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Akt2 Regulates All Akt Isoforms and Promotes Resistance to Hypoxia through Induction of miR-21 upon Oxygen Deprivation

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Abstract

The growth and survival of tumor cells in an unfavorable hypoxic environment depend upon their adaptability. Here, we show that both normal and tumor cells expressing the protein kinase Akt2 are more resistant to hypoxia than cells expressing Akt1 or Akt3. This is due to the differential regulation of microRNA (miR) 21, which is upregulated by hypoxia only in Akt2-expressing cells. By upregulating miR-21 upon oxygen deprivation, Akt2 downregulates PTEN and activates all three Akt isoforms. miR-21 also targets PDCD4 and Sprouty 1 (Spry1), and the combined downregulation of these proteins with PTEN is sufficient to confer resistance to hypoxia. Furthermore, the miR-21 induction by Akt2 during hypoxia depends upon the binding of NF-κB, cAMP responsive element–binding protein (CREB), and CBP/p300 to the *miR-21* promoter, in addition to the regional acetylation of histone H3K9, all of which are under the control of Akt2. Analysis of the Akt2/miR-21 pathway in hypoxic MMTV-PyMT–induced mouse mammary adenocarcinomas and human ovarian carcinomas confirmed the activity of the pathway *in vivo*. Taken together, this study identifies a novel Akt2-dependent pathway that is activated by hypoxia and promotes tumor resistance via induction of miR-21. *Cancer Res; 71(13); 4720–31. ©2011 AACR.*

Introduction

Tumor growth depends on the development of networks of new blood vessels, which bring nutrients and oxygen to the proliferating tumor cells. The structural and functional defects of the new blood vessels and the lagging nature of tumor angiