Loss of *PTEN* facilitates HIF-1-mediated gene expression

Wayne Zundel,[1] Cornelia Schindler,[1] Daphne Haas-Kogan,[2] Albert Koong,[1] Fiona Kaper,[1] Eunice Chen,[1] Alexander R. Gottschalk,[2] Heather E. Ryan,[3] Randall S. Johnson,[3] Anne B. Jefferson,[4] David Stokoe,[2] and Amato J. Giaccia[1,5]

[1]Mayer Cancer Biology Research Laboratory, Department of Radiation Oncology, Stanford University, Stanford, California 94305-5468 USA; [2]Department of Radiation Oncology, University of California, San Francisco, California 94115 USA; [3]Department of Biology, University of California, San Diego, La Jolla, California 92093-0366 USA; [4]Chiron Corporation, Emeryville, California 94608 USA

In glioblastoma-derived cell lines, *PTEN* does not significantly alter apoptotic sensitivity or cause complete inhibition of DNA synthesis. However, in these cell lines PTEN regulates hypoxia- and IGF-1-induced angiogenic gene expression by regulating Akt activation of HIF-1 activity. Restoration of wild-type *PTEN* to glioblastoma cell lines lacking functional PTEN ablates hypoxia and IGF-1 induction of HIF-1-regulated genes. In addition, Akt activation leads to HIF-1α stabilization, whereas PTEN attenuates hypoxia-mediated HIF-1α stabilization. We propose that loss of *PTEN* during malignant progression contributes to tumor expansion through the deregulation of Akt activity and HIF-1-regulated gene expression.

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The *PTEN* tumor suppressor gene was originally isolated from a homozygous deletion on human chromosome 10q23 in glioblastoma multiformes (GBMs) (Li et al. 1997; Steck et al. 1997). Germ-line mutations in *PTEN* result in autosomal dominant syndromes (Cowden disease, Bannayan–Zonana syndrome) associated with an elevated risk for cancer (Liaw et al. 1997; Marsh et al. 1997). *PTEN* encodes a dual-specificity phosphatase, and both somatic and germ-line mutations cluster within conserved regions of the phosphatase domain (Li et al. 1997; Liaw et al. 1997; Marsh et al. 1997; Steck et al. 1997). Surprisingly, physiological targets of PTEN were found to be lipid products of the PI(3)K proto-oncogene, PIP2(3,4) and PIP3(3,4,5) (Maehama and Dixon 1998). Taken together, PTEN negatively regulates downstream effectors of PI(3)K, an enzyme reported to affect multiple aspects of tumorigenesis (Fruman et al. 1998).

The proto-oncogene Akt is a Ser/Thr kinase that is a critical effector of PI(3)K and exhibits transforming capacity (Aoki et al. 1998). Akt activity is essential for transducing growth factor and integrin-mediated anti-apoptotic effects (Datta et al. 1997; Khwaja et al. 1997). Several direct phosphorylation targets of Akt have been identified: Bad, GSK-3β, and Forkhead transcription factors (Datta et al. 1997; Pap and Cooper 1998; Brunet et al. 1999; Kops et al. 1999). In some cell types, Akt has been reported to modulate G_1 progression via inactivation of GSK-3β (Diehl et al. 1998). Additionally, Akt has been reported to mediate vascular endothelial growth factor (*VEGF*) induction under hypoxia (Mazure et al. 1997). Thus, Akt has multiple roles in tumorigenesis through the deregulation of cell cycle, enhancement of apoptotic resistance, and alteration of angiogenic potential.

Glioblastoma has one of the highest incidences of *PTEN* mutation (25%–60%) and *PTEN* mutation has been strongly associated with tumor differentiation (for review, see Cantley and Neel 1999). Importantly, low-grade gliomas rarely possess *PTEN* mutations, but, loss of heterozygosity (LOH) at 10q23 is found in ~70% of advanced glioblastoma (Cantley and Neel 1999). Occurrence of *PTEN* mutations late in tumorigenesis suggests that PTEN loss of function may provide a selective advantage for tumor expansion.

Previous studies have characterized the regulation of apoptotic sensitivity in genetically matched *PTEN* wild-type and null fibroblasts (Di Cristofano et al. 1998; Stambolic et al. 1998). As predicted, *PTEN* loss allows hyperactivation of the PI(3)K/Akt survival pathway and leads to increased apoptotic resistance (Davies et al. 1998; Haas-Kogan et al. 1998; Li et al. 1998; Stambolic et al. 1998). Glioblastomas are one of the most difficult tumors to treat as they are resistant to chemotherapy (Petersdorf et al. 1994) and are refractory to killing by many apoptotic stimuli (W. Zundel, unpubl.). Thus, apoptotic potential is one clear phenotypic manifestation thought to be conferred to cells on loss of *PTEN* function.

All tumors require angiogenesis for tumor expansion (Wesseling et al. 1997). Glioblastoma, in particular, is one of the most vascularized tumors and exhibits increased expression of many proangiogenic genes such as *VEGF* and fibroblast growth factor (FGF) (Wesseling et al. 1997). Because PI(3)K and Akt previously have been implicated in the induction of *VEGF* expression by hypoxia (Mazure et al. 1997), we hypothesized that wild-type *PTEN* expression in *PTEN* mutant glioblastoma cell lines could alter the cellular response to hypoxia and subsequent *VEGF* expression.

Results

PTEN *expression attenuates hypoxia-mediated activation of Akt*

Hypoxia and growth factors (e.g., IGF-1, insulin, PDGF)

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[5]Corresponding author.
E-MAIL giaccia@leland.stanford.edu; FAX (650) 723-7382.

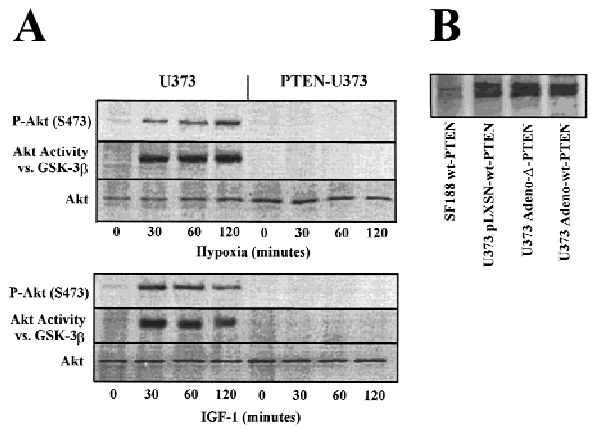

Figure 1. Hypoxia induces Akt activity that is regulated by PTEN. (*A*) U373 cells were retrovirally infected with wild-type *PTEN* and subjected to hypoxia or IGF-1 (50 ng/ml) for the indicated times. Cells were harvested and the lysates allocated for immunoblotting using α-Phospho-Akt (S473) (*top*) α-Akt antibodies or Akt kinase activity using GST–GSK-3β as a substrate. (*B*) U373 cells were either retrovirally (wt–*PTEN*) or adenovirally (wt or phosphatase inactive-Δ *PTEN*) infected. The U373-infected cells or a glioblastoma cell line possessing two wt–*PTEN* alleles (SF188) as control were immunoprecipitated and immunoblotted with Santa Cruz SC-571 and UBI-ID 07016, respectively.

are critical modulators of tumor angiogenesis (Warren et al. 1996; Mazure et al. 1997; Zelzer et al. 1998; Wang et al. 1999). If these stimuli activate proangiogenic gene expression through a PI(3)K/Akt-dependent pathway (Fruman et al. 1998), *PTEN* should block gene expression by inhibiting Akt activation in response to hypoxia. To test this hypothesis, the *PTEN* mutant glioblastoma cell line U373 was used to assess the effects of wild-type *PTEN* expression on hypoxia- and IGF-1-stimulated Akt phosphorylation and kinase activity (Fig. 1A). Hypoxia and IGF-1 stimulated Akt phosphorylation on Ser-473 and Akt kinase activity toward a GST–GSK-3β substrate within 30 min, and that activity remained sustained for >2 hr. Expression of wild-type *PTEN* in the same cells completely blocked hypoxia- and IGF-1-induced Akt phosphorylation and kinase activity, consistent with the inhibition of Akt by wild-type PTEN in serum-stimulated glioblastomas (Haas-Kogan et al. 1998).

We next assayed PTEN levels 72 hr postinfection (Fig. 1B). Viral-mediated gene transfer into U373 yielded ~11%–22%-increase in PTEN levels. Retroviral infection yielded ~85%–94% infectivity whereas the adenoviral-mediated GFP–*PTEN* infection efficiency was 52%–90%.

PTEN *overexpression does not alter serum deprivation or hypoxia-mediated apoptosis and only partially alters DNA synthesis*

To evaluate the role of *PTEN* expression on DNA synthesis we analyzed [^{3}H]-thymidine incorporation in serum-deprived or mitogen-stimulated U373 cells (Fig. 2A). Wild-type *PTEN*, but not phosphatase-inactive *PTEN*, reduced DNA synthesis by ~60%. Although significant, DNA synthesis was not completely attenuated and the *PTEN*-infected cells did survive and replicate. Although these cells were under selection for *PTEN* expression, it is possible that the DNA synthesis observed is mediated by 10%–40% of uninfected cells. Hypoxia completely inhibited DNA synthesis in U373 and *PTEN*-expressing U373, whether in the presence or absence of serum, indicating that hypoxia-mediated cell cycle arrest is dominant even in cells possessing deregulated and active Akt.

Because the role of PI(3)K and Akt in anti-apoptotic functions is based on developmental models and transformed or immortalized cell lines, we evaluated the sensitivity of U373 to the proapoptotic stimuli of serum deprivation and hypoxia (Fig. 2B). We found that U373 and other glioblastoma cell lines are extremely resistant to many apoptotic stimuli (data not shown). However, expression of *PTEN* even at highly elevated levels (Fig. 1B) fails to alter apoptotic sensitivity of U373 cells to serum deprivation or hypoxia at 72 hr, despite having no detectable Akt activity (Fig. 1A). Other apoptotic stimuli,

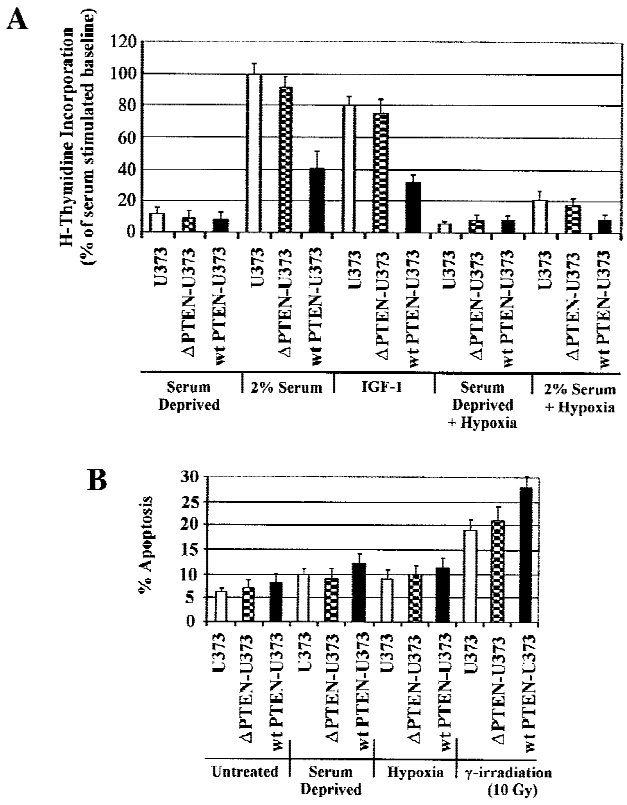

Figure 2. *PTEN* overexpression incompletely inhibits DNA synthesis and has minimal effects on apoptosis. U373 cells were infected with adenovirus containing wild-type or phosphatase inactive-Δ *PTEN*. (*A*) Forty-eight hours postinfection, serum-starved U373-infected cells were labeled with [^{3}H]-thymidine, treated as indicated for 24 hr, and assayed for [^{3}H]-thymidine incorporation. (*B*) Forty-eight hours postinfection, U373-infected cells were treated as indicated and assayed for apoptosis.

