[CANCER RESEARCH 63, 2675–2680, May 15, 2003]

Up-Regulation of Hypoxia-inducible Factor 2α in Renal Cell Carcinoma Associated with Loss of *Tsc-2* Tumor Suppressor Gene[1]

Matt Yu Liu, Lorenz Poellinger, and Cheryl L. Walker[2]

Science Park-Research Division, The University of Texas M. D. Anderson Cancer Center, Smithville, Texas 78957 [M. Y. L., C. L. W.] and Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institutet, S-171 77 Stockholm, Sweden [L. P.]

ABSTRACT

In the Eker rat model, inactivation of the Tuberous Sclerosis-2 (*Tsc-2*) tumor suppressor gene leads to high frequency of spontaneous renal cell carcinoma (RCC). By analogy to human RCC in which mutations in the von Hippel-Lindau (*VHL*) tumor suppressor gene result in accumulation of hypoxia-inducible factor α (HIFα) and up-regulation of vascular endothelial growth factor (VEGF), we investigated the regulation of HIF and its target gene *VEGF* in rat RCC resulting from Tsc-2 defects. To examine HIFα activity, a panel of rat renal epithelial cells were analyzed for expression of HIF1α and the homologous protein, HIF2α, under normoxic and hypoxic conditions. RCC-derived cell lines exhibited high basal levels of HIF activity as determined using hypoxia response element-luciferase reporter constructs. HIF2α was stabilized in RCC-derived cell lines and in five of six primary tumors compared with normal kidney, which was consistent with the high levels of hypoxia response element-reporter activity observed in the cell lines. Primary RCCs that developed in Eker rats were highly vascularized, which was similar to their human counterparts. Furthermore, reverse-transcriptase PCR and immunoblotting demonstrated that VEGF was abundantly expressed in both rat RCC cell lines and primary tumors. The 120-, 164-, and 188-amino-acid isoforms of VEGF were expressed at the RNA and protein levels in RCC-derived cell lines, although only a single band was observed in primary tumors. Taken together, these data suggest that RCC caused by loss of the *Tsc-2* tumor suppressor gene (which retain wild-type *Vhl*) up-regulate VEGF via a HIF2α-mediated mechanism. Thus, loss of *Tsc-2* and *VHL* tumor suppressor gene function appears to have similar consequences in Eker rats and humans respectively, identifying dysregulation of HIFα and VEGF expression as a common pathway for the development of RCC in different species and in tumors with different molecular etiologies.

INTRODUCTION

The ability to adapt to changes in oxygen availability is critical for tumor angiogenesis, metastasis, and physiological and pathological processes such as development and wound healing. The basic-helix-loop-helix PAS[3] domain transcription factor hypoxia-inducible factor 1 (HIF1) plays a critical role in oxygen homeostasis (1). It is composed of two subunits, HIF1α and HIF1β. HIF1α is the oxygen-regulated component that determines HIF activity (2). HIF1α accumulation occurs during hypoxia as a result of inhibition of its proteolytic degradation through the ubiquitin proteasome pathway (3–5). HIF regulates many genes involved in maintaining O_2 homeostasis and the physiological response to O_2 deprivation, such as erythropoietin, glucose transporters, glycolytic pathway enzymes, *VEGF*, heme oxygenase, and inducible nitric oxide synthase (1). Sharing 48% homology with HIF1α, HIF2α is also called endothelial PAS domain protein-1 (EPAS1; see Ref. 6), HIF1α-like factor (HLF; see Ref. 7), HIF-related factor (HRF; see Ref. 8), or MOP-2 ("member of PAS superfamily"; see Ref. 9) and is also present in a number of cell types and tissues (10). Although they differ in abundance and distribution, HIF-1α and HIF2α appear to function similarly. Both become stabilized in response to hypoxia and function as a heterodimer with HIF1β, transactivating the expression of reporter genes containing HRE in DNA (6).

Comprised of at least five known isoforms, VEGF is a potent endothelial cell-specific mitogen that promotes the growth and maintenance of vascular endothelial cells and is the major angiogenesis inducer *in vivo* (11). VEGF expression can be regulated at the transcriptional level or by stabilization of VEGF mRNA. Binding of HIF1 to the consensus HRE in the VEGF promoter promotes VEGF gene expression (12, 13). Studies on regulation of VEGF and erythropoietin by hypoxia (12, 14, 15) revealed that for both genes, hypoxia-inducibility is conferred by homologous enhancer sequences. A 28-bp region in the 5′ promoter of rat and human VEGF (15) has high homology and similar protein binding characteristics as the HIF1 binding sites (HRE sites) in *EPO* (16). Many studies have shown that high levels of VEGF are produced by various types of tumors [for review see Ref. 17)]. These and other studies have established the role of VEGF in tumor angiogenesis and underscore the importance of identifying the regulatory mechanisms of VEGF expression in tumors.

RCC arises from the epithelial cells of the renal nephron and is characterized by its many different variants (18). Alterations in the von Hippel-Lindau (*VHL*) tumor suppressor gene are associated with the clear-cell variant of this disease (19, 20), which accounts for ~75% of all RCC (21). Both HIF1α and HIF2α are regulated by VHL-mediated ubiquitination and degradation and are short-lived under normoxic conditions. Under normoxic conditions, HIF1α/2α is a major target for the specific E3 ubiquitin ligase activity of pVHL (1). Proline hydroxylation of HIF in the presence of oxygen targets this protein for recognition by VHL, ubiquitination, and proteolysis (22–28). Mutations in VHL in human RCC result in accumulation of HIF1α/2α and further up-regulation of VEGF mRNA expression (23, 29–33).

In contrast to human RCC, the *Tsc-2* tumor suppressor gene is the primary target for RCC in rodents. A hereditary form of RCC occurs in a line of Long-Evans rats carrying a mutation in the *Tsc-2* gene (*Tsc-2*$^{Ek/+}$; see Ref. 34). The Eker mutation, a result of a retroviral insertion event in the *Tsc-2* gene, predisposes these animals to a high frequency of spontaneous and carcinogen-induced RCC (35–38), which develop in these animals subsequent to loss of the wild-type *Tsc-2* allele (39, 40). *Tsc-2* knockout mice similarly develop spontaneous RCC (41, 42) in contrast to *Vhl* knockout mice, which do not develop these tumors (43, 44). In addition, carcinogen-induced rat RCC also exhibits mutations in the *Tsc-2* gene (40, 45, 46). Phenotypically, murine RCC differs from its human counterpart in that these tumors are predominantly chromophilic and, although predominantly solid, often have a prominent cystic component (47, 48). RCC that develops in the Eker rat expresses wild-type *Vhl* (49, 50), and *Vhl* mutations in rat RCC are uncommon, although when they occur, they are associated with rare RCC with clear-cell cytology (51).

