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Negative regulation of hypoxia-inducible genes by the von Hippel–Lindau protein

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ABSTRACT **Inactivation of the von Hippel–Lindau protein (pVHL) has been implicated in the pathogenesis of renal carcinomas and central nervous system hemangioblastomas. These are highly vascular tumors which overproduce angiogenic peptides such as vascular endothelial growth factor/vascular permeability factor (VEGF/VPF). Renal carcinoma cells lacking wild-type pVHL were found to produce mRNAs encoding VEGF/VPF, the glucose transporter GLUT1, and the platelet-derived growth factor B chain under both normoxic and hypoxic conditions. Reintroduction of wild-type, but not mutant, pVHL into these cells specifically inhibited the production of these mRNAs under normoxic conditions, thus restoring their previously described hypoxia-inducible profile. Thus, pVHL appears to play a critical role in the transduction of signals generated by changes in ambient oxygen tension.**

Germ-line mutations of the von Hippel–Lindau (*VHL*) gene produce a hereditary cancer syndrome characterized by the development of renal carcinomas, retinal angiomas, central nervous system hemangioblastomas, and pheochromocytomas (1, 2). Tumor development in this setting is associated with inactivation or loss of the remaining wild-type *VHL* allele (2). In keeping with Knudson's model, mutation or transcriptional silencing of both *VHL* alleles appears to be a common event in sporadic renal carcinomas and cerebellar hemangioblastomas (3–7).

Renal carcinomas and central nervous system hemangioblastomas are highly vascular tumors which overproduce angiogenic peptides such as vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) (8–10). Furthermore, these tumors, and pheochromocytomas, may cause paraneoplastic erythrocytosis due to ectopic erythropoietin production (11). These observations suggested that the *VHL*-encoded protein (pVHL) might regulate the expression of hypoxia-inducible genes such as VEGF/VPF (12, 13) and erythropoietin (14, 15).

MATERIALS AND METHODS

Cell Culture. 786-O renal carcinoma cells and Hep3B human hepatoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% defined/supplemented bovine calf serum (HyClone). Renal carcinoma 786-O subclones stably transfected with pRc/CMV, pRc/CMV-VHL(wt), and pRc/CMV-VHL(1–115) plasmids were grown as described previously (16). Cells were grown in 100-mm dishes in 10 ml of medium unless otherwise indicated. Cells cultured under hypoxic conditions (5% CO_2/1% O_2/94% N_2) were grown in an ESPEC triple gas incubator (Tabai-Espec Corp, Osaka, Japan). All experiments were performed when cells reached ≈70-90% confluence.

Northern Blot Analysis. Northern blot analysis was performed essentially as described previously (12). Total RNA was prepared using RNA STAT-60 (Tel-Test "B", Friendswood, TX) and isolated according to the supplier's protocol. Total RNA (15 μg per lane) was denatured in formaldehyde, electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and a trace amount of ethidium bromide, and transferred to a GeneScreen*Plus* nylon filter (New England Nuclear) using 10× standard saline citrate (1× = 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). Human VEGF cDNA was a gift of Judith Abraham at Scios Nova (Mountain View, CA), human platelet-derived growth factor (PDGF) B chain cDNA was a gift of Chuck Stiles (Dana–Farber Cancer Institue, Boston), human GLUT1 cDNA was a gift of Barbara Kahn (Beth Israel Hospital, Boston), human c-myc cDNA was a gift of Chi Van Dang (Johns Hopkins Hospital, Baltimore), and the cyclophilin cDNA was a gift of Ursula Kaiser (Brigham and Women's Hospital, Boston).

Metabolic Labeling and Immunoprecipitation. Equal numbers of viable cells, as determined by trypan blue exclusion, were plated in 100-mm dishes. Forty-eight hours later, cells were washed twice with phosphate-buffered saline (PBS) and once with methionine-free medium, and then grown in 4 ml of medium containing [^{35}S]methionine [500 μCi·ml^{-1} (1 μCi = 37 kBq); EXPRE^{35}S^{35}S, New England Nuclear] for 3 or 6 hr. Aliquots (1 ml) of supernatant were precleared by serial passage over protein A-Sepharose. Aliquots of precleared tissue culture supernatant containing ≈5 × 10^6 trichloroacetic acid-precipitable cpm were incubated with 0.5 μg of anti-VEGF (Santa Cruz Biotechnology) or control [anti-VHL (R98)] affinity-purified polyclonal antibody for 1 hr with rocking at 4°C. Immunoprecipitation with protein A-Sepharose was as described previously (17).

VEGF/VPF Radioimmunoassay. A 1-ml sample of medium was removed from each 100-mm dish at 1, 2, and 6 hr and frozen at −20°C for later analysis. At the conclusion of the experiment, all the medium was removed, the plates were washed with PBS, and the adherent cells were scraped, pelleted, and frozen at −20°C. VEGF ELISA was performed on each sample, in duplicate, using a commercially available kit (R & D Systems) according to the manufacturer's protocol. Error bars represent 1 SEM (three independent samples). Protein content of the cell pellets was determined in duplicate by using the Bradford protein assay (Bio-Rad).

VEGF Promoter Assay. VEGF promoter assays were performed essentially as described previously (18). Briefly, ≈4 × 10^6 cells were electroporated with 20 μg of a reporter plasmid containing 1.7 kb of rat VEGF 5′ flanking sequence fused upstream of the luciferase gene. Cells from a single cuvette were then split and grown on plastic dishes in 1% or 21% oxygen for 18 hr prior to the determination of luciferase activity.

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Abbreviations: pVHL, von Hippel–Lindau protein; VEGF/VPF, vascular endothelial growth factor/vascular permeability factor; PDGF, platelet-derived growth factor.

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VEGF/VPF RNase Protection Assay. Cells were grown to 50% confluency and all measurements were done in triplicate from three separate flasks. Actinomycin D (Sigma) was added to cultures to a final concentration of 5 μg/ml. Total RNA was isolated by the modified acid/phenol method of Chomczynski using RNAzol (Tel-Test). Ten micrograms of RNA from each sample was analyzed by RNase protection assay using a VEGF165 isoform-specific riboprobe (19) and a riboprobe for 18S rRNA (Ambion) for normalization. The RNase protection assay was performed as described previously (19) and quantitated by PhosphorImager (Molecular Dynamics) analysis and IMAGE-QUANT software.

RESULTS

Renal carcinoma 786-O cells contain a frameshift *VHL* mutation and fail to produce endogenous, wild-type, pVHL (16). In a previous study, we generated multiple stable, independent, 786-O subclones producing hemagglutinin-tagged wild-type pVHL [HA-pVHL(wt)] or HA-pVHL(1–115) (16). The latter is a C-terminal truncation mutant lacking residues which are frequently altered or deleted by tumor-derived *VHL* mutations. The introduction of HA-VHL(wt) into 786-O cells had no apparent effect on their growth *in vitro* but dramatically inhibited their growth as tumors in nude mice (16).

The 786-O cells, as well as 786-O cells producing HA-pVHL(1–115), produced high levels of VEGF/VPF mRNA under both hypoxic (1% O_2) and normoxic (21% O_2) conditions as determined by Northern blot analysis (Fig. 1). In contrast, the presence of HA-VHL(wt) inhibited VEGF mRNA production under normoxic, but not hypoxic, conditions (Fig. 1). Comparable loading of RNA in each lane was confirmed by ethidium bromide staining. Wild-type pVHL had similar effects on the abundance of mRNAs encoded by the hypoxia-inducible GLUT1 glucose transporter (20–22) and PDGF B-chain genes (23) (Fig. 1). Thus, reintroduction of wild-type pVHL restored the hypoxia-inducible pattern described previously for these mRNAs in a variety of cell types, such as the Hep3B cells used as a control in this experiment (Fig. 1) (14, 15). In contrast, the presence or absence of wild-type pVHL had no apparent effect on the expression of constitutively transcribed mRNAs, such as cyclophilin and actin mRNAs, or on the expression of the cell-growth-regulated c-myc mRNA (Fig. 1). As is typical of many mRNAs, the abundance of the cyclophilin mRNA was somewhat diminished under hypoxic conditions in a VHL-independent manner (Fig. 1 and C.J., A.P.L., and M.A.G., unpublished data). Erythropoietin mRNA was not detected in 786-O cells, under either normoxic or hypoxic conditions, by Northern blotting and RNase protection assays (C.J., A.P.L., and M.A.G., unpublished data). Paraneoplastic erythrocytosis occurs in only 1–5% of renal carcinomas (11).