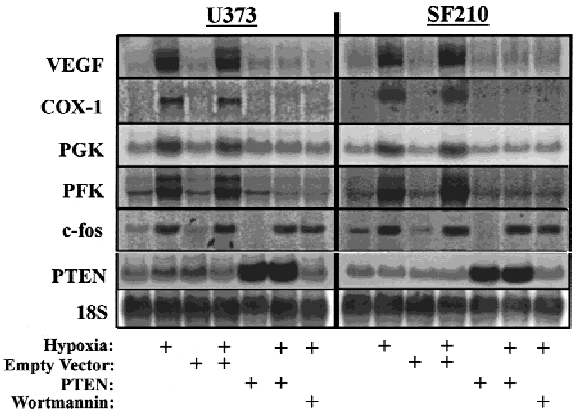

Figure 3. PTEN regulates the expression of HIF-1-regulated genes under hypoxia. U373 or SF210 cells were retrovirally infected with wild-type *PTEN* or empty vector control. Thirty-six hours postinfection, U373 and SF210 cells expressing *PTEN* or empty vector control and parental cells ± 100 nM wortmannin were subjected to 9 hr of hypoxia. mRNA was isolated and analyzed by Northern blot using *VEGF*, *COX-1*, *PGK-1*, *PFK*, c-*fos*, and *PTEN* probes.

such as γ-irradiation, only minimally sensitized glioblastoma cells to apoptosis by *PTEN* expression. These results suggest that glioblastomas have acquired anti-apoptotic mutations other than loss of *PTEN* that are sufficient to protect the cell from some physiological insults. Figure 2A and B, indicates that loss of *PTEN* function facilitates tumor expansion in a manner independent from its anti-apoptotic and -proliferative activities.

PTEN *restoration regulates expression of endogenous hypoxia-inducible genes*

To investigate whether *PTEN* regulates endogenous expression of hypoxia-induced genes implicated in angiogenesis, we analyzed *VEGF* and *COX-1* mRNA expression in hypoxia-treated U373 and SF210 (Fig. 3). The expression levels of other hypoxia-inducible genes required for glycolysis (*PGK-1*, *PFK*) were also analyzed. Wild-type *PTEN* expression and the PI(3)K inhibitor wortmannin both blocked endogenous *VEGF*, *COX-1*, *PGK-1*, and *PFK* induction in response to hypoxia when compared with empty vector controls. This effect of PTEN is not due to global down-regulation of transcription as seen by the ribosomal 18S panel. Furthermore, the negative regulation of PTEN on hypoxia-inducible genes is specific to the PI(3)K/PTEN/Akt/HIF pathway because hypoxia-induced c-*fos* mRNA levels (which are regulated by the MAPK pathway) are insensitive to PTEN expression. These results clearly establish a role for *PTEN* in the regulation of not just angiogenic factor expression but hypoxia-inducible genes in general.

PI(3)K, Akt, and HIF-1α are required for VEGF *expression that is negatively regulated by wild-type* PTEN

Figure 3 illustrates that wild-type *PTEN* inhibits various hypoxia-inducible genes shown previously to be dependent on hypoxia-inducible factor 1 (HIF-1) (Semenza 1998). HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β/ARNT subunit and a hypoxia-stabilized HIF-1α subunit (Semenza 1998). Normally, HIF-1α is expressed constitutively but rapidly degraded under oxic conditions by ubiquitin-mediated degradation (Semenza 1998). To evaluate the role of PI(3)K and Akt in HIF-1α-dependent transactivation, we used a reporter construct containing five tandem repeats of the hypoxia-responsive element (*HRE*) from the *erythropoietin* gene, which has been shown previously to be HIF-1 responsive (Semenza et al. 1991; Mazure et al. 1997). Similar patterns of VEGF and HRE reporter activity were induced by hypoxic exposure and inhibited by wild-type PTEN expression (Fig. 4A). We also found that constitutively active PI(3)K (p110*) and Akt (myr–Akt) were sufficient to transactivate both *VEGF* and *HRE* reporter constructs to levels comparable to IGF-1 stimulation (Fig. 4A). However, whereas the p110* reporter stimulation could be attenuated by PTEN, myr–Akt could not. It has been reported previously that myr–Akt is unrespon-

