

# Modulation of Hypoxia-inducible Factor 1 $\alpha$ Expression by the Epidermal Growth Factor/Phosphatidylinositol 3-Kinase/PTEN/AKT/FRAP Pathway in Human Prostate Cancer Cells: Implications for Tumor Angiogenesis and Therapeutics<sup>1</sup>

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## Abstract

Dysregulated signal transduction from receptor tyrosine kinases to phosphatidylinositol 3-kinase (PI3K), AKT (protein kinase B), and its effector FKBP- $\gamma$ -rapamycin-associated protein (FRAP) occurs via autocrine stimulation or inactivation of the tumor suppressor PTEN in many cancers. Here we demonstrate that in human prostate cancer cells, basal-, growth factor-, and mitogen-induced expression of hypoxia-inducible factor 1 (HIF-1)  $\alpha$ , the regulated subunit of the transcription factor HIF-1, is blocked by LY294002 and rapamycin, inhibitors of PI3K and FRAP, respectively. HIF-1-dependent gene transcription is blocked by dominant-negative AKT or PI3K and by wild-type PTEN, whereas transcription is stimulated by constitutively active AKT or dominant-negative PTEN. LY294002 and rapamycin also inhibit growth factor- and mitogen-induced secretion of vascular endothelial growth factor, the product of a known HIF-1 target gene, thus linking the PI3K/PTEN/AKT/FRAP pathway, HIF-1, and tumor angiogenesis. These data indicate that pharmacological agents that target PI3K, AKT, or FRAP in tumor cells inhibit HIF-1 $\alpha$  expression and that such inhibition may contribute to therapeutic efficacy.

## Introduction

Tumor progression involves the selection of cells with somatic mutations that activate oncogenes and inactivate tumor suppressor genes. These mutations have the effect of driving cells through the cell cycle in an uncontrolled manner and preventing apoptosis. Two adaptations that are universal characteristics of solid tumors, indicating that they are necessary for tumor progression, are increased glycolytic metabolism and angiogenesis (reviewed in Ref. 1). These adaptations are also driven by genetic alterations in tumor cells, but their molecular basis has remained obscure. Loss of function mutations in tumor suppressor genes or activating mutations in oncogenes have been shown to dysregulate signal transduction pathways leading from growth factors (such as EGF<sup>3</sup>) and their cognate receptor tyrosine kinases to PI3K, which catalyzes the conversion of phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-

triphosphate, respectively (reviewed in Ref. 2). These products are allosteric activators of phosphatidylinositol-dependent kinase 1, which phosphorylates and activates AKT (protein kinase B). Targets of AKT include BAD, an inhibitor of apoptosis, and FRAP, an activator of p70<sup>S6k</sup>, which is required for ribosomal biogenesis and cell cycle progression (reviewed in Ref. 2). These findings have delineated mechanisms by which the PI3K/AKT pathway promotes cell proliferation and inhibits cell death. This pathway is negatively regulated by PTEN, which dephosphorylates phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-triphosphate (reviewed in Ref. 2). In PCA, PTEN loss of function correlates with increased angiogenesis and appears to be critical for progression to hormone-refractory metastatic disease (3–6). However, the basis for these correlations has not been determined. The role of HIF-1 as an essential transcriptional activator of genes encoding glucose transporters, glycolytic enzymes, and VEGF is well established (reviewed in Ref. 7). In this study, we demonstrate that modulation of the EGF/PI3K/AKT/FRAP pathway alters the expression of HIF-1 $\alpha$  protein, HIF-1-dependent transcriptional activity, and VEGF protein in human PCA cells. These results provide a mechanism contributing to the overexpression of HIF-1 $\alpha$  in PCA and other solid cancers (8) and have important implications regarding cancer progression and therapy.

## Materials and Methods

**Tissue Culture.** The human PCA cell lines DU145, PC-3, PPC-1, and TSU were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS (complete media). Cells were exposed to hypoxia as described previously (9–11).

