserts and wells, respectively. Cells were allowed to attach for 4 hours and were cultured for 2 days. Uninvaded cells in the upper chamber were removed with cotton swabs and invaded cells were fixed with 100% methanol for 2 minutes and stained with 0.5% crystal violet in 2% methanol for 2 minutes. For each insert, at least five random microscopic fields (x200 magnification) were counted. Experiments were carried out in triplicates.

2.5 Tail vein assay of lung cancer cell colonization

Cells were dissociated into a single cell suspension and the density of cell suspension was adjusted with PBS to 10^7 cells/ml. Athymic mice were purchased from Harlan Laboratories (Indianapolis, Indiana) and were maintained on a 12h:12 h light-dark cycle in the Biologic Resources Laboratory. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Illinois at Chicago following National Institutes of Health guidelines. Mice were placed in a mouse tail illuminator (Braintree Scientific Inc., Braintree, MA) with the tail protruding through the opening in the wall of the restraining device. 1-ml syringe was filled with the cell suspension and 0.2 ml of the cell suspension was slowly injected into the lateral tail vein of nude mice with a 27G1/2 needle. After 5 weeks, the animals were euthanized and their lungs were removed. Left lungs were rinsed in PBS and placed in a beaker containing Bouin's solution *(*0.9% (v/v) picric acid, 9% (v/v) formaldehyde, 5% (v/v) acetic acid) overnight. After removing the excess Bouin's solution by rinsing the lungs with PBS, the numbers of pulmonary tumor colonies were counted with the aid of a dissecting microscope. Right lungs were fixed in 10% formaldehyde and processed for H & E staining and representative microscopic images were obtained with AxioVision LE software.

2.5 Statistical analysis

Statistical analysis was done using GraphPad Prism 4 (GraphPad, San Diego, CA, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA) when applicable; *t* tests were performed and significant difference values were set at 0.05.

3. Results

3.1 Knockdown of pVHL promotes epithelial-mesenchymal transition (EMT) and migration in lung cancer cells

To determine whether loss of pVHL affects lung cancer cell phenotypes, we established an A549 cell line with suppression of pVHL by shRNA as described previously [24]. As shown in Fig 1A that pVHL expression was suppressed in the A549-sh-VHL cells. Since A549 cells express both HIF-1α and HIF-2α and loss of pVHL stabilizes HIF-α [24], we determined the expression of HIF-1α and HIF-2α in A549-sh-VHL cells. As shown in Fig 1B, knockdown of pVHL stabilized both HIF-1α and HIF-2α. Previous studies reported that in renal carcinoma cells loss of pVHL decreased expression of E-cadherin, which is associated with EMT [10,26-28]. Thus we investigated whether loss of pVHL in A549 cells alters expression of EMT markers. As shown in Fig 1B, knockdown of pVHL increased expression of α-SMA and vimentin and decreased expression levels of E-cadherin. Since EMT is known to increase cell motility [9-11], we performed scratch assay to determine cell motility in these cells and as shown in Fig 1C and 1D, knockdown of pVHL increased A549 cell migration. In addition, A549-sh-VHL cells were more invasive than control cells (Fig 1E).

3.2 Knockdown of pVHL decreases A549 cell proliferation and colonization in lung

We compared the cell proliferation rates of A549, A549-sh-dHIF and A549-sh-VHL and as shown in Fig 2A, cells with suppression of pVHL proliferated at a slower rate than control cells. To investigate the consequence of loss of pVHL in the colonization of A549 cells, we injected these cells into the lateral tail vain of athymic nude mice and five weeks later we determined the numbers of tumor nodules in the lungs of these mice. As shown in Fig 2B & 2C, knockdown of pVHL decreased the number of the tumor nodules, suggesting that knockdown of pVHL in lung cancer cells inhibits lung cancer cell colonization.

3.3 Overexpression of the constitutive active HIF increases lung cancer cell colonization

Since the loss of pVHL is associated with activation of HIF (Fig 1B) [29,30], we then determined whether the loss-of-function of pVHL in A549 cells is due to the gain-of-function of HIF. We established a cell line with constitutive expression of nondegradable HIF-1α [25] and determined their proliferation rates and the colonization potential in the tail vein assay. As shown in Fig 3, overexpression of the constitutive active HIF-1α did not significantly alter cell proliferation in lung cancer cells, whereas increased the number of tumor nodules in nude mice, suggesting that overexpression of constitutive active HIF increases lung cancer cell colonization.