To ask whether the differences in VEGF/VPF mRNA abundance observed under normoxic conditions were manifest at the protein level, 786-O subclones producing wild-type or mutant HA-pVHL were metabolically labeled with [^{35}S]methionine under normoxic conditions. Newly synthesized VEGF/VPF was assayed by immunoprecipitation of tissue culture supernatants followed by gel electrophoresis and autoradiography (Fig. 2). The presence of wild-type HA-pVHL inhibited VEGF/VPF secretion in this assay, although it did not have a global effect on protein secretion as determined by the amount of trichloroacetic acid-precipitable ^{35}S present in the tissue culture supernatants (Fig. 2 and O.I., C. Corless, A. Kibel, and W.G.K., unpublished data). In parallel, VEGF/VPF secretion by unlabeled 786-O subclones was measured by ELISA (Fig. 3). This assay also showed that the decrease in VEGF/VPF mRNA abundance in the presence of wild-type pVHL under normoxic conditions was associated with a decrease in VEGF/VPF secretion.

Hypoxia regulates the production of VEGF mRNA at both the transcriptional and posttranscriptional levels. Specifically, hypoxia activates the HIF1 transcription factor, leading to a 2- to 5-fold increase in VEGF/VPF transcription (18, 25, 26). In

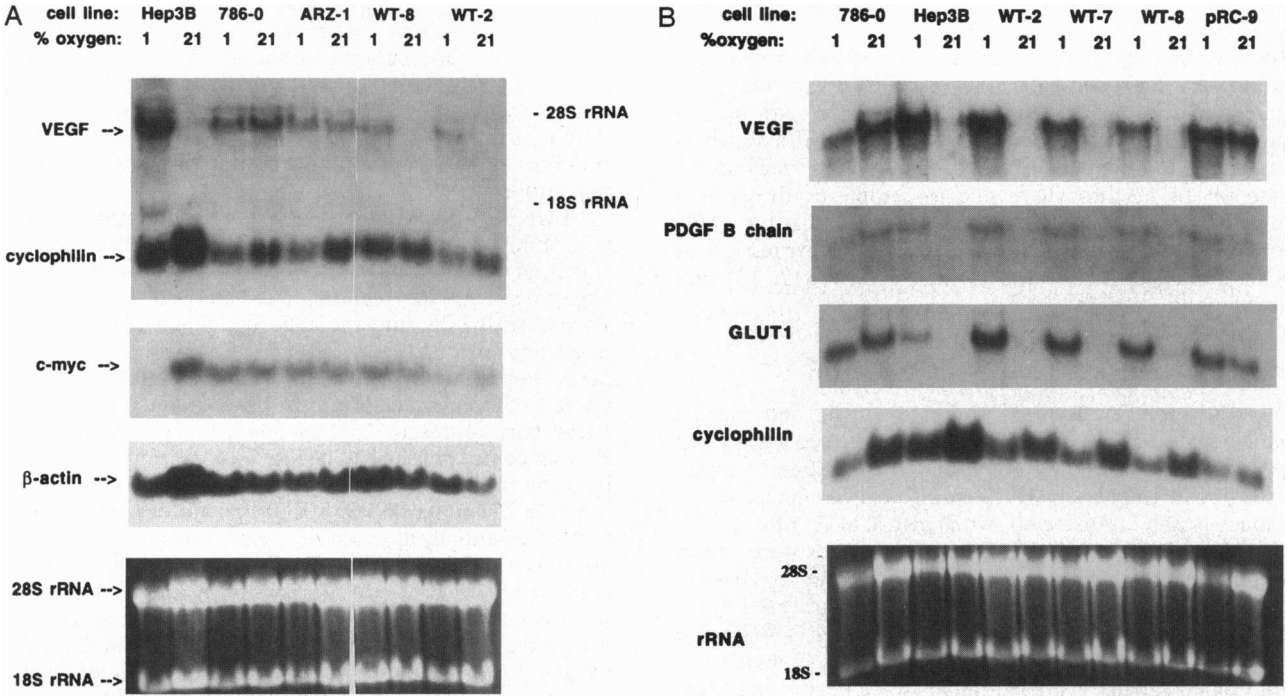