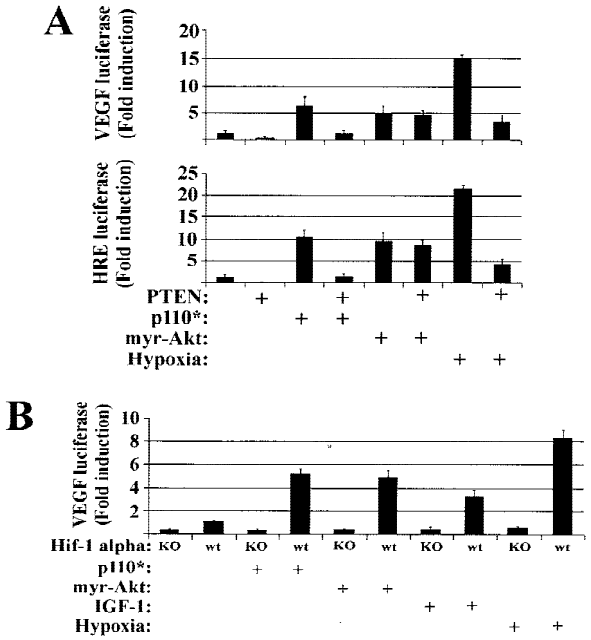

Figure 4. PTEN regulates PI(3)K-induced *VEGF* expression upstream of Akt in a HIF-1α dependent fashion. (*A*) U373 cells were cotransfected with either a VEGF–luciferase (*top*) or a HRE–luciferase (*bottom*) reporter in combination with either a constitutively active PI(3)K (*p110**) or a constitutively active Akt (*myr–Akt*). Portions of the parental U373 and the *p110** and *myr–Akt*-transfected cells were then retrovirally infected with *PTEN*. Thirty-six hours postinfection, the cells were treated under oxic or hypoxic conditions for 12 hr, followed by lysis and luciferase activity quantitation. (*B*) *HIF-1α* homozygous null and HIF-1α wild-type MEFs were cotransfected with VEGF–luciferase in combination with either a constitutively active PI(3)K (*p110**) or a constitutively active Akt (*myr–Akt*). Portions of these cells were then retrovirally infected with *PTEN*. Thirty-six hours postinfection, the cells were treated with IGF-1 or exposed to hypoxic conditions for 12 hr followed by lysis and luciferase activity quantitation.

sive to *PTEN* expression (Li et al. 1998), supporting the concept that PIP(3,4)/PIP(3,4,5) binding serves primarily as a localization signal (Kohn et al. 1996).

To genetically link hypoxia and IGF-1 activation of PI(3)K/Akt to HIF-mediated transactivation, we studied the ability of the PI(3)K/Akt pathway to modulate *VEGF* reporter activity in mouse embryo fibroblasts (MEFs) derived from homozygous null *HIF-1α* mice (Ryan et al. 1998). Hypoxia, IGF-1, and constitutively active forms of PI(3)K and Akt were all dependent on HIF-1α for *VEGF* reporter transactivation (Fig. 4B). Thus, hypoxia and IGF-1 stimulate PI(3)K and Akt, which leads to HIF-1α-dependent *VEGF* transactivation.

PTEN and Akt mediate degradation/stabilization of HIF-1α

Under low oxygen conditions, HIF-1α is stabilized by an undetermined mechanism that is necessary for translocation, heterodimerization, and transactivation (Semenza 1998). To determine whether *PTEN* expression altered the stability of HIF-1α PTEN-infected cells were exposed to hypoxic challenge for various periods of time (Fig. 5A). PTEN completely suppressed the stabilization of HIF-1α protein by hypoxia, indicating a possible role for PI(3)K stabilization of HIF-1α (Fig. 5A). To explore the role of Akt in HIF-1α stabilization, we infected cells with an inducible constitutively active form of Akt (myr–Akt–ER) or an inactive control (myr–A2–ER) under oxic conditions (Fig. 5B). Activation of myr–Akt–ER by 4-hydroxytamoxifen (4-HT) resulted in stabilization of HIF-1α, whereas activated myr–A2–ER had no effect. These results strongly indicate that hypoxia mediates HIF-1α stabilization through an Akt-dependent pathway in glioblastoma cells.