3.4 Knockdown of pVHL decreases integrin/Focal Adhesion kinase (FAK) signaling

Our results showed that loss of pVHL prevented lung cell colonization (Fig 2) whereas constitutive HIF promotes lung cancer colonization (Fig 3), thus we reason that the HIF-independent function of pVHL overrides the HIF-dependent function during lung cancer cell colonization in pVHL null lung cancer cells. Previously we have shown that overexpression of pVHL promotes fibroblast proliferation via intergrin/FAK signaling independent of HIF function [31]. Thus we investigated the expression levels of integrin and FAK phosphorylation. As shown in Fig 4, knockdown of pVHL decreased expression levels of integrin α4 and integrin β1. Accordingly, the phosphorylation of FAK, a downstream signaling molecule of integrin pathway was reduced (Fig 4C).

4. Discussion

Although it is well established that pVHL acts as a tumor suppressor in many organs especially in kidney [32], the role of pVHL in lung cancer development is largely unknown. In our study, we provide evidence that although lung cancer cells with knockdown of pVHL appear to acquire the EMT phenotype, such as increased migration, invasion, and elevated expression of

α-SMA and vimentin, loss of pVHL inhibits lung cancer cell proliferation and colonization in a HIF-independent manner.

Our results that knockdown of pVHL results in the EMT phenotype (Fig 1) are in line with previous reports that knockdown of pVHL decreases the expression of tight junction components, including occludin and claudin 1 [33] and that in renal carcinoma cell knockdown of pVHL results in the loss of E-cadherin expression and EMT [26-28]. Together, these data suggest that pVHL participates in the maintenance of epithelial cell junction and epithelial phenotype in lung. Cells undergoing EMT gain increased motility and indeed we have shown that knockdown of pVHL increases lung cancer cell motility and invasiveness (Fig 1). Similarly, a previous report shows that overexpression of pVHL inhibits cell motility via formation of focal adhesion and decreased stabilizing of actin organization [34].

In renal carcinoma cells, loss of pVHL promotes EMT, resulting in elevated invasion and cancer progression [26-28]. However, our study suggests that knockdown of pVHL also promotes EMT but prevents lung cancer proliferation and cell colonization (Fig 2), suggesting that the role of pVHL in cancer development may vary in different tissue types. Indeed, loss of pVHL also reduces cell proliferation in fibroblasts, mammary epithelial cells, and chondrocytes [35-37]. Interestingly, cells with HIF-DPA have increased lung cancer cell colonization (Fig 3) and dominant negative HIF inhibits lung cancer colonization [38]. These results suggest a HIF-independent function of pVHL in lung cancer progression.

To understand the mechanism underlining the HIF-independent function of pVHL in our model, we have investigated the effects of knockdown of pVHL on integrin/FAK signaling pathway, which is critical for cell proliferation and cancer development and has been implicated in pVHL-mediated fibroblast proliferation and extracellular matrix assembly [31,39-42]. We have found that knockdown of pVHL decreases expression levels of integrin α4 and β1 and decreases FAK phosphorylation in A549 lung cancer cells (Fig 4). Our results are in line with previous studies that FAK is overexpressed in lung cancer cells and inhibition of FAK prevents

lung cancer cell proliferation and lung cancer progression[43-45]. Consistently, Zhao et al reported that inhibition of integrin α4 reduces lung colonization of melanoma cells [46]. However, in other studies activation of FAK is associated with increased migration [47,48]. It is worth to point out that activation of FAK by integrin αVβ3 induces migration [47], whereas we showed that loss of integrin α4β1/FAK correlated with elevated migration and invasion, suggesting that the function of FAK on cell migration, invasion, and colonization may depend on the type of integrin that activates it.

Taken together, we provide evidence that HIF-independent function of pVHL overrides HIF-dependent function to regulate lung cancer progression, similar to that in mammary epithelium [36]. It also suggests that pVHL suppression alone may not be sufficient to induce cancer in some tissues such as lung or mammary gland [36], mostly because in these tissues loss of pVHL induces cell growth arrest and decreases the proliferation of cells [35-37]. However, non small cell lung cancer patients frequently contains loss of *VHL* allele [21-23] and loss of *VHL* allele increases lung cancer susceptibility [49], we speculate that it is in conjunction with the mutation of other tumor suppressor genes that pVHL deletion/mutation may contribute to lung cancer progression and further studies are warranted.