FIG. 1. Regulation of hypoxia-inducible gene expression by pVHL. Hep3B human hepatoma cells, 786-O renal carcinoma cells, and 786-O subclones stably transfected with mammalian expression plasmids encoding pVHL(wt) (cell lines WT-2, WT-7, and WT- 8) or pVHL(1–115) (cell line ARZ-1) (16), or with the backbone expression plasmid pRc-CMV (cell line pRC-9) (16), were grown in the presence of 1% or 21% oxygen. Total RNA was isolated 24 (*A*) or 37 (*B*) hr later and analyzed by Northern blotting using the indicated probes. Comparable loading of RNA was confirmed by ethidium bromide staining.

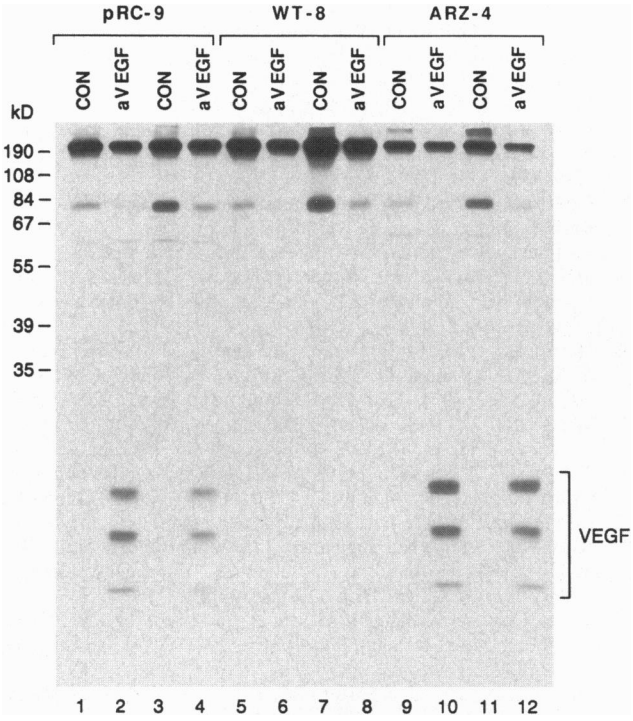

FIG. 2. Detection of newly synthesized VEGF protein in renal carcinoma cell culture supernatants. Renal carcinoma 786-O clonal cell lines stably transfected with mammalian expression plasmids encoding pVHL(wt) or pVHL(1–115) (WT-8 and ARZ-4, respectively) (16) or with the backbone expression plasmid pRc-CMV (pRC-9) (16) were grown in the presence of 21% oxygen and metabolically labeled with [^{35}S]methionine. Three (lanes 1, 2, 5, 6, 9, and 10) and 6 (lanes 3, 4, 7, 8, 11, and 12) hr later, aliquots of tissue culture supernatant were removed and immunoprecipitated with affinity-purified polyclonal anti-VEGF or anti-VHL (control) sera. Immunoprecipitates were analyzed by SDS/15%PAGE and were detected by autoradiography. Secreted VEGF/VPF migrates as a family of polypeptides due to alternative splicing and posttranslational modification (24).