Received 8/21/02; accepted 3/13/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in parts by NIH Grants CA63613 and ES08263 (to C. L. W.) and ES07784.

[2] To whom requests for reprints should be addressed, at Science Park-Research Division, The University of Texas M. D. Anderson Cancer Center, Smithville, TX 78957. Phone: (512) 237-9550; Fax: (512) 237-2475.

[3] The abbreviations used are: PAS, Per-Arnt-Sim; VEGF, vascular endothelial growth factor; HRE, hypoxia response element; VHL, von Hippel-Lindau; Tsc-2, tuberous sclerosis complex-2; DFX, desferrioxamine; ALLN, Ac-LLnL-CHO; RT, reverse transcriptase; aa, amino acid(s).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/10/2675/2503456/ch1003002675.pdf by guest on 12 August 2024

To determine whether dysregulation of HIF1α/2α and VEGF expression occurs in RCC resulting from inactivation of the *Tsc-2* tumor suppressor gene, we examined HIF1α/2α expression and activity in normal and neoplastic rat renal cells under normoxic and hypoxic conditions. Cells derived from *Tsc-2* null rat RCC showed constitutively high expression of HIF2α and HIF transcriptional activity. Furthermore, HIF2α accumulation in primary tumors correlated with up-regulation of VEGF and tumor vascularization.

MATERIALS AND METHODS

Reagents. The hypoxia mimetics DFX and $CoCl_2$ and proteasomal protease inhibitor ALLN were from Sigma (St. Louis, MO). Mouse monoclonal anti-HIF1α (NB100-105, clone H1α67), rabbit polyclonal anti-HIF2α (NB100-122) antibody (Novus, Littleton, CO), and rabbit polyclonal anti-VEGF (Santa Cruz, Santa Cruz, CA) were used for Western analysis. For reporter assays, luciferase reporter constructs PL949 (a generous gift from Dr. Chris Bradfield, Madison, WI) and HRE-Luc with six or three tandem copies of HRE of the erythropoietin gene were used. pGL3 promoter vector and pRL-TK Renila constructs were obtained from Promega (Madison, WI).

Cells Lines and Culture Conditions. The ERC15 and ERC18 cell lines were derived from Eker rat RCC (38). As described previously, neither of them expresses the *Tsc-2* gene product tuberin (52). The TRKE2 cell line was established by *in vitro* transformation of kidney epithelial cells with *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (53) and has no mutations within the *Tsc-2* coding region (unpublished data). EKT2 carries two copies of the mutant *Tsc-2* allele ($Tsc\text{-}2^{EK/EK}$) and does not express tuberin (52). ERC15, ERC18, EKT2, and TRKE2 were maintained as described previously (52). The human renal tumor cell lines A498, 786-O derived from clear-cell RCCs, and 112, ACHN derived from non-clear-cell RCC were maintained as previously described (54).

Western Analysis. Cells were harvested in TEN (40 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl) buffer and then lysated in cell extraction WCE buffer (10 mM HEPES, 400 mM NaCl, 0.1 mM EDTA, 5% glycerol) with protease inhibitors. After centrifugation, the supernatant containing the total cell lysate was quantitated. Proteins separated in SDS-polyacrylamide gels were transferred onto polyvinylidene difluoride membranes, and blocked for 1 h in 5% milk with washing buffer (Tris 5 mM, NaCl 35 mM, 0.1% Tween 20). Primary antibodies were hybridized for 2 h in 2% milk for 2 h at room temperature. A secondary antibody conjugated to horseradish peroxidase (1:2000 dilution) was hybridized for 1 h in 2% milk with washing buffer. After extensive rinses with washing buffer, the complexes were visualized using KPL LumiGLO (KPL, Gaithersburg, MD). Tumors and normal tissue samples were pulverized under liquid N_2 using mortar and pestle and processed as above.

Luciferase Reporter Assays. Twenty-four-well plates were seeded with 10,000 cells/well and grown overnight in media containing 10% serum. For dual-reporter assay, a Fugene 6 Transfectant Reagent Kit (Roche, Indianapolis, IN) was used to transfect cells with pGL3 promoter vector or PL949 construct together with pRL-TK Renila construct, according to the manufacturer's instructions. Alternatively, cells were cotransfected with HRE-Luc and pCMV-β-galactosidase construct as a control. After 24 h of transfection, cells were treated with 250 μM DFX for 6 h. A Microtiter Plate Luminometer (Thermo Lab Systems, Helsinki, Finland) was used to determine the Firefly and Renila luciferase activity using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI), or Firefly and β-galactosidase activity with a luciferase assay kit by Tropix (Bedford, MA).

RT-PCR. Primers U-14 (5′ CTGCTCTCTTGGGTGCACTGG 3′) and L-556 (5′ CACCGCCTTGGCTTGTCACAT 3′) were designed to discriminate the various isoforms of rat VEGF. cDNA from renal cell carcinomas and normal kidney were synthesized by reverse transcription of total RNA. Reverse transcription was performed at 42°C for 60 min. VEGF cDNA fragments were amplified by 30 rounds of PCR at 1 min at 95°C, 1 min at 58°C, 1 min at 72°C with a Perkin-Elmer Thermal Cycler (Perkin-Elmer, Boston, MA), and *Taq* DNA polymerase (Applied Biosystems, Foster City, CA).

ELISA. Rat RCC and normal kidney samples were extracted with WCE buffer (10 mM HEPES, 400 mM NaCl, 9.1mMEDIA, 5% glycerol, protease inhibitors) and protein concentration quantified using BCA Protein Assay kit (Pierce, Rockford, IL). The VEGF content per 10 μg of total protein was determined using the VEGF ELISA assay kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

Immunohistochemistry. RCC specimens were fixed with 10% formalin and embedded in paraffin according to routine procedures. Formalin-fixed, paraffin-embedded sections of the tumor tissue were examined immunohistochemically with goat polyclonal anti-CD-31 antibody (Santa Cruz, Santa Cruz, CA). Immunoreactive products were visualized with 3,3′-diaminobenzidine (Sigma, St. Louis, MO). Light microscopy was used to visual vessels and micrographs were taken at ×100 magnification (×10 objective and ×10 ocular).