**Immunoblot Assays.** Cells ( $0.5\text{--}1.0 \times 10^6$ ) were seeded onto 150-mm tissue culture dishes (Falcon) and incubated for 36–48 h in complete media (except for AKT assays, in which cells were plated directly in media with 0.1% FBS). The cells were incubated in media with 0–0.1% FBS for 24 h and then given fresh media with 0–0.1% FBS alone or with 10% FBS, EGF (Life Technologies, Inc.), PMA, or 4 $\alpha$ -PMA, either alone or with LY294002, PD098059, rapamycin, or wortmannin (Alexis Corp.), for 6–8 h. For analysis of HIF-1 $\alpha$  expression, nuclear extracts were prepared, and aliquots were analyzed using monoclonal antibody H1 $\alpha$ 67 (Novus Biologicals, Inc.) as described previously (8). Blots were stripped and incubated with anti-topoisomerase I antibodies (TopoGEN). Aliquots of whole cell lysates were subjected to immunoblot assay using anti-AKT and phospho-AKT antibodies (New England Biolabs). All immunoblots were developed using enhanced chemiluminescence reagents (Amersham).

**Transient Transfection Assays.** DU145 cells were seeded onto 24-well culture plates at a density of  $4 \times 10^4$  cells/well and incubated for 24 h in complete media. The cells were transfected with 12.5 ng of control plasmid pTK-RL (Promega) containing the herpes simplex virus thymidine kinase promoter and *Renilla reniformis* (sea pansy) luciferase coding sequences; 100 ng of reporter plasmid p2.1 containing a 68-bp hypoxia response element from the *ENO1* gene, an SV40 promoter, and *Photinus pyralis* (firefly) luciferase

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<sup>3</sup> The abbreviations used are: EGF, epidermal growth factor; PI3K, phosphatidylinositol 3-kinase; FRAP, FKBP- $\gamma$ -rapamycin-associated protein; PCA, prostate cancer; HIF-1, hypoxia-inducible factor 1; VEGF, vascular endothelial growth factor; FBS, fetal bovine serum; IGF, insulin-like growth factor; PMA, phorbol 12-myristate 13-acetate.

coding sequences (12); and 500 ng of pCEP4 (Invitrogen) or expression vector encoding AKT-MYR, AKT (K179M), wild-type PTEN, PTEN (C124S), or p85 $\Delta$  (13–16). KD-AKT, C124S PTEN, and p85 $\Delta$  have each been shown to have dominant negative effects in cells expressing the respective wild-type protein. Cells were exposed to plasmid DNA for 8 h in 1  $\mu$ l of Fugene-6 (Boehringer Mannheim). Cells were then incubated in DMEM with 0.1% FBS for 16 h, followed by exposure to 10% FBS, 100 nM PMA, and 1% O<sub>2</sub> or no treatment for 24 h. Cells were lysed in 100  $\mu$ l of buffer, and Dual-Luciferase (Promega) reporter assays were performed on 20- $\mu$ l aliquots.

**VEGF ELISA Assays.** TSU cells were seeded onto 6-well culture plates at a density of 4  $\times$  10<sup>4</sup> cells per well, incubated for 24 h in complete media, and then given serum-free media for 16 h, followed by fresh serum-free media, either alone or with 10% FBS, EGF, or PMA alone or with LY294002 or rapamycin, for 24 h. Conditioned media were removed for storage at -80°C, and cells were counted. VEGF protein concentration in the media was determined by ELISA using a commercial kit (R&D Systems).