addition, the half life of the VEGF/VPF mRNA, which under normoxic conditions is 30–45 min, is increased 3- to 10-fold by hypoxia (22, 25, 27–29). To determine whether the effect of pVHL on VEGF mRNA abundance was transcriptional, post-transcriptional, or both, additional experiments were performed. In the first set of experiments, 786-O clones which did or did not produce exogenous wild-type pVHL were transfected with a reporter plasmid containing approximately 1.7 kb of VEGF/VPF 5′ flanking sequence fused to a luciferase cDNA (18). The reporter plasmid used in these studies contains the hypoxia-responsive 5′ regulatory elements, including the HIF1 binding site, which have been identified to date in the VEGF/VPF gene (18). Following transfection the cell cultures were split and cultured under normoxic or hypoxic conditions. The presence of wild-type pVHL had no apparent effect on the induction of luciferase activity by hypoxia (Fig. 4*A*). In addition, nuclear run-on experiments performed to date have failed to detect an effect of wild-type pVHL on VEGF/VPF transcription under normoxic conditions (ref. 30 and O.I., C. Corless, A. Kibel, and W.G.K., unpublished data). These observations, taken together, suggested that the effect of pVHL on VEGF/VPF mRNA abundance might be posttranscriptional.

To address this possibility, parental 786-O cells and a 786–O subclone producing wild-type, exogenous, pVHL (WT-8) were grown under normoxic conditions. VEGF mRNA abundance was determined by RNase protection assay at various times following the addition of actinomycin D. At time 0, the VEGF mRNA/18S rRNA ratio was ≈5-fold greater in the parental

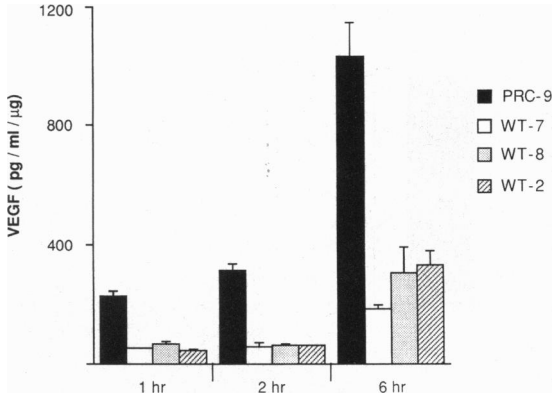

FIG. 3. Measurement of VEGF protein in renal carcinoma cell culture supernatants. The 786-O renal carcinoma clonal cell lines stably transfected with a mammalian expression plasmid encoding pVHL(wt) (WT-2, WT-7, and WT-8) (16) or with the backbone expression plasmid pRc-CMV (pRC-9) (16) were plated in triplicate and grown in the presence of 21% oxygen. Aliquots of tissue culture supernatant were removed at the indicated times after media change and VEGF concentration was determined by ELISA. At the conclusion of the experiment the cells were washed, pelleted, and analyzed for total protein content. Values shown are VEGF concentration normalized for the amount of protein in the cell pellet.

786-O cells than in the WT-8 cells, in keeping with the Northern blot data shown in Fig. 1. The half-life of the VEGF mRNA in the parental cells was ≈4 hr, whereas it was ≈1 hr in the WT-8 cells (Fig. 4*B*). Thus, the effect of pVHL on VEGF mRNA abundance under normoxic conditions can be largely, if not exclusively, accounted for by changes in the apparent stability of the VEGF mRNA. In particular, the VEGF mRNA in VHL -/- cells appears to be inappropriately stable under normoxic conditions, with a half-life which would be typical for hypoxic cells.