RESULTS

HIF1α and HIF2α Expression. Loss of VHL in RCC has been reported to lead to accumulation of HIF1α/2α because of the abrogated ubiquitin mediated degradation of HIF1α/2α. To confirm these data and establish conditions for using hypoxia mimetics DFX and $CoCl_2$, a panel of RCC cell lines derived from clear-cell (mutant *VHL*) and non-clear-cell (wild-type *VHL*) tumors was examined for expression of HIF1α/2α under normoxic or hypoxic conditions (Fig. 1*A*). In *VHL*-null 786-O and A498 cells, HIF1α could not be detected, but HIF2α was constitutively stabilized under both normoxic and hypoxic conditions. *VHL* wild-type cells, ACHN and 112, did not express HIF1α or HIF2α under normoxia, but accumulated both HIF1α and HIF2α in response to either 250 μM DFX or 250 μM $CoCl_2$ treatment. Treatment with the proteasome inhibitor ALLN did not result in stabilization of HIF2α in ACHN or 112 cells, although a slight stabilization of HIF1α was observed in these cells, primarily in ACHN cells, which was consistent with a previous report that HIF1α is regulated under normoxic conditions through the proteasome mediated pathway (5).

We next analyzed HIF1α and HIF2α expression in a series of rat renal epithelial cell lines containing wild type *Vhl* (49) under normoxic and chemically induced hypoxic conditions. Under normoxic conditions, Eker rat tumor-derived cell lines ($Tsc\text{-}2^{Ek/-}$) ERC15, ERC18, homozygous mutant ($Tsc\text{-}2^{Ek/Ek}$) EKT2 and TRKE2 ($Tsc\text{-}2^{+/+}$) cell lines demonstrated undetectable or low levels endogenous HIF1α expression (Fig. 1*B*). After treatment with 250 μM DFX or 250 μM $CoCl_2$, HIF1α accumulation increased dramatically in ERC18, TRKE2, and EKT2, whereas HIF1α expression in ERC15 remained undetectable. Treatment with 200 μM ALLN for 6 h also stabilized HIF1α protein in ERC18, TRKE2, and EKT2 cell lines, suggesting ubiquitin-mediated degradation of HIF1α occurred under normoxic conditions in these cells (Fig. 1*C*). Primary RCC from Eker rats and normal kidney were also analyzed for HIF1α expression, and as shown in Fig. 1*D*, HIF1α expression was undetectable in all six tumors and in normal kidney.

In contrast to HIF1α, under normoxic conditions, HIF2α was stabilized in ERC15, ERC18, EKT2, and TRKE2 cells as shown in Fig. 1*B*. Treatment with 250 μM DFX or 250 μM $CoCl_2$ did not significantly increase levels of HIF2α compared with normoxia, although ALLN treatment for 6 h did increase levels of HIF2α moderately in these cells, suggesting that further HIF2α stabilization could be achieved by blocking proteasome-mediated degradation (data not shown). In addition to these cell lines, five of six primary Eker rat RCCs also exhibited stabilization of HIF2α, whereas none of the six normal kidney samples examined showed detectable HIF2α expression (Fig. 1*D*).

HIF Transcriptional Activity. To confirm the transcriptional activity of stabilized HIF2α in rat RCC cells, luciferase reporter assays were performed under normoxic and hypoxic conditions. Constitutively high levels of HIF activity were observed in ERC15,TRKE2, and A498 (VHL^{-}) cell lines, which was consistent with the endogenous high level of HIF2α expression detected by Western analysis

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/10/2675/2503456/ch1003002675.pdf by guest on 12 August 2024

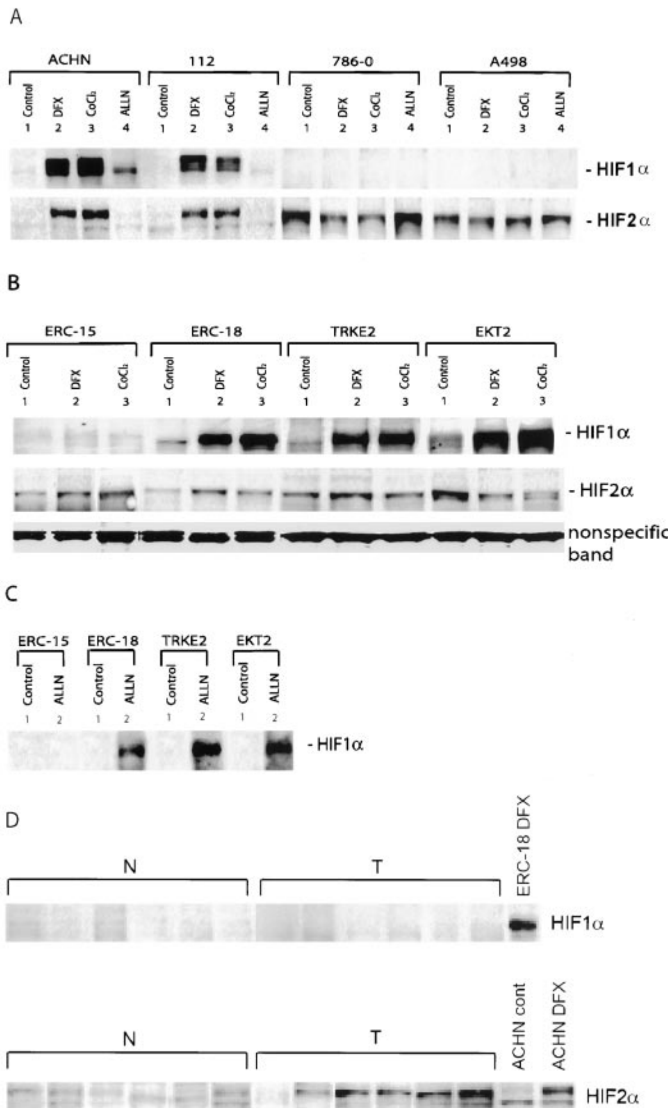

Fig. 1. HIF1α/-2α expression in renal epithelial cells. *A*, immunoblot of total cell lysates from human renal epithelial cell lines. ACHN, 112 non-clear-cell RCC lines with wild-type *VHL*. 786-O, A498 clear-cell RCC lines, $VHL^{-/-}$. *1*, normoxia (control); *2*, treatment with 250 μM DFX for 6 h; *3*, treatment with 250 μM $CoCl_2$ for 6 h; *4*, treatment with 100 μM ALLN for 6 h. *B*, immunoblot of total cell lysates from rat renal epithelial cells. A nonspecific band at ~50 kDa was used as a loading control. *1*, normoxia (control); *2*, treatment with 250 μM DFX for 6 h; *3*, treatment with 250 μM $CoCl_2$ for 6 h. *C*, stabilization of HIF1α in rat renal epithelial cells after treatment with 200 μM ALLN for 6 h. *D*, stabilization of HIF2α in *Tsc-2*-null RCC. Immunoblot of 100 μg of total cell lysate from six Eker rat RCC and six normal kidney samples. *N*, normal kidney; *T*, RCC.