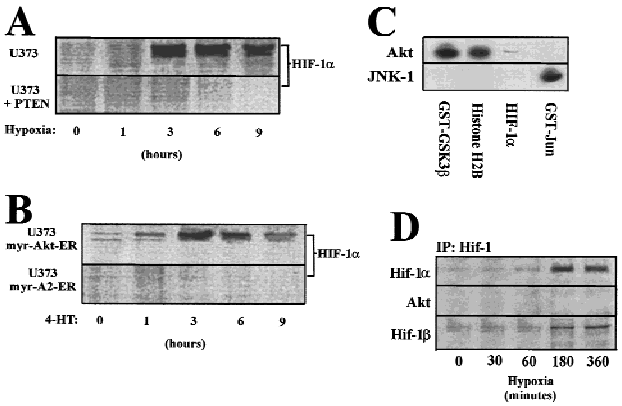

Figure 5. Akt stimulates HIF-1α stabilization. (*A*) U373 cells were retrovirally infected with wild-type PTEN. Thirty-six hours postinfection, U373 parental cells and *PTEN*-expressing U373 were subjected to hypoxia for the indicated time. Cells were lysed at the indicated times followed by SDS-PAGE, transfer, and immunoblotting with anti-HIF-1α. (*B*) Cells were retrovirally infected with *myr–Akt–ER* or *myr–A2–ER*. Thirty-six hours postinfection, the cells were subjected to induction by 4-HT for the indicated time and analyzed as in *A*. (*C*) U373 cells were serum-deprived and subjected to 1 hr of hypoxia to activate Akt or to UV-C (10 J/m^2) for JNK-1 activation for use in immune complex kinase assays. Reactions were performed using 500 ng of either histone H2B, GST–jun, GST–GSK-3β, or HIF-1α as substrates and Akt or JNK-1 immunoprecipitations for in vitro kinase assays. Kinase reactions were subjected to SDS-PAGE, and gels were dried and visualized by PhosphorImaging. (*D*) Cells were subjected to hypoxia for the indicated times, lysed, immunoprecipitated using anti-HIF-1α, and subjected to SDS-PAGE, transfer, and immunoblot using anti-HIF-1α anti-Akt, or anti-HIF-1β antibodies.

Akt phosphorylates proteins containing a RXRXXS/T consensus (Datta et al. 1997; Pap and Cooper 1998; Brunet et al. 1999; Kops et al. 1999). HIF-1α does not contain this motif or any significant amino acid sequence having a similar pattern. To confirm that Akt-induced HIF-1α stabilization and transactivation is not a result of direct phosphorylation of HIF-1α, we used HIF-1α as a substrate in in vitro kinase assays (Fig. 5C). HIF-1α was a poor substrate for Akt compared with known Akt substrates GSK-3β and histone H2B. JNK-1 kinase activity is included as a negative control to confirm the specificity of Akt kinase activity. These results suggest that Akt modulates an undetermined downstream effector that regulates HIF-1α stabilization.

Often signaling complexes are formed that allow for the identification of pathway components. We immunoprecipitated HIF-1α from hypoxia-stimulated cells at various time points to detect any possible Akt and HIF-1α complex formation (Fig. 5D). No detectable Akt/HIF-1α interaction was detectable, whereas HIF-1α and HIF-1β complexes formed as expected. Although it is possible that Akt and HIF-1α interactions are too transitory or labile to be detected, the lack of an Akt phosphorylation site, coupled with no significant in vitro phosphorylation, strongly suggests that Akt is modulating other protein(s) that increase HIF-1α stabilization.

Discussion

In this study we observed that *PTEN* inactivation leads to hypoxia-inducible gene expression in glioblastoma lines irrespective of PTEN's effects on apoptosis and cell cycle control. We have shown that PTEN can regulate hypoxia- and growth-factor stimulated transcription of VEGF and HRE promoters. Interestingly, stimulation of Akt by growth factors, p110*, or expression of myr–Akt generated approximately one-fifth to one-third of the reporter response compared with hypoxia. This suggests that hypoxia potentiates the transactivation of HIF-regulated genes by other mechanisms in addition to Akt. We have shown additionally that HIF-1α is not a direct substrate for Akt but that HIF-1α stabilization is signaled through Akt. Cumulatively, these findings suggest a new role for *PTEN* mutations in the regulation of hypoxia-inducible gene expression and give insight into why these mutations are observed predominantly in the late stages of tumor development.