DISCUSSION

VEGF/VPF has been implicated in tumor angiogenesis and, in model systems, inhibition of VEGF/VPF can inhibit tumorigenesis (31–34). Our data suggest a model wherein the development of tumors in von Hippel–Lindau disease is due, at least in part, to loss of pVHL function and consequent deregulation of hypoxia-inducible genes such as those encoding VEGF/VPF and PDGF B chain. An antiangiogenic effect of pVHL may account for the earlier observation that pVHL inhibits 786-O cell growth *in vivo* but not *in vitro* (16). As a corollary, these data raise the possibility that inhibition of angiogenic peptides such as VEGF/VPF might mimic the ability of pVHL to suppress tumor formation *in vivo*.

A frequently mutated region of pVHL (residues 157–172) binds to elongin B and C and prevents their association with elongin A, thus inhibiting the activity of the elongin/SIII transcripitional elongation complex (35–37). How might elongin binding play a role in the regulation of hypoxia-inducible genes described here? One possibility is that elongin/SIII regulates the transcription of a gene encoding a protein which regulates the stability of hypoxia-inducible mRNAs. A second possibility is that the presence or absence of elongin/SIII during the elongation of hypoxia-inducible mRNAs influences their subsequent stability. Alternatively, it is possible that the elongins directly influence mRNA stability independent of their role in elongation. Such a view might account for the observation that pVHL, elongin C, and pVHL/elongin complexes are detected primarily in the cytoplasm (ref. 16 and O.I., C. Corless, A. Kibel, and W.G.K., unpublished data). Finally, it is possible that the regulation of hypoxia-inducible mRNA stability by pVHL represents a previously unrecognized function unrelated to its ability to bind to

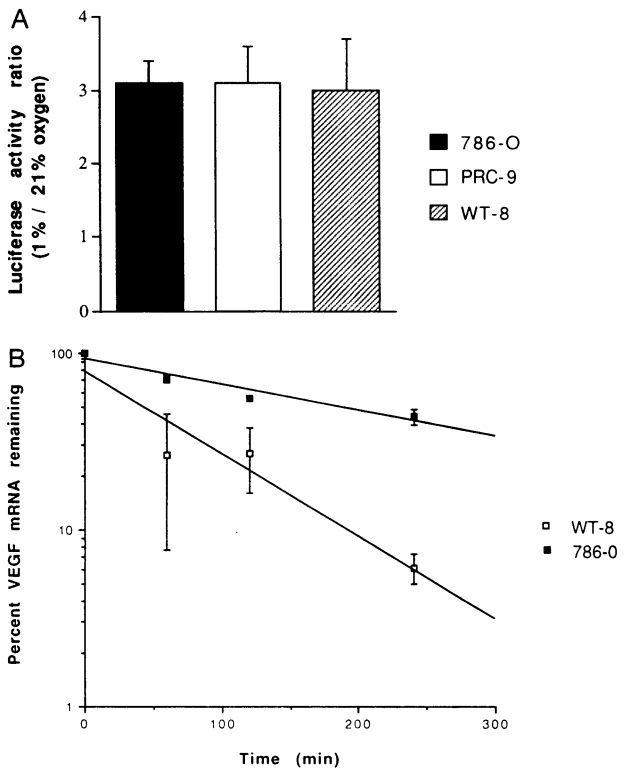

FIG. 4. Evidence for posttranscriptional control of VEGF mRNA abundance by pVHL. (*A*) Parental 786-O renal carcinoma cells or 786-O subclones stably transfected with a mammalian expression plasmid encoding pVHL(wt) (WT-8) (16) or with the backbone expression plasmid pRc-CMV (pRC-9) (16) were electroporated with a reporter plasmid containing 1.7 kb of rat VEGF 5′ flanking sequence subcloned upstream of the luciferase gene. Cells from a single cuvette were then split into two portions and grown on plastic dishes in 1% or 21% oxygen for 18 hr. Data shown are luciferase values for cells grown in the presence of 1% oxygen relative to cells grown in 21% oxygen. (*B*) Parental 786-O cells or 786-O cells stably producing wild-type pVHL (WT-8) were grown under normoxic conditions. VEGF mRNA and 18S rRNA levels were measured by RNase protection assay in triplicate at the indicated times following the addition of actinomycin D. The abundance of the VEGF mRNA at time = 0 was set to 100% for both the 786-O cells and WT-8 cells. Error bars indicate SEM. Linear regression analysis was used to plot the decay in VEGF mRNA abundance over time.