**Results**

We demonstrated previously that human PCA lines, most notably PC-3 cells, express HIF-1 $\alpha$  protein and HIF-1 DNA-binding activity under nonhypoxic conditions, and expression is further increased in response to hypoxia (11). Potential clinical implications of these findings were underscored by the immunohistochemical demonstration that HIF-1 $\alpha$  is overexpressed (relative to adjacent normal tissue) in common human solid tumors, including PCA (8). HIF-1 $\alpha$  expression was also induced in transformed cells exposed to EGF, fibroblast growth factor 2, IGF-1, or IGF-2 (10). Because of the known role of EGF signaling via the PI3K pathway (reviewed in Ref. 2), we investigated whether up-regulation of this pathway contributed to increased HIF-1 $\alpha$  expression in PCA cells. As an initial means of modulating the activity of this pathway, we examined the effect of serum starvation and stimulation. TSU, PC-3, DU145, and PPC-1 cells were cultured at low density in serum-free medium for 24 h and then exposed to 0% or 10% FBS for 6 h. All four cell lines demonstrated some degree of HIF-1 $\alpha$  expression under serum-free conditions that increased in response to serum stimulation (Fig. 1A). To examine responses to specific mitogens, cells were exposed to 100 nM PMA or 20 ng/ml EGF. PMA strongly induced HIF-1 $\alpha$  expression in DU145, TSU, and PPC-1 cells (Fig. 1B). Exposure of TSU cells to 20 ng/ml EGF also markedly induced HIF-1 $\alpha$  expression, whereas the effect of EGF on DU145 and PPC-1 cells was more modest. In DU145 and TSU cells, similar levels of HIF-1 $\alpha$  expression were induced by exposure to PMA or hypoxia, whereas the biologically inactive 4 $\alpha$ -PMA had no effect (Fig. 1C).

To determine whether PI3K pathway activity was required for HIF-1 $\alpha$  expression, PCA cells were exposed to LY294002 or wortmannin, inhibitors of PI3K, or to rapamycin, an inhibitor of FRAP (17), a signaling molecule downstream of PI3K (Fig. 2A). PC-3 cells were cultured in 0.1% FBS in the presence of varying concentrations of LY294002 under hypoxic (1% O<sub>2</sub>) or nonhypoxic (20% O<sub>2</sub>) conditions. HIF-1 $\alpha$  expression under nonhypoxic conditions was partially inhibited by 1  $\mu$ M LY294002 and completely inhibited by 10  $\mu$ M LY294002 (Fig. 2B, top panel). In contrast, hypoxia-induced HIF-1 $\alpha$  expression was only partially inhibited by 10  $\mu$ M LY294002 and was more completely inhibited by 50  $\mu$ M LY294002. Wortmannin was a more potent inhibitor in nonhypoxic cells because partial inhibition and complete inhibition of HIF-1 $\alpha$  expression were observed in the presence of 10 and 100 nM wortmannin, respectively, in nonhypoxic cells, whereas only modest inhibition was observed with 200 nM wortmannin in hypoxic cells (Fig. 2B, middle panel). Rapamycin was the most potent drug tested; it inhibited HIF-1 $\alpha$  expression at concentrations of 10 and 50 nM in nonhypoxic and hypoxic cells, respectively (Fig. 2B, bottom panel). Induction of HIF-1 $\alpha$  expression in PC-3 or TSU cells exposed to either 10% FBS, 100 nM PMA, or 20