Although multiple studies have implicated a role of PTEN in apoptosis through Akt, apoptosis and angiogenesis need not be mutually exclusive events. As tumor growth exceeds vascular density, the tumor develops

nonvascularized areas in which metabolic byproducts, acidosis, low growth factor and nutrients, as well as hypoxia, stimulate apoptosis (Yuan and Glazer 1998). Thus, apoptosis driven by the tumor microenvironment could potentially select for loss of negative regulators of apoptosis, such as *PTEN*, as has been shown for other tumor suppressors that regulate apoptosis, such as *p53* (Graeber et al. 1996). Therefore, loss of *PTEN* would both increase cell survival in an adverse tumor microenvironment and increase responsiveness to hypoxia-induced HIF-1 activity that would stimulate angiogenic gene expression as well as other essential genes required to survive under low oxygen conditions. This study suggests that glioblastoma cell lines are very apoptotically resistant in a *PTEN*-independent fashion, further inferring alterations in apoptotic genes irrespective of *PTEN* loss and provides alternative explanations for the importance of *PTEN* mutations in tumor expansion.

PTEN is not the only tumor suppressor gene implicated in HIF-1α regulation. The tumor suppressor protein Von-Hippel–Lindau (VHL) also regulates HIF-1α expression by modulating its protein stability, presumably via its E3 ubiquitin ligase activity (Iwai et al. 1999; Maxwell et al. 1999). Tumor cells that have mutant forms of *VHL* exhibit increased expression of many HIF-regulated genes under aerobic conditions. In contrast, *PTEN* mutations have minimal effect on oxic expression of HIF-regulated genes but potentiate their induction following hypoxia or growth factor stimulation.

The frequency of *PTEN* mutation underscores how the most frequently mutated genes in cancer often control multiple facets of tumorigenesis. For instance, *p53* is implicated in apoptosis, cell cycle control, and genomic instability (Giaccia and Kastan 1998), whereas *ras* is implicated in cell cycle, apoptosis, and angiogenesis (Campbell et al. 1998). In contrast, the ultimate effectors of apoptosis, the caspases, are infrequently mutated in cancers (Mandruzzato et al. 1997). *PTEN* mutations result in a well-documented deregulation of critical lipid second messengers that control pivotal steps in pathways that suppress apoptosis, increase proliferation, and stimulate angiogenesis. Thus, the finding that wild-type PTEN controls multiple avenues of tumor function makes it a likely target for therapeutic intervention in tumors such as glioblastoma or prostate cancer in which gene therapy approaches are feasible.

Materials and methods

Cell culture and reagents

U373, SF188, and SF210 glioblastoma cell lines were maintained in DMEM containing 10% (vol/vol) FBS (GIBCO BRL). All experiments were performed at 80%–100% confluence. The *HIF-1α* nullizygous and parental cell lines (Ryan et al. 1998) were maintained in DMEM containing 15% (vol/vol) FBS. IGF-1 (GIBCO BRL) and wortmannin (Biomol) were prepared as 1000× stock solutions. The antibodies used were Akt and Akt/Ser-473 (New England Biolabs no. 9272 and 9271, respectively), JNK-1 (Santa Cruz SC-571), PTEN (Santa Cruz SC-571, UBI-ID 07016), and HIF-1α and HIF-1β (Transduction Labs. H72320 and A78420, respectively).

Apoptosis

Apoptosis was quantified as described previously (Graeber et al. 1996). Briefly, following treatment, cells were incubated with 2 μg/ml each of bis-benzamide (Hoechst no. 33342, Sigma) and propidium iodide (Sigma) for 15 min. Viability ratios (number of apoptotic cells/total number of cells) were determined by scoring low-magnification fields of randomly selected fields for cells with condensed and fragmented nuclei and loss of membrane integrity. Fields of cells expressing GFP–*PTEN* under low magnification were referenced to Hoechst/propidium iodide on the same field by switching fluorescent filters.