elongin B and C. If so, this observation might account for the observation that some tumor-derived, loss of function, pVHL mutants retain the ability, at least when overproduced, to bind to elongin B and C (38).

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1. McKusick, V. A. (1992) *Mendelian Inheritance in Man* (Johns Hopkins Univ. Press, Baltimore).
2. Linehan, W. M., Lerman, M. I. & Zbar, B. (1995) *J. Am. Med. Assoc.* **273,** 564–570.
3. Kanno, H., Kondo, K., Ito, S., Yamamoto, I., Fujii, S., Torigoe, S., Sakai, N., Hosaka, M., Shuin, T. & Yao, M. (1994) *Cancer Res.* **54,** 4845–4847.
4. Whaley, J. M., Naglich, J., Gelbert, L., Hsia, Y. E., Lamiell, J. M., Green, J. S., Collins, D., Neumann, H. P. H., Laidlaw, J., Li, F. P., Klein-Szanto, A. J. P., Seizinger, B. R. & Kley, N. (1994) *Am. J. Hum. Genet.* **55,** 1092–1102.
5. Foster, K., Prowse, A., van den Berg, A., Fleming, S., Hulsbeek, M. M. F., Crossey, P. A., Richards, F. M., Cairns, P., Affara, N. A., Ferguson-Smith, M. A., Buys, C. H. C. M. & Maher, E. R. (1994) *Hum. Mol. Genet.* **3,** 2169–2173.
6. Gnarra, J. R., Tory, K., Weng, Y., Schmidt, L., Wei, M. H., Li, H., Latif, F., Liu, S., Chen, F., Duh, F.-M., Lubensky, I., Duan, D. R., Florence, C., Pozzatti, R., Walther, M. M., Bander, N. H., Grossman, H. B., Brauch, H., Pomer, S., Brooks, J. D., Isaacs, W. B., Lerman, M. I., Zbar, B. & Linehan, W. M. (1994) *Nat. Genet.* **7,** 85–90.
7. Herman, J. G., Latif, F., Weng, Y., Lerman, M. I., Zbar, B., Liu, S., Samid, D., Duan, D.-S. R., Gnarra, J. R., Linehan, W. M. & Baylin, S. B. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9700–9704.
8. Wizigmann-Voos, S., Breier, G., Risau, W. & Plate, K. (1995) *Cancer Res.* **55,** 1358–1364.
9. Takahashi, A., Sasaki, H., Kim, S., Tobisu, K., Kakizoe, T., Tsukamoto, T., Kumamoto, Y., Sugimura, T. & Terada, M. (1994) *Cancer Res.* **54,** 4233–4237.
10. Sato, K., Terada, K., Sugiyama, T., Takahashi, S., Saito, M., Moriyama, M., Kakinuma, H., Suzuki, Y., Kato, M. & Kato, T. (1994) *Tohoku J. Exp. Med.* **173,** 355–360.
11. Golde, D. W. & Hocking, W. G. (1981) *Ann. Intern. Med.* **95,** 71–87.
12. Goldberg, M. A. & Schneider, T. J. (1994) *J. Biol. Chem.* **269,** 4355–4359.
13. Shweiki, D., Itin, A., Soffer, D. & Keshet, E. (1992) *Nature (London)* **359,** 843–845.
14. Goldberg, M. A., Glass, G. A., Cunningham, J. M. & Bunn, H. F. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7972–7976.
15. Goldberg, M. A., Dunning, S. P. & Bunn, H. F. (1988) *Science* **242,** 1412–1415.
16. Iliopoulos, O., Kibel, A., Gray, S. & Kaelin, W. G. (1995) *Nat. Med.* **1,** 822–826.
17. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J.-Y., Huang, C.-M., Lee, W.-H., Marsilio, E., Paucha, E. & Livingston, D. M. (1988) *Cell* **54,** 275–283.
18. Levy, A. P., Levy, N. S., Wegner, S. & Goldberg, M. A. (1995) *J. Biol. Chem.* **270,** 13333–13340.
19. Levy, A. P., Levy, N. S., Loscalzo, J., Calderone, A., Takahashi, N., Yeo, K.-T., Koren, G., Colucci, W. S. & Goldberg, M. A. (1995) *Circ. Res.* **76,** 758–766.
20. Bashan, N., Burdett, E., Hundal, H. S. & Klip, A. (1992) *Am. J. Physiol.* **262,** C682–C690.
21. Loike, J. D., Cao, L., Brett, J., Ogawa, S., Silverstein, S. C. & Stern, D. (1992) *Am. J. Physiol.* **263,** C326–C333.
22. Stein, I., Neeman, M., Shweiki, D., Itin, A. & Keshet, E. (1995) *Mol. Cell. Biol.* **15,** 5363–5368.
23. Kourembanas, S., Hannan, R. L. & Faller, D. V. (1990) *J. Clin. Invest.* **86,** 670–674.
24. Shima, D. T., Adamis, A. P., Ferrara, N., Yeo, K.-T., Yeo, T.-K., Allende, R., Folkman, J. & D'Amore, P. A. (1995) *Mol. Med.* **1,** 182–193.
25. Ikeda, E., Achen, M. G., Breier, G. & Risau, W. (1995) *J. Biol. Chem.* **270,** 19761–19766.
26. Liu, Y., Cox, S. R., Morita, T. & Kourembanas, S. (1995) *Circ. Res.* **77,** 638–643.
27. Levy, A. P., Levy, N. S. & Goldberg, M. A. (1996) *J. Biol. Chem.* **271,** 2746–2753.
28. Shima, D. T., Deutsch, U. & D'Amore, P. A. (1995) *FEBS Lett.* **370,** 203–208.
29. White, F. C., Carroll, S. M. & Kamps, M. P. (1995) *Growth Factors* **12,** 289–301.
30. Gnarra, J. R., Zhou, S., Merrill, M. J., Wagner, J., Krumm, A., Papavassiliou, E., Oldfield, E. H., Klausner, R. D. & Linehan, W. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10589–10594.
31. Kolch, W., Martiny-Baron, G., Kieser, A. & Marme, D. (1995) *Breast Cancer Res. Treat.* **36,** 139–155.
32. Kondo, S., Asano, M. & Suzuki, H. (1993) *Biochem. Biophys. Res. Commun.* **194,** 1234–1241.
33. Kim, K., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. & Ferrara, N. (1993) *Nature (London)* **362,** 841–846.
34. Millauer, B., Shawver, L. K., Plate, K. H., Risau, W. & Ullrich, A. (1994) *Nature (London)* **367,** 576–579.

35. Kibel, A., Iliopoulos, O., DeCaprio, J. D. & Kaelin, W. G. (1995) *Science* **269,** 1444–1446.
36. Duan, D. R., Pause, A., Burgress, W., Aso, T., Chen, D. Y. T., Garrett, K. P., Conaway, R. C., Conaway, J. W., Linehan, W. M. & Klausner, R. D. (1995) *Science* **269,** 1402–1406.
37. Aso, T., Lane, W. S., Conaway, J. W. & Conaway, R. C. (1995) *Science* **269,** 1439–1443.
38. Duan, D. R., Humphrey, J. S., Chen, D. Y. T., Weng, Y., Sukegawa, J., Lee, S., Gnarra, J. R., Linehan, W. M. & Klausner, R. D. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6495–6499.