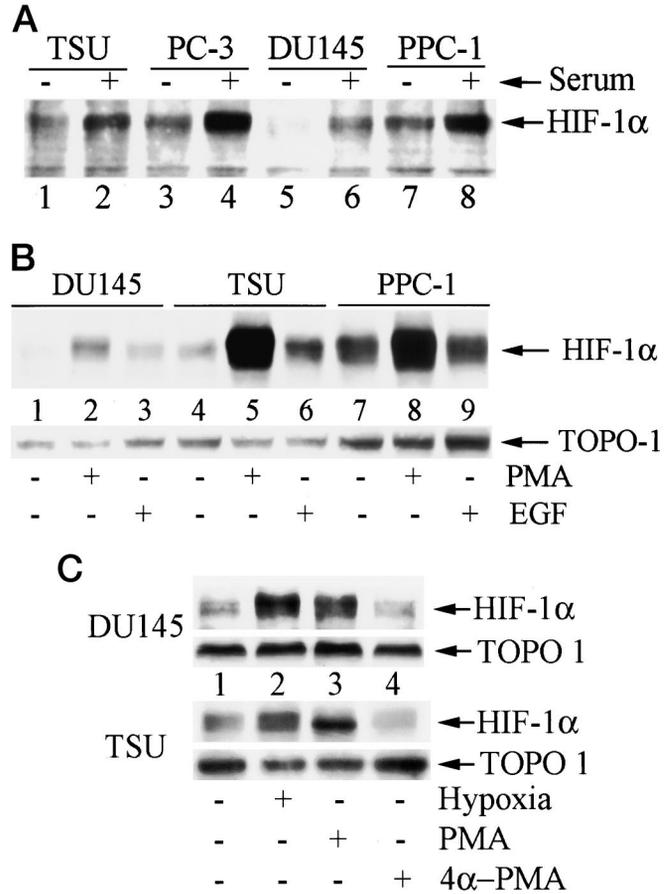


Fig. 1. Induction of HIF-1 $\alpha$  expression in PCA cells exposed to serum, PMA, EGF, or hypoxia. A, effect of serum starvation and stimulation. Cells were cultured in serum-free media for 24 h and then incubated in media containing 0% or 10% FBS for 6 h before immunoblot assay using an anti-HIF-1 $\alpha$  monoclonal antibody. B, effect of PMA and EGF stimulation. Cells cultured in media containing 0.1% FBS for 24 h were untreated (Lanes 1, 4, and 7) or exposed to 100 nM PMA (Lanes 2, 5, and 8) or 20 ng/ml EGF (Lanes 3, 6, and 9) for 8 h before immunoblot assay for HIF-1 $\alpha$  (top panel). The blot was then stripped and assayed for topoisomerase I (bottom panel) as a control for sample loading and transfer. C, effect of hypoxia and PMA. DU145 (top panels) and TSU (bottom panels) cells cultured in media containing 0.1% FBS for 20 h were untreated (Lane 1) or exposed to 1% O<sub>2</sub> (Lane 2), 100 nM PMA (Lane 3), or 100 nM 4 $\alpha$ -PMA (Lane 4) for 8 h before HIF-1 $\alpha$  and topoisomerase I immunoblot assays.

ng/ml EGF was completely inhibited by 50  $\mu$ M LY294002 (Fig. 2C). PMA-induced HIF-1 $\alpha$  expression was completely inhibited in the presence of 10  $\mu$ M LY294002 or 10 nM rapamycin (data not shown). Under the experimental conditions used, none of the inhibitors caused cell death during the study period as determined by analysis of cellular ATP concentration, morphology, or trypan blue exclusion (data not shown). Taken together, these results suggest that basal and mitogen-induced HIF-1 $\alpha$  expression in PCA cells is highly dependent on PI3K activity, whereas other signaling pathways stimulate hypoxia-induced expression.

AKT lies between PI3K and FRAP in this signaling pathway (Fig. 2A). In TSU cells cultured in serum-free media, a modest degree of AKT phosphorylation was detected, which increased in response to EGF stimulation (Fig. 3, top panel, Lanes 1 and 2). These data are consistent with previous reports of EGF-stimulated AKT activity in PCA cells (18, 19). Both basal and EGF-induced AKT phosphorylation were blocked by LY294002 (Lane 3). In contrast, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor PD098059 (Fig. 2A) had no inhibitory effect (Fig. 3, top panel, Lane 4), suggesting that mitogen-activated protein kinase activity is not required in these cells. In PC-3 cells, which show the highest level of HIF-1 $\alpha$  expression under nonhypoxic conditions (Fig.