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References

[1] Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T. and Thun, M.J. (2008). Cancer statistics, 2008. CA: a cancer journal for clinicians 58, 71-96.

[2] Erler, J.T. and Giaccia, A.J. (2006). Lysyl oxidase mediates hypoxic control of metastasis. Cancer Research 66, 10238-41.

[3] Harris, A.L. (2002). Hypoxia--a key regulatory factor in tumour growth. Nature Reviews. Cancer 2, 38-47.

[4] Peinado, H. and Cano, A. (2008). A hypoxic twist in metastasis.[comment]. Nature Cell Biology 10, 253-4.

[5] Chambers, A.F., Groom, A.C. and MacDonald, I.C. (2002). Dissemination and growth of cancer cells in metastatic sites. Nature reviews Cancer 2, 563-72.

[6] Pantel, K. and Brakenhoff, R.H. (2004). Dissecting the metastatic cascade. Nature reviews Cancer 4, 448-56.

[7] Gupta, G.P. and Massague, J. (2006). Cancer metastasis: building a framework. Cell 127, 679-95.

[8] MacDonald, I.C., Groom, A.C. and Chambers, A.F. (2002). Cancer spread and micrometastasis development: quantitative approaches for in vivo models. BioEssays : news and reviews in molecular, cellular and developmental biology 24, 885-93.

[9] Zavadil, J. and Bottinger, E.P. (2005). TGF-beta and epithelial-to-mesenchymal transitions. Oncogene 24, 5764-74.

[10] Thiery, J.P. and Sleeman, J.P. (2006). Complex networks orchestrate epithelial-mesenchymal transitions. Nat Rev Mol Cell Biol 7, 131-42.

[11] Peinado, H., Olmeda, D. and Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer 7, 415-28.

[12] Hugo, H., Ackland, M.L., Blick, T., Lawrence, M.G., Clements, J.A., Williams, E.D. and Thompson, E.W. (2007). Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression. Journal of cellular physiology 213, 374-83.

[13] Tse, J.C. and Kalluri, R. (2007). Mechanisms of metastasis: epithelial-to-mesenchymal transition and contribution of tumor microenvironment. Journal of cellular biochemistry 101, 816-29.

[14] Iliopoulos, O., Kibel, A., Gray, S. and Kaelin, W.G. (1995). Tumour suppression by the human von Hippel-Lindau gene product. Nature medicine 1, 822-6.

[15] Bishop, T. et al. (2004). Genetic analysis of pathways regulated by the von Hippel-Lindau tumor suppressor in Caenorhabditis elegans. PLoS biology 2, e289.

[16] Ivan, M. et al. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science (New York, N Y) 292, 464-8.

[17] Jaakkola, P. et al. (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science (New York, N Y) 292, 468-72.

[18] Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001). Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. The EMBO journal 20, 5197-206.

[19] Corless, C.L., Kibel, A.S., Iliopoulos, O. and Kaelin, W.G. (1997). Immunostaining of the von Hippel-Lindau gene product in normal and neoplastic human tissues. Human pathology 28, 459-64.

[20] Los, M., Jansen, G.H., Kaelin, W.G., Lips, C.J., Blijham, G.H. and Voest, E.E. (1996). Expression pattern of the von Hippel-Lindau protein in human tissues. Laboratory investigation; a journal of technical methods and pathology 75, 231-8.

[21] Ho, W.L., Chang, J.-W., Tseng, R.-C., Chen, J.-T., Chen, C.-Y., Jou, Y.-S. and Wang, Y.-C. (2002). Loss of heterozygosity at loci of candidate tumor suppressor genes in

microdissected primary non-small cell lung cancer. Cancer detection and prevention 26, 343-9.

[22] An, Q. et al. (2002). Deletion of tumor suppressor genes in Chinese non-small cell lung cancer. Cancer letters 184, 189-95.

[23] Pitterle, D.M., Jolicoeur, E.M. and Bepler, G. (1998). Hot spots for molecular genetic alterations in lung cancer. In vivo (Athens, Greece) 12, 643-58.

[24] Zhou, G., Dada, L.A., Chandel, N.S., Iwai, K., Lecuona, E., Ciechanover, A. and Sznajder, J.I. (2008). Hypoxia-mediated Na-K-ATPase degradation requires von Hippel Lindau protein. FASEB J 22, 1335-42.