(Fig. 1, *A* and *B*). As shown in Fig. 2*A*, under normoxic conditions, high levels of HRE-driven luciferase activity were observed with PL949 in ERC15, TRKE2, and A498 lines ranging from 3.2–4.5-fold relative to control cells transfected with pGL3, in contrast to ACHN cells (wild-type *VHL*), which exhibited no significant increase relative to controls under normoxic conditions. Similarly, high levels of HRE-Luc activity were observed in ERC15, $TRKE_2$, and A498 cells ranging from 5–22-fold relative to control under normoxic conditions with HRE-Luc construct (data not shown). However, as expected, after treatment with 250 μM DFX for 6 h, ACHN cells exhibited a >5.5-fold increase in HIF-mediated luciferase activity with PL949 relative to normoxic conditions as shown in Fig. 2*B*. In contrast, ERC-15, TRKE2, and A498 exhibited no significant increase in HIF luciferase activity in response to treatment with 250 μM DFX relative to high normoxic levels of HIF activity. A slight increase in HIF activity was observed in TRKE2 cells under hypoxic condition, probably as a result of the accumulation of HIF1α that occurs in these cells (Fig. 1*B*).

Expression of VEGF in RCC. To determine whether the stabilization of HIF2α in *Tsc-2*-null RCC was associated with up-regulation of VEGF and angiogenesis, we next characterized VEGF expression in RCC-derived cell lines and primary tumors. Three isoforms of VEGF were detected in RCC and normal kidney by RT-PCR using isoform-specific primers as shown in Fig. 3*A*. RCC-derived ERC cell lines expressed the 188-, 164-, and 120-aa-specific isoforms of VEGF, although the 188-aa isoform was barely detectable in these cells as shown in Fig. 3*B*. By Western analysis, three VEGF isoforms could be detected in the total cell lysates of ERC15, ERC18, TRKE2, and EKT2 cells (Fig. 3*C*). In seven of seven primary RCCs examined, VEGF was also expressed, although only a single VEGF isoform was present, which was highly expressed relative to normal kidney (Fig. 3*D*). VEGF protein expression was confirmed by ELISA assay to quantitate VEGF production in tumors and normal kidney. Primary rat RCC ($n = 6$) contained 10.2 ± 3.1 pg of VEGF/μg of protein, whereas normal kidney ($n = 6$) contained 0.78 ± 0.34 pg of VEGF/μg of protein (data not shown). Consistent with VEGF overexpression, Eker rat RCC were also highly vascularized, exhibiting larger and more irregular vascular area as compared with the normal kidney (Fig. 4).

DISCUSSION

In this study, we have determined that the transcription factor HIF2α is stabilized in rat RCC expressing the wild-type *VHL* tumor suppressor gene. Primary RCC arising in Eker rats and RCC-derived cell lines expressed HIF2α had constitutively high HIF activity, exhibited VEGF accumulation, and were highly vascularized.

Angiogenesis and extensive tumor vascularization are characteristic of clear-cell RCC, a fact that has been attributed to stabilization of HIF1α/2α and expression of the proangiogenic growth factor VEGF resulting from loss of VHL function (55). In the present study, using

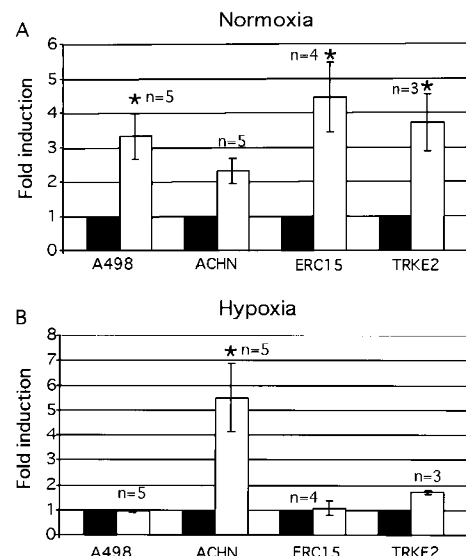

Fig. 2. HRE reporter activity. *A*, fold induction of reporter activity in cells transfected with PL949 (*open bars*) and cells transfected with pGL3 promoter plasmid (*closed bars*) under normoxic conditions. *, denotes significant difference from pGL3 promoter transfected cells. *B*, fold induction of reporter activity (PL949) relative to normoxic levels under hypoxic conditions (DFX treatment for 6 h). *n*, represents individual experiment number. *, indicates significant difference from the cells under normoxic condition. Firefly luciferase activity was normalized to Renila luciferase activity. Statistical significance was determined using ANOVA ($P < 0.05$).

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/10/2675/2503456/ch1003002675.pdf by guest on 12 August 2024