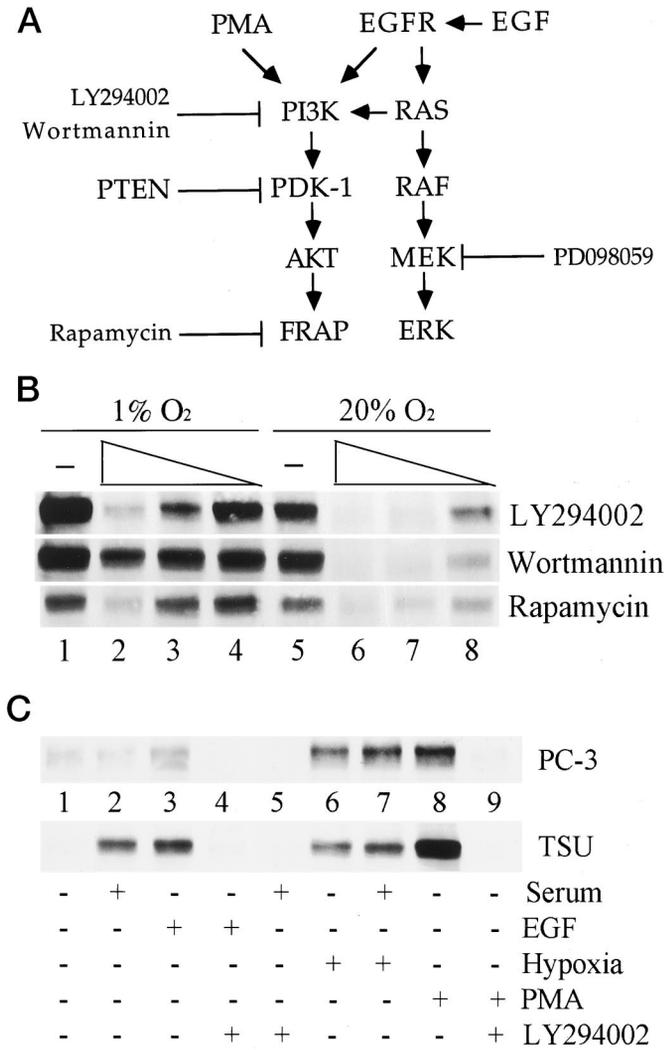


Fig. 2. Effect of pharmacologic inhibitors on HIF-1 $\alpha$  expression. **A**, relevant signal transduction pathways activated by EGF and PMA. **B**, effect of PI3K and FRAP inhibitors on basal and hypoxia-induced HIF-1 $\alpha$  expression. PC-3 cells were cultured in media containing 0.1% FBS for 20 h, followed by the addition of LY294002 (0, 50, 10, or 1  $\mu$ M), wortmannin (0, 200, 100, or 10 nM), or rapamycin (0, 50, 10, or 1 nM) and incubation in 1% (Lanes 1–4) or 20% (Lanes 5–8) O<sub>2</sub> for 8 h before HIF-1 $\alpha$  immunoblot assay. **C**, effect of PI3K inhibition on serum-, EGF-, and PMA-induced HIF-1 $\alpha$  expression. PC-3 and TSU cells were incubated in media containing 0.1% FBS for 24 h and then exposed to 10% FBS, 20 ng/ml EGF, 100 nM PMA, or 1% O<sub>2</sub> in the presence or absence of 50  $\mu$ M LY294002 for 6 h before HIF-1 $\alpha$  immunoblot assay.

1A; Ref. 11), the extent of AKT phosphorylation was even greater than that in TSU cells but was nevertheless completely blocked by treatment with LY294002 (Fig. 3, top panel, Lanes 5 and 6), whereas treatment with rapamycin (which inhibits the pathway downstream of AKT) or PD098059 had no inhibitory effect (Lanes 7 and 8). Hypoxia had no effect on AKT phosphorylation in PC-3 cells (Lanes 9–12). Total AKT protein levels were not affected by EGF, LY294002, PD098059, or hypoxia (Fig. 3, bottom panel).

To determine whether PI3K pathway activity affects HIF-1-mediated gene transcription, DU145 cells were cotransfected with the reporter gene p2.1 (12) containing a 68-bp hypoxia response element from the promoter of the human *ENO1* gene (which encodes the glycolytic enzyme enolase) and expression vectors encoding wild-type or mutant components of the PI3K pathway. Reporter gene activity increased 2.4-fold in response to PMA stimulation (Fig. 4A) in the presence of empty expression vector. In contrast, reporter gene activity was below basal levels in PMA-stimulated cells transfected

with vector encoding KD-AKT, a catalytically-inactive (kinase-dead) form of AKT containing a K179M missense mutation (14); wild-type PTEN; or p85 $\Delta$ , a dominant-negative form of the PI3K p85 regulatory subunit (16). Reporter activity was induced 17-fold by hypoxia, and this response was partially inhibited by KD-AKT, wild-type PTEN, or PI3K-p85 $\Delta$  (Fig. 4B). These results, which are consistent with the effects of the PI3K inhibitor LY294002 on HIF-1 $\alpha$  expression reported above (Fig. 2), demonstrate that PI3K and PTEN-regulated AKT activity are required for HIF-1-mediated transcription in response to PMA.