[^{3}H] Thymidine incorporation

Cells were seeded in 35-mm plates and infected with adenoviral wild-type *PTEN* or phosphatase-deficient *PTEN*. Forty-eight hours after infection, the cells were serum deprived for 48hr in DMEM plus 300 μg/ml G418 (GIBCO BRL). The cells were treated as indicated and cultured in 2 ml of fresh medium containing 1 μCi/ml [methyl-^{3}H] thymidine 5′-triphosphate (NEN Life Science Products), 300 μg/ml G418 for another 18 hr. Serum and hypoxia were administered simultaneously to the cultures. To eliminate the possibility that variations in cell density between plates would cause variations in ^{3}H incorporation, two additional dishes were plated and used to count cell number for each transfection at the time when [^{3}H] thymidine was added. For harvesting cells, the growth medium containing [^{3}H] thymidine was removed, and the cells were washed twice with PBS. The cells were rinsed twice with 2 ml of ice-cold 5% TCA and lysed by incubation in 1.5 ml of 0.25 M NaOH for 15 min at room temperature. A 0.6 ml-aliquot of each lysate was used for counting [^{3}H] thymidine incorporation. For all experiments, triplicate plates were used and mean values were graphed.

Immunoblots and kinase assays

Akt and phospho-Akt were immunoprecipitated, resolved by 12.5% SDS-PAGE, transferred to PVDF, blotted using PhosphoPlus Akt (Ser-473) Antibody Kit (New England Biolabs), visualized using a Vistra Western ECF Blotting Kit (Amersham L.S.), and quantitated by Fluorimager (Molecular Dynamics). In vitro kinase reactions were performed for 40 min at 30°C with constant shaking in 30-μl reaction volumes containing 500 ng of histone H2B, GST–jun, GST–GSK-3β, or immunoprecipitated HIF-1α as substrates, 10 μCi of [^{32}P] ATP, and immunoprecipitations of active Akt or JNK-1 as described (Datta et al. 1997). Kinase reactions were stopped by the addition of SDS-PAGE loading buffer and boiling for 5 min. The kinase reaction products were resolved by 12.5% SDS-PAGE, and the gels were washed for 5 min in dH_2O, dried, and visualized by PhosphorImaging (Molecular Dynamics).

Constructs, expression, and reporter assays

Construction and preparation of the VEGF and HRE luciferase reporter constructs, *p110**, *PLXN–PTEN*, *myr–Akt*, *myr–Akt–ER*, and *myr–A2–ER* were described previously (Hu et al. 1995; Mazure et al. 1997; Haas-Kogan et al. 1998; Kohn et al. 1998). Retroviral infection was performed by three sequential 6-hr incubations containing 5 μg/ml Polybrene, followed by recovery for 2–6 days. Adenoviruses expressing the *PTEN* or *PTENΔ* genes and capable of replicating in the "packaging" 293 cell line were made using the pAdEasy protocol. The virus was stored in single-use aliquots at –80°C. U373 cells were routinely infected at an MOI of 10 and cells were harvested 48 hr post infection. Transfections were performed using Lipofectamine Plus (GIBCO BRL). Luciferase assays were performed using the TB161 Luciferase Reporter Kit (Promega) and quantitated using a Monolight 2010 luminometer (Analytical Luminescence Laboratory).

RNA isolation and Northern blotting

Total RNA was isolated with TRIzol (GIBCO BRL). Total RNA/lane (10 μg) was denatured with glyoxal and size fractionated by electrophoresis on 1.4% agarose/sodium phosphate gels. RNA was transferred to nylon membranes and UV cross-linked. *PGK*, *PFK*, and *COX-1* were purchased as GEM clones (Genome Systems). Probes were cut out of the inserts with *Eco*RI and *Not*I from pINCY. The *VEGF* probe comprises a 600-bp fragment of the 5′UTR of *VEGF*. Radiolabeled probes were generated by random priming (Rediprime, Amersham) of cDNAs representing the complete coding sequences of *PGK*, *PFK*, c-*fos*, and *COX-1*. Blots were prehybridized and hybridized in ExpressHyb solution (Clontech) at 65°C, washed several times in 2× SSC/0.05% SDS and 0.2× SSC/0.1% SDS at 65°C, exposed to a PhosphorImager plate overnight, and visualized on a Storm 860 PhosphorImager (Molecular Dynamics).

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