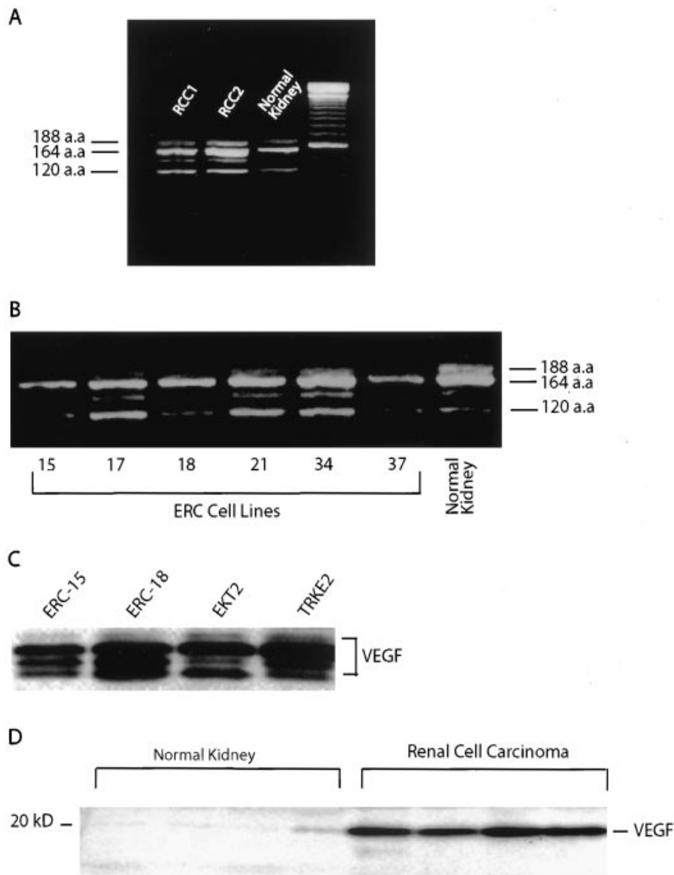

Fig. 3. Identification of VEGF isoforms expressed in rat RCC. *A*, RT-PCR of mRNA from Eker rat RCC and normal kidney. *B*, gel electrophoresis of RT-PCR of RCC-derived cell lines: ERC15, -17, -18, -21, -34, and -37. Expected VEGF amplification products of 408, 540, and 601 bp correspond to VEGF isoforms $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$, respectively. *C*, immunoblot of total cell lysates from rat RCC-derived cell lines (ERC) and transformed rat kidney epithelial cells (EKT2 and TRKE2). Antibodies used recognized three isoforms of VEGF: 120, 164, and 188 aa. *D*, VEGF protein expression in rat RCC. Immunoblot of total cell lysates from seven Eker rat RCC (three; data not shown) and four normal kidney samples under denaturing conditions.

the Eker rat model, *Tsc-2* tumor suppressor gene defects and development of RCC also resulted in HIF stabilization and up-regulation of VEGF. VEGF is a specific mitogen and survival factor for endothelial cells and a vital promoter of angiogenesis physiologically and pathologically. Increased expression of VEGF in RCC has been demonstrated previously (56). Interestingly, VEGF189 isoform in human RCC has been shown to be tightly correlated with the stage and vascularization status of this disease (57). Tumor size is also significantly correlated with VEGF189 isoform expression, with tumors expressing the VEGF189 isoform being more vascularized than those lacking this isoform (57). Rodent VEGF isoforms are one aa shorter than their human homologs (58), and we identified three isoforms of VEGF at the mRNA level in Eker rat RCC-derived cell lines corresponding to the 188-, 164-, and 120-aa isoforms, which were also expressed in primary tumors. In all cases, RNA expression in tumors and cell lines correlated with the up-regulation of VEGF protein as determined by ELISA assay and Western analysis. These data indicate that in RCC associated with loss of *Tsc-2* tumor suppressor gene function, stabilization of HIF2α correlates with tumor angiogenesis.

Rat RCC differs histologically from the human disease, having primarily a chromophilic rather than clear-cell cytology (47, 48). These tumors often have a prominent cystic component and can also have a tubulopapillary appearance. Expression of HIF1α and -2α in human RCC is tightly correlated with the clear-cell variant, particularly HIF2α expression (31). Whereas 17 of 17 clear-cell tumors exhibited stabilization of HIF2α, none of the papillary, chromophobe, or collecting duct tumors examined expressed this gene (31). Whereas human RCC of the clear-cell type results primarily from alterations in the *VHL* tumor suppressor gene, murine RCC results from loss of function of the *Tsc-2* tumor suppressor gene. However, murine and human RCC have the same cell of origin, the proximal tubule epithelial cell of the renal nephron (47, 48). Therefore, expression of HIF2α in both VHL^- human and $Tsc\text{-}2^-$ rat tumors represents a point of etiologic convergence between tumor development via these two pathways. That *TSC-2*- and *VHL*-mediated tumorigenic pathways intersect is further supported by the finding that clear-cell RCC in tuberous sclerosis patients have defects in *TSC-2* but retain wild-type *VH*L (59). Dissecting pathways involved in *TSC-2*- and *VHL*-related RCC might provide important information linking the molecular events that participate in the development of different subsets of RCC.

The VHL tumor suppressor protein targets HIFα subunits for ubiquitin-mediated proteolysis, and in RCC bearing inactivating mutations in both *VHL* alleles, HIFα subunits are stabilized and accumulate at high levels irrespective of oxygen levels. *Tsc-2*-associated tumors and tumor-derived cell lines express wild-type *VHL* (49, 50), however, suggesting that mechanism(s) other than loss of *Vhl* function are responsible for HIF stabilization. Interestingly, the TRKE-2 cell line, which expresses wild-type tuberin, the product of the *Tsc-2* gene, also exhibited HIF2α stabilization, suggesting either that loss of tuberin is not directly responsible for HIF stabilization in *Tsc-2*-null cells, or that in the TRKE cell line, which was transformed *in vitro* by MNNG (53), contains other alterations in the cellular pathway(s) in which tuberin participates. Alternate pathways for HIFα stabilization other than loss of VHL function include loss of p*53* function and cell-signaling via PI3K (60). Interestingly, although tuberin-null tumors and cell lines contain wild-type *p53* (61, 62), tuberin has recently been shown to be a critical down-stream regulator of PI3K signaling (63). This suggests the possibility that stabilization of HIFα in tuberin-null cells may be a result of disrupted PI3K signaling downstream from AKT.

VHL-null clear-cell RCC and cell lines exhibit stabilization of HIF2α. Recent data have suggested functional differences in the role of HIF1α and -2α in RCC. These two proteins share 48% homology and a common *VHL*-mediated degradation pathway. Both of these

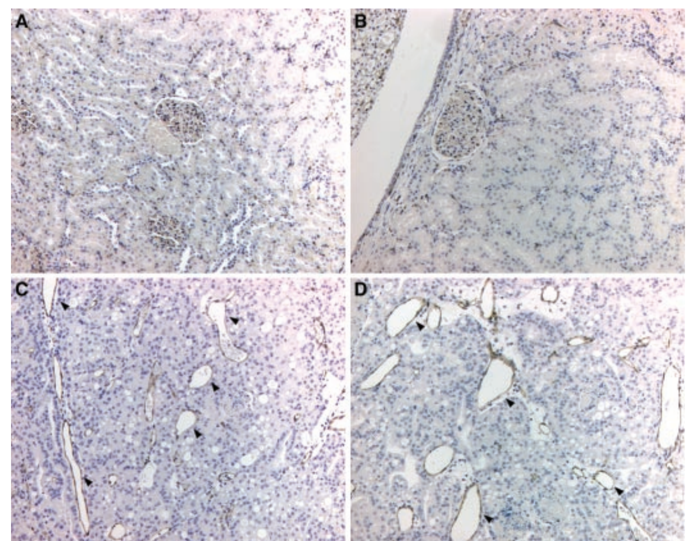

Fig. 4. CD31 staining of primary tumors. CD-31 staining of vascular area in RCC (*C* and *D*) and normal kidney (*A* and *B*). *Arrows* indicate CD-31-positive vessels in tumor sections.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/10/2675/2503456/ch1003002675.pdf by guest on 12 August 2024