To determine whether activation of the PI3K pathway was sufficient to activate HIF-1-mediated gene transcription, DU145 cells that express wild-type PTEN (5) were cotransfected with reporter p2.1 and expression vectors encoding AKT-MYR, a myristoylated and constitutively active form of AKT (13), or a catalytically inactive form of PTEN containing a C124S missense mutation. The transfected cells were incubated in 10% FBS (Fig. 4C), 0.1% FBS (Fig. 4D), or 0.1% FBS with 100 nM PMA (Fig. 4E). Under all three conditions, both constitutively active AKT and dominant-negative PTEN induced reporter gene expression, with the greatest response observed in PMA-stimulated cells.

To demonstrate that the PI3K-mediated induction of HIF-1 transcriptional activity results in biological activity, the secretion of VEGF protein by TSU cells was analyzed by ELISA. Cells were serum-starved for 6 h and then exposed to no treatment, 10% FBS, 50 nM PMA, or 20 ng/ml EGF for 24 h. Despite the short incubation time, FBS, PMA, and EGF each increased VEGF protein levels in the tissue culture supernatant, and PMA resulted in the greatest induction (Fig. 4F), as was also observed with respect to HIF-1 $\alpha$  expression (Fig. 1). Treatment with low concentrations of either LY294002 (10  $\mu$ M) or rapamycin (10 nM) markedly inhibited the induction of VEGF expression by FBS, PMA, or EGF (Fig. 4F), similar to the effect of these inhibitors on mitogen-induced HIF-1 $\alpha$  expression (Fig. 2). Thus, both HIF-1 $\alpha$ -dependent gene transcription (Fig. 4, A–E) and the expression of a HIF-1-regulated gene product (Fig. 4F) are modulated by the activity of the PI3K/AKT/FRAP pathway in PCA cells.

**Discussion**

In this study, we demonstrate that activation of the PI3K/PTEN/AKT/FRAP pathway by EGF, PMA, serum, or autocrine stimulation results in increased expression of HIF-1 $\alpha$  protein, HIF-1 transcriptional activity, and VEGF protein expression in PCA cells. HIF-1 $\alpha$  protein expression is regulated by ubiquitination and proteasomal degradation (reviewed in Ref. 7). Additional studies are required to determine whether this process is modulated by PI3K/AKT/FRAP activity and, if so, whether such modulation involves direct phosphorylation of HIF-1 $\alpha$ .

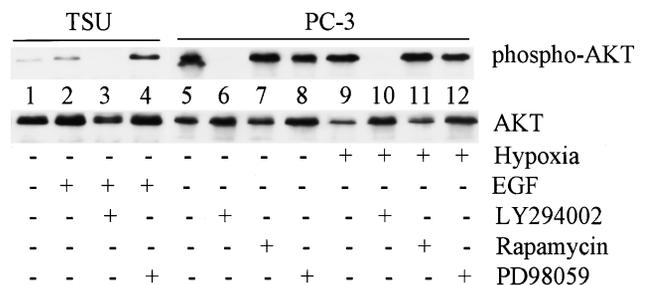


Fig. 3. Analysis of AKT phosphorylation. TSU and PC-3 cells cultured in serum-free media for 24 h were untreated or exposed to 20 ng/ml EGF, 50  $\mu$ M LY294002, 20 nM rapamycin, or 100  $\mu$ M PD098059 under nonhypoxic (20% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) conditions as indicated for 8 h before immunoblot assay using antibodies against phosphorylated (top panel) or total (bottom panel) AKT protein.