[25] Zhou, G., Dada, L.A., Wu, M., Kelly, A., Trejo, H., Zhou, Q., Varga, J. and Sznajder, J.I. (2009). Hypoxia-induced alveolar epithelial-mesenchymal transition requires mitochondrial ROS and hypoxia-inducible factor 1. American Journal of Physiology - Lung Cellular & Molecular Physiology 297, L1120-30.

[26] Esteban, M.A. et al. (2006). Regulation of E-cadherin expression by VHL and hypoxia-inducible factor. Cancer research 66, 3567-75.

[27] Evans, A.J. et al. (2007). VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail. Molecular and cellular biology 27, 157-69.

[28] Krishnamachary, B., Zagzag, D., Nagasawa, H., Rainey, K., Okuyama, H., Baek, J.H. and Semenza, G.L. (2006). 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 research 66, 2725-31.

[29] Biju, M.P., Neumann, A.K., Bensinger, S.J., Johnson, R.S., Turka, L.A. and Haase, V.H. (2004). Vhlh gene deletion induces Hif-1-mediated cell death in thymocytes. Molecular and cellular biology 24, 9038-47.

[30] Ohh, M. et al. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nature cell biology 2, 423-7.

[31] Zhou, Q. et al. Role of von Hippel-Lindau protein in fibroblast proliferation and fibrosis. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology 25, 3032-44.

[32] Kaelin, W.G. and Maher, E.R. (1998). The VHL tumour-suppressor gene paradigm. Trends in genetics : TIG 14, 423-6.

[33] Harten, S.K., Shukla, D., Barod, R., Hergovich, A., Balda, M.S., Matter, K., Esteban, M.A. and Maxwell, P.H. (2009). 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. Molecular biology of the cell 20, 1089-101.

[34] Kamada, M., Suzuki, K., Kato, Y., Okuda, H. and Shuin, T. (2001). von Hippel-Lindau protein promotes the assembly of actin and vinculin and inhibits cell motility. Cancer research 61, 4184-9.

[35] Mack, F.A., Patel, J.H., Biju, M.P., Haase, V.H. and Simon, M.C. (2005). Decreased growth of Vhl-/- fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. Molecular and cellular biology 25, 4565-78.

[36] Seagroves, T.N., Peacock, D.L., Liao, D., Schwab, L.P., Krueger, R., Handorf, C.R., Haase, V.H. and Johnson, R.S. VHL deletion impairs mammary alveologenesis but is not sufficient for mammary tumorigenesis. The American journal of pathology 176, 2269-82.

[37] Pfander, D. et al. (2004). Deletion of Vhlh in chondrocytes reduces cell proliferation and increases matrix deposition during growth plate development. Development (Cambridge, England) 131, 2497-508.

[38] Zhou, Q., Chen, T., Ibe, J.C.F., Raj, J.U. and Zhou, G. Loss of either hypoxia inducible factor 1 or 2 promotes lung cancer cell colonization. Cell cycle (Georgetown, Tex) 10, 2233-4.

[39] Hynes, R.O. (2002). A reevaluation of integrins as regulators of angiogenesis. Nature medicine 8, 918-21.

[40] Ohh, M. et al. (1998). The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. Molecular cell 1, 959-68.

[41] Kurban, G., Hudon, V., Duplan, E., Ohh, M. and Pause, A. (2006). Characterization of a von Hippel Lindau pathway involved in extracellular matrix remodeling, cell invasion, and angiogenesis. Cancer research 66, 1313-9.

[42] Tang, N., Mack, F., Haase, V.H., Simon, M.C. and Johnson, R.S. (2006). pVHL function is essential for endothelial extracellular matrix deposition. Molecular and cellular biology 26, 2519-30.

[43] Meng, X.N. et al. (2009). Characterisation of fibronectin-mediated FAK signalling pathways in lung cancer cell migration and invasion. British journal of cancer 101, 327-34.

[44] Shieh, J.-M. et al. Mitochondrial apoptosis and FAK signaling disruption by a novel histone deacetylase inhibitor, HTPB, in antitumor and antimetastatic mouse models. PloS one 7, e30240.

[45] Kruewel, T., Schenone, S., Radi, M., Maga, G., Rohrbeck, A., Botta, M. and Borlak, J. Molecular characterization of c-Abl/c-Src kinase inhibitors targeted against murine tumour progenitor cells that express stem cell markers. PloS one 5, e14143.