proteins form heterodimers with HIF1β and bind to the same HRE in the promoter region of target genes to transactivate target gene expression. However, differences do exist in their regulation and function. Structurally, the two proteins differ at a critical cysteine residue, which determines HIF2α susceptibility to redox regulation (64). HIF1α and -2α also exhibit different tissue distributions. HIF1α mRNA is ubiquitously expressed, whereas HIF2α message is expressed most abundantly in the highly vascularized adult tissues, such as lung, heart, liver, and placenta and endothelial cells of the embryonic and adult mouse (6, 7). Targeted disruption of these genes in mice also leads to different phenotypes (65, 66). In our experiments, both HIF2α (ERC-15, ERC-18) and HIF1α (ERC-18) accumulated in response to the proteasome inhibitor ALLN, indicating the existence of an intact proteolysis pathway in *Tsc-2*-null RCC derived cells, although cell line specificity was observed. Stabilization specifically of HIF2α, rather than HIF1α, under normoxic conditions in these cells suggests that different regulatory mechanisms may exist for the stabilization of HIF1α and HIF2α. Recently, HIF2α has been shown to promote tumor growth, whereas overexpression of HIF1α alone in *VHL* wild-type RCC-derived cells does not reproduce the tumorigenic phenotype (67, 68), suggesting that of the two, HIF2α is the more relevant target for tumorigenesis. Furthermore, the phenotype of tumors in which HIF stabilization was induced by expression of a peptide containing the oxygen-dependent degradation domain of HIF exhibited a poorly differentiated histology, distinct from that of clear-cell type (67), suggesting that VHL function(s) other than stabilization of HIF are responsible for the clear-cell histological phenotype. Specific stabilization of HIF2α, but not HIF1α in spontaneous RCC exhibiting loss of the *Tsc-2* tumor suppressor gene with a chromophilic, rather than clear-cell histology, would support both these hypotheses. The identification of these two different pathways for regulation of HIF in RCC should facilitate future studies aimed at understanding the role of this transcription factor in tumorigenesis and mechanism(s) for selective stabilization of HIF2α *versus* HIF1α.

ACKNOWLEDGMENTS

We thank Dr. C. Bradfield for the PL949 luciferase construct, Dr. J. Gnarra for helpful discussions, and B. Brooks and S. Henninger for manuscript preparation.

REFERENCES

1. Semenza, G. L. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu. Rev. Cell Dev. Biol., *15:* 551–578, 1999.
2. Huang, L. E., Arany, Z., Livingston, D. M., and Bunn, H. F. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α subunit. J. Biol. Chem., *271:* 32253–32259, 1996.
3. Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. Regulation of hypoxia-inducible factor 1β is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. USA, *95:* 7987–7992, 1998.
4. Salceda, S., and Caro, J. Hypoxia-inducible factor 1α (HIF-1aα) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J. Biol. Chem., *272:* 22642–22647, 1997.
5. Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y., and Poellinger, L. Regulation of the hypoxia-inducible transcription factor 1α by the ubiquitin-proteasome pathway. J. Biol. Chem., *274:* 6519–6525, 1999.
6. Tian, H., McKnight, S. L., and Russell, D. W. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev., *11:* 72–82, 1997.
7. Ema, M., Taya, S., Yokotani, N., Sogawa, K., Matsuda, Y., and Fujii-Kuriyama, Y. A novel bHLH-PAS factor with close sequence similarity to hypoxia- inducible factor 1α regulates the VEGF expression and is potentially involved in lung and vascular development. Proc. Natl. Acad. Sci. USA, *94:* 4273–4278, 1997.
8. Flamme, I., Frohlich, T., von Reutern, M., Kappel, A., Damert, A., and Risau, W. HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 α and developmentally expressed in blood vessels. Mech. Dev., *63:* 51–60, 1997.
9. Hogenesch, J. B., Chan, W. K., Jackiw, V. H., Brown, R. C., Gu, Y. Z., Pray-Grant, M., Perdew, G. H., and Bradfield, C. A. Characterization of a subset of the basic-helix-loop-helix-PAS superfamily that interacts with components of the dioxin signaling pathway. J. Biol. Chem., *272:* 8581–8593, 1997.
10. Talks, K. L., Turley, H., Gatter, K. C., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., and Harris, A. L. The expression and distribution of the hypoxia-inducible factors HIF-1α and HIF-2α in normal human tissues, cancers, and tumor-associated macrophages. Am. J. Pathol., *157:* 411–421, 2000.
11. Ferrara, N. VEGF: an update on biological and therapeutic aspects. Curr. Opin. Biotechnol., *11:* 617–624, 2000.
12. Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D., and Semenza, G. L. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol. Cell. Biol., *16:* 4604–4613, 1996.
13. Maxwell, P. H., Dachs, G. U., Gleadle, J. M., Nicholls, L. G., Harris, A. L., Stratford, I. J., Hankinson, O., Pugh, C. W., and Ratcliffe, P. J. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc. Natl. Acad. Sci. USA, *94:* 8104–8109, 1997.
14. Goldberg, M. A., and Schneider, T. J. Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. J. Biol. Chem., *269:* 4355–4359, 1994.
15. Levy, A. P., Levy, N. S., Wegner, S., and Goldberg, M. A. Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. J. Biol. Chem., *270:* 13333–13340, 1995.
16. Madan, A., and Curtin, P. T. A 24-base-pair sequence 3′ to the human erythropoietin gene contains a hypoxia-responsive transcriptional enhancer. Proc. Natl. Acad. Sci. USA, *90:* 3928–3932, 1993.
17. Kerbel, R. S. Tumor angiogenesis: past, present and the near future. Carcinogenesis (Lond.), *21:* 505–515, 2000.
18. Thoenes, W., Storkel, S., and Rumpelt, H. J. Histopathology and classification of renal cell tumors (adenomas, oncocytomas and carcinomas). The basic cytological and histopathological elements and their use for diagnostics. Pathol. Res. Pract., *181:* 125–143, 1986.
19. Zbar, B., and Lerman, M. Inherited carcinomas of the kidney. Adv. Cancer Res., *75:* 163–201, 1998.
20. Gnarra, J. R., Tory, K., Weng, Y., Schmidt, L., Wei, M. H., Li, H., Latif, F., Liu, S., Chen, F., Duh, F. M., *et al*. Mutations of the VHL tumour suppressor gene in renal carcinoma. Nat. Genet., *7:* 85–90, 1994.
21. Kovacs, G., Akhtar, M., Beckwith, B. J., Bugert, P., Cooper, C. S., Delahunt, B., Eble, J. N., Fleming, S., Ljungberg, B., Medeiros, L. J., Moch, H., Reuter, V. E., Ritz, E., Roos, G., Schmidt, D., Srigley, J. R., Storkel, S., van den Berg, E., and Zbar, B. The Heidelberg classification of renal cell tumours. J. Pathol., *183:* 131–133, 1997.
22. Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat. Cell Biol., *2:* 423–427, 2000.
23. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature (Lond.), *399:* 271–275, 1999.
24. Tanimoto, K., Makino, Y., Pereira, T., and Poellinger, L. Mechanism of regulation of the hypoxia-inducible factor-1α by the von Hippel-Lindau tumor suppressor protein. EMBO J., *19:* 4298–4309, 2000.
25. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science (Wash. DC), *292:* 464–468, 2001.
26. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science (Wash. DC), *292:* 468–472, 2001.
27. Bruick, R. K., and McKnight, S. L. A conserved family of prolyl-4-hydroxylases that modify HIF. Science (Wash. DC), *294:* 1337–1340, 2001.
28. Yu, F., White, S. B., Zhao, Q., and Lee, F. S. HIF-1α binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc. Natl. Acad. Sci. USA, *98:* 9630–9635, 2001.
29. Wiesener, M. S., Munchenhagen, P. M., Berger, I., Morgan, N. V., Roigas, J., Schwiertz, A., Jurgensen, J. S., Gruber, G., Maxwell, P. H., Loning, S. A., Frei, U., Maher, E. R., Grone, H. J., and Eckardt, K. U. Constitutive activation of hypoxia-inducible genes related to overexpression of hypoxia-inducible factor-1α in clear cell renal carcinomas. Cancer Res., *61:* 5215–5222, 2001.
30. Gnarra, J. R., Zhou, S., Merrill, M. J., Wagner, J. R., Krumm, A., Papavassiliou, E., Oldfield, E. H., Klausner, R. D., and Linehan, W. M. Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. Proc. Natl. Sci. USA, *93:* 10589–10594, 1996.
31. Turner, K. J., Moore, J. W., Jones, A., Taylor, C. F., Cuthbert-Heavens, D., Han, C., Leek, R. D., Gatter, K. C., Maxwell, P. H., Ratcliffe, P. J., Cranston, D., and Harris, A. L. Expression of hypoxia-inducible factors in human renal cancer: relationship to angiogenesis and to the von Hippel-Lindau gene mutation. Cancer Res., *62:* 2957–2961, 2002.
32. Krieg, M., Haas, R., Brauch, H., Acker, T., Flamme, I., and Plate, K. H. Up-regulation of hypoxia-inducible factors HIF-1α and HIF-2α under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. Oncogene, *19:* 5435–5443, 2000.
33. Iliopoulos, O., Levy, A. P., Jiang, C., Kaelin, W. G., Jr., and Goldberg. M. A. Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. Proc. Natl. Acad. Sci. USA, *93:* 10595–10599, 1996.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/10/2675/2503456/ch1003002675.pdf by guest on 12 August 2024