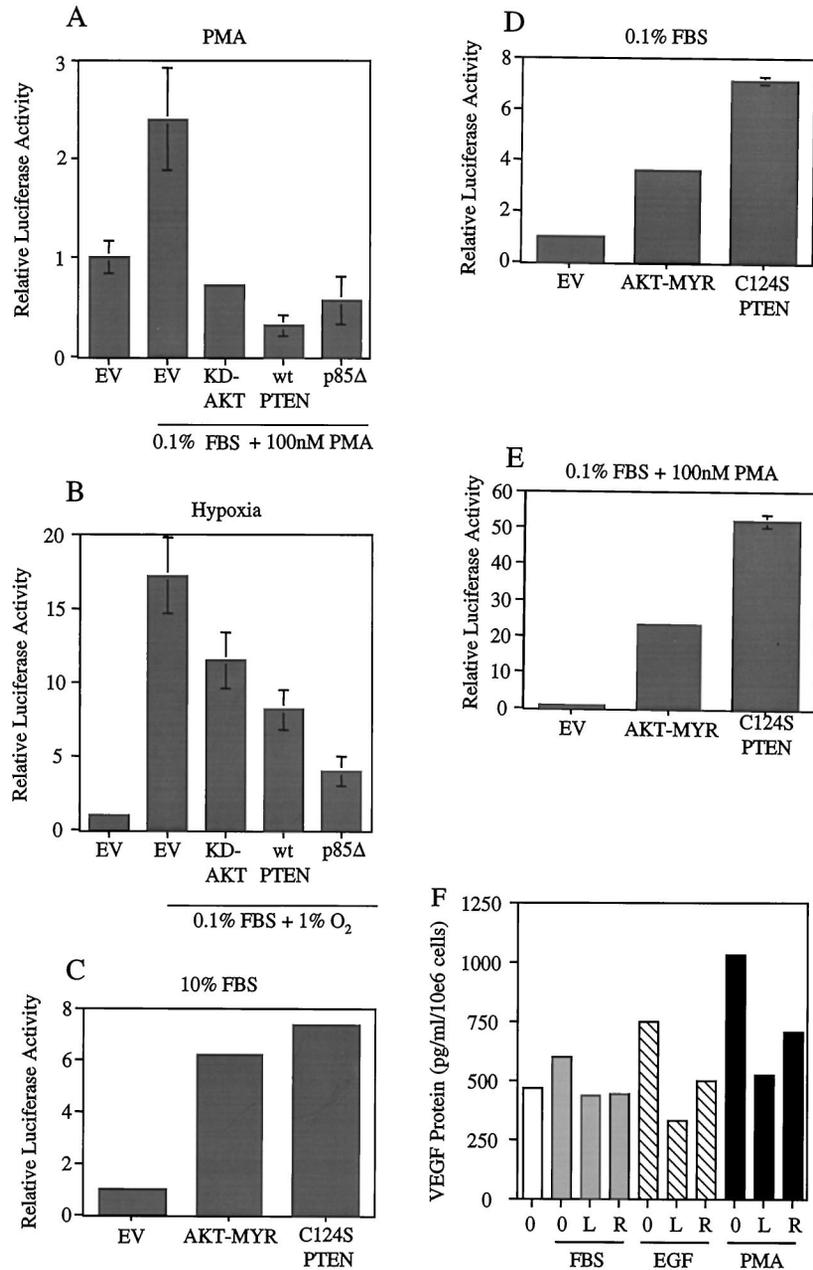


Fig. 4. Effect of altered PI3K, PTEN, or AKT activity on HIF-1-dependent gene expression. A–E, DU145 cells were cotransfected with a control plasmid containing the HSV TK promoter upstream of *Renilla* luciferase coding sequences, a reporter plasmid containing a hypoxia response element upstream of a SV40 promoter and firefly luciferase coding sequences, and expression vector containing no insert (EV) or cDNA encoding catalytically inactive (kinase-dead; KD) or constitutively active (myristoylated; MYR) AKT, wild-type (wt) or mutant (C124S) PTEN, or a deleted form of the p85 subunit of PI3K (p85 $\Delta$ ). Eight h after transfection, the cells were incubated in 10% (C) or 0.1% (D) FBS for 16 h and then harvested, or serum-deprived cells were exposed to PMA (A and E) or 1% O<sub>2</sub> (B) for an additional 24 h. For each sample, the ratio of firefly to *Renilla* luciferase was determined and normalized to the value obtained from untreated cells transfected with empty vector (EV) to generate the relative luciferase activity. Data represent the mean and SE for three independent transfections. Note that the scale of the y axis differs between graphs. F, VEGF protein secretion by TSU cells. After 16 h of serum starvation, cells were untreated (□) or exposed to 10% FBS (■), 20 ng/ml EGF (▨), or 50 nM PMA (■) for 24 h, either alone (O) or in the presence of 10  $\mu$ M LY294002 (L) or 10 nM rapamycin (R). VEGF protein concentrations in conditioned media were determined by ELISA and corrected for cell number. Each bar represents the mean of VEGF concentrations from duplicate plates of cells, which differed by  $\leq$ 20% in all cases.

These results provide a molecular basis for the previously reported expression of HIF-1 $\alpha$  under nonhypoxic conditions in PCA cells (11). It is likely that, *in vivo*, increased activity of the PI3K pathway contributes to the dramatic overexpression of HIF-1 $\alpha$  in PCA and other human cancers (8). The tumor suppressor PTEN, which negatively regulates the PI3K pathway, is a target for mutation in PCA, breast cancer, gliomas, and other tumor types (3–6, 19–21). In PCA, inactivation of PTEN expression is associated with disease progression and angiogenesis (3, 4). It is well established that HIF-1 activates genes encoding glucose transport-