[46] Zhao, J. et al. (2008). Inhibition of alpha(4) integrin mediated adhesion was involved in the reduction of B16-F10 melanoma cells lung colonization in C57BL/6 mice treated with gambogic acid. European journal of pharmacology 589, 127-31.

[47] Fong, Y.-C. et al. (2009). Osteopontin increases lung cancer cells migration via activation of the alphavbeta3 integrin/FAK/Akt and NF-kappaB-dependent pathway. Lung cancer (Amsterdam, Netherlands) 64, 263-70.

[48] Bourboulia, D., Jensen-Taubman, S., Rittler, M.R., Han, H.Y., Chatterjee, T., Wei, B. and Stetler-Stevenson, W.G. Endogenous angiogenesis inhibitor blocks tumor growth via direct and indirect effects on tumor microenvironment. The American journal of pathology 179, 2589-600.

[49] Zanesi, N. et al. (2005). Lung cancer susceptibility in Fhit-deficient mice is increased by Vhl haploinsufficiency. Cancer research 65, 6576-82.

Figure legends:

Figure 1. Knockdown of pVHL promotes lung cancer cells epithelia-mesenchymal transition (EMT) and migration. Cultured A549, A549-sh-VHL (sh-VHL), and A549-sh-dHIF (sh-dHIF) were lysed and aliquots containing the same amount of proteins were subjected to SDS-PAGE, followed by Western blot analysis for the detection of pVHL (A), HIF-1α, HIF-2α, E-cadherin, α-smooth muscle actin (α-SMA), and vimentin (B). Actin (A) and Tubulin (B) were used as control for equal loading. C) Cells were cultured on 35 mm dishes overnight to reach confluence. Wounds were created by scratching a straight line with a 250 ul tip and the images of original wound were taken and the starting width were measured with AxioVision LE software (Zeiss). After 24 hours incubation, the width of the wounds was measured as described above and the difference between the width before and after migration was presented as the migration distance. Representative images of the width of the wounds before and after migration were shown and the quntitation of the migration distances was shown in D). Data were expressed as mean ± SEM. *n* =5. **, $p<0.01$. E) Cells were cultured on BD Matrigel invasion chambers for 48 hours. Invaded cells were stained and the number of invaded cells in each field was counted under microscopic fields at x200 magnification. Experiments were carried out in triplicates and repeated three times. The results were compared to that of A549 cells and data are expressed as mean+SEM. **, $p<0.01$.

Figure 2. Knockdown of pVHL decreases A549 cell proliferation and colonization. A) Cells were cultured in dishes with starting number of $2x10^5$/dish. 1, 3, 5, and 7 days after incubation, the total numbers of cells in each dish were counted. The experiments were carried out in triplicates and repeated three times. Data were expressed as mean ± SEM. *n* =3. **, $p<0.01$. B) Two million cells were injected into athymic nude mice via the lateral tail vein. After five weeks, lungs of these mice were removed and stained with Bouin's solution and the tumor nodules on the left lungs were counted under a dissecting microscope. Data were expressed as mean ± SEM. *n*

=8. **, p<0.01. Representative microscopic images of the H&E staining of the right lungs of these mice were shown in C). Arrows indicate the location of the tumor nodules.

Figure 3. Overexpression of constitutive HIF increases lung cancer cell colonization. A) Cells were cultured in dishes with starting number of $2x10^5$/dish. 1, 3, 5, and 7 days after incubation, the total numbers of cells in each dish were counted. The experiments were carried out in triplicates and repeated three times. Data were expressed as mean ± SEM. *n* =3. B) Two million cells were injected into athymic nude mice via the lateral tail vein. After five weeks, lungs of these mice were removed and stained with Bouin's solution and the tumor nodules on the left lungs were counted under a dissecting microscope. Data were expressed as mean ± SEM. *n* =8. *, p<0.05. Representative microscopic images of the H&E staining of the right lungs of these mice were shown in C). Arrows indicate the location of the tumor nodules.

Figure 4. Knockdown of pVHL decreases expression of integrin α4β1 and downregulates FAK phosphorylation. Cultured A549-sh-VHL, and A549-sh-dHIF were lysed and aliquots containing the same amount of proteins were subjected to SDS-PAGE, followed by Western blot analysis for the detection of integrin α4 (A), integrin β1 (B), total FAK and phosphoryalted FAK (C). Tubulin was used as control for equal loading.