34. Eker, R., and Mossige, J. A dominant gene for renal adenomas in the rat. Nature (Lond.), *189:* 858–859, 1961.
35. Walker, C., Goldsworthy, T. L., Wolf, D. C., and Everitt, J. Predisposition to renal cell carcinoma due to alteration of a cancer susceptibility gene. Science (Wash. DC), *255:* 1693–1695, 1992.
36. Yeung, R. S., Xiao, G. H., Jin, F., Lee, W. C., Testa, J. R., and Knudson, A. G. Predisposition to renal carcinoma in the Eker rat is determined by germ- line mutation of the tuberous sclerosis 2 (*TSC2*) gene. Proc. Natl. Acad. Sci. USA, *91:* 11413–11416, 1994.
37. Kobayashi, T., Hirayama, Y., Kobayashi, E., Kubo, Y., and Hino, O. A germline insertion in the tuberous sclerosis (*Tsc2*) gene gives rise to the Eker rat model of dominantly inherited cancer. Nat. Genet., *9:* 70–74, 1995.
38. Hino, O., Klein-Szanto, A. J., Freed, J. J., Testa, J. R., Brown, D. Q., Vilensky, M., Yeung, R. S., Tartof, K. D., and Knudson, A. G. Spontaneous and radiation-induced renal tumors in the Eker rat model of dominantly inherited cancer. Proc. Natl. Acad. Sci. USA, *90:* 327–331, 1993.
39. Yeung, R. S., Xiao, G. H., Everitt, J. I., Jin, F., and Walker, C. L. Allelic loss at the tuberous sclerosis 2 locus in spontaneous tumors in the Eker rat. Mol. Carcinog., *14:* 28–36, 1995.
40. Kobayashi, T., Urakami, S., Hirayama, Y., Yamamoto, T., Nishizawa, M., Takahara, T., Kubo, Y., and Hino, O. Intragenic *Tsc2* somatic mutations as Knudson's second hit in spontaneous and chemically induced renal carcinomas in the Eker rat model. Jpn. J. Cancer Res., *88:* 254–261, 1997.
41. Kobayashi, T., Minowa, O., Kuno, J., Mitani, H., Hino, O., and Noda, T. Renal carcinogenesis, hepatic hemangiomatosis, and embryonic lethality caused by a germ-line *Tsc2* mutation in mice. Cancer Res., *59:* 1206–1211, 1999.
42. Onda, H., Lueck, A., Marks, P. W., Warren, H. B., and Kwiatkowski, D. J. Tsc2(+/−) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. J. Clin. Investig., *104:* 687–695, 1999.
43. Gnarra, J. R., Ward, J. M., Porter, F. D., Wagner, J. R., Devor, D. E., Grinberg, A., Emmert-Buck, M. R., Westphal, H., Klausner, R. D., and Linehan, W. M. Defective placental vasculogenesis causes embryonic lethality in VHL-deficient mice. Proc. Natl. Acad. Sci. USA, *94:* 9102–9107, 1997.
44. Haase, V. H., Glickman, J. N., Socolovsky, M., and Jaenisch, R. Vascular tumors in livers with targeted inactivation of the von Hippel-Lindau tumor suppressor. Proc. Natl. Acad. Sci. USA, *98:* 1583–1588, 2001.
45. Urakami, S., Tokuzen, R., Tsuda, H., Igawa, M., and Hino, O. Somatic mutation of the tuberous sclerosis (*Tsc2*) tumor suppressor gene in chemically induced rat renal carcinoma cell. J. Urol., *158:* 275–278, 1997.
46. Satake, N., Urakami, S., Hirayama, Y., Izumi, K., and Hino, O. Biallelic mutations of the *Tsc2* gene in chemically induced rat renal cell carcinoma. Int. J. Cancer, *77:* 895–900, 1998.
47. Wolf, D. C., Whiteley, H. E., and Everitt, J. I. Preneoplastic and neoplastic lesions of rat hereditary renal cell tumors express markers of proximal and distal nephron. Vet. Pathol., *32:* 379–386, 1995.
48. Everitt, J. I., Goldsworthy, T. L., Wolf, D. C., and Walker, C. L. Hereditary renal cell carcinoma in the Eker rat: a rodent familial cancer syndrome. J. Urol., *148:* 1932–1936, 1992.
49. Walker, C., Ahn, Y. T., Everitt, J., and Yuan, X. Renal cell carcinoma development in the rat independent of alterations at the *VH*L gene locus. Mol. Carcinog., *15:* 154–161, 1996.
50. Shiao, Y. H., Diwan, B. A., Perantoni, A. O., Calvert, R. J., Zbar, B., Lerman, M. I., and Rice, J. M. Polymerase chain reaction-single-strand conformation polymorphism analysis for the *VHL* gene in chemically induced kidney tumors of rats using intron-derived primers. Mol. Carcinog., *19:* 230–235, 1997.