ers, glycolytic enzymes, heme oxygenase-1, IGF-2, IGF-binding proteins, inducible nitric oxide synthase, transferrin, and VEGF, all of which have been implicated in tumor progression (reviewed in Ref. 7). In particular, the association between PTEN loss of function and angiogenesis may be explained by the induction of HIF-1 $\alpha$ , leading to increased VEGF expression. Colon cancer cells transfected with a HIF-1 $\alpha$  expression vector demonstrated increased VEGF mRNA expression as well as increased growth and angiogenesis of tumor xenografts (22).

In addition to PTEN, loss of function mutations in tumor suppressor

genes encoding VHL (23) and p53 (8, 22) result in increased expression of HIF-1 $\alpha$  and VEGF. Gain of function mutations in oncogenes also induce HIF-1 $\alpha$  expression, as demonstrated for *v-src* (24) and inferred for autocrine activation of EGF and IGF-I receptors, based on the results presented above and in previous studies (9, 10). Induction of transcription via the *VEGF* gene promoter by activated H-RAS also requires PI3K/AKT activity and an intact HIF-1 binding site (16). Thus, V-SRC, H-RAS, and receptor tyrosine kinases all lead to increased activity of both the PI3K/AKT pathway (2, 18, 19, 25) and HIF-1.

Several conclusions can be drawn from the available data. First, in human tumors, increased expression of HIF-1 $\alpha$  is induced by genetic alterations as well as by physiological stimulation. Second, expression of HIF-1 may play a major role in promoting angiogenesis and metabolic adaptation in PCA and other common solid tumors. In addition to the data regarding the effects of increased HIF-1 $\alpha$  expression cited above, loss of HIF-1 expression in tumor cells is associated with decreased xenograft growth and angiogenesis (24, 26). Third, whereas genetic alterations affecting signal transduction pathways are highly variable among human tumors, increased expression of HIF-1 $\alpha$  may represent a common final pathway. Fourth, if HIF-1 mediated angiogenesis and metabolic adaptation play important roles in tumor progression, as suggested by previous studies (7, 8, 22–24, 26), then pharmacological inhibition of HIF-1 activity may represent a useful treatment strategy. Furthermore, the effect of PI3K/AKT/FRAP pathway inhibitors on HIF-1 $\alpha$  expression may provide a basis for therapeutic efficacy.

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