51. Shiao, Y. H., Rice, J. M., Anderson, L. M., Diwan, B. A., and Hard, G. C. von Hippel-Lindau gene mutations in N-nitrosodimethylamine-induced rat renal epithelial tumors. J. Natl. Cancer Inst., *90:* 1720–1723, 1998.
52. Kleymenova, E., Ibraghimov-Beskrovnaya, O., Kugoh, H., Everitt, J., Xu, H., Kiguchi, K., Landes, G., Harris, P., and Walker, C. Tuberin-dependent membrane Localization of polycystin-1. A functional link between polycystic kidney disease and the *TSC2* tumor suppressor gene. Mol. Cell, *7:* 823–832, 2001.
53. Walker, C., and Ginsler, J. Development of a quantitative *in vitro* transformation assay for kidney epithelial cells. Carcinogenesis (Lond.), *13:* 25–32, 1992.
54. Perera, A. D., Kleymenova, E. V., and Walker, C. L. Requirement for the von Hippel-Lindau tumor suppressor gene for functional epidermal growth factor receptor blockade by monoclonal antibody C225 in renal cell carcinoma. Clin. Cancer Res., *6:* 1518–1523, 2000.
55. Kaelin, Jr., W. G. Molecular basis of the VHL hereditary cancer syndrome. Nat. Rev. Cancer, *2:* 673–682, 2002.
56. Brown, L. F., Berse, B., Jackman, R. W., Tognazzi, K., Manseau, E. J., Dvorak, H. F., and Senger, D. R. Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. Am. J. Pathol., *143:* 1255–1262, 1993.
57. Tomisawa, M., Tokunaga, T., Oshika, Y., Tsuchida, T., Fukushima, Y., Sato, H., Kijima, H., Yamazaki, H., Ueyama, Y., Tamaoki, N., and Nakamura, M. Expression pattern of vascular endothelial growth factor isoform is closely correlated with tumour stage and vascularisation in renal cell carcinoma. Eur. J. Cancer, *35:* 133–137, 1999.
58. Ferrara, N., and Davis-Smyth, T. The biology of vascular endothelial growth factor. Endocr. Rev., *18:* 4–25, 1997.
59. Duffy, K., Al-Saleem, T., Karbowniczek, M., Ewalt, D., Prowse, A. H., and Henske, E. P. Mutational analysis of the von Hippel-Lindau gene in clear cell renal carcinomas from tuberous sclerosis complex patients. Mod. Pathol., *15:* 205–210, 2002.
60. Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. Activation of the HIF pathway in cancer. Curr. Opin. Genet. Dev., *11:* 293–299, 2001.
61. Horesovsky, G., Recio, L., Everitt, J., Goldsworthy, T., Wolf, D. C., and Walker, C. p53 status in spontaneous and dimethylnitrosamine-induced renal cell tumors from rats. Mol. Carcinog., *12:* 236–240, 1995.
62. Nishiyama, Y., Suwa, H., Okamoto, K., Fukumoto, M., Hiai, H., and Toyokuni, S. Low incidence of point mutations in H-, K- and N-*ras* oncogenes and *p53* tumor suppressor gene in renal cell carcinoma and peritoneal mesothelioma of Wistar rats induced by ferric nitrilotriacetate. Jpn. J. Cancer Res., *86:* 1150–1158, 1995.
63. McManus, E. J., and Alessi, D. R. TSC1-TSC2: a complex tale of PKB-mediated S6K regulation. Nat. Cell Biol., *4:* E214–216, 2002.
64. Lando, D., Pongratz, I., Poellinger, L., and Whitelaw, M. L. A redox mechanism controls differential DNA binding activities of hypoxia-inducible factor (HIF) 1α and the HIF-like factor. J. Biol. Chem., *275:* 4618–4627, 2000.
65. Tian, H., Hammer, R. E., Matsumoto, A. M., Russell, D. W., and McKnight, S. L. The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev., *12:* 3320–3324, 1998.
66. Iyer, N. V., Kotch, L. E., Agani, F., Leung, S. W., Laughner, E., Wenger, R. H., Gassmann, M., Gearhart, J. D., Lawler, A. M., Yu, A. Y., and Semenza, G. L. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 α. Genes Dev., *12:* 149–162, 1998.
67. Maranchie, J. K., Vasselli, J. R., Riss, J., Bonifacino, J. S., Linehan, W. M., and Klausner, R. D. The contribution of VHL substrate binding and HIF1-α to the phenotype of VHL loss in renal cell carcinoma. Cancer Cell, *1:* 247–255, 2002.
68. Kondo, K., Klco, J., Nakamura, E., Lechpammer, M., and Kaelin, W. G. Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell, *1:* 237–246, 2002.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/63/10/2675/2503456/ch1003002675.pdf by guest on 12 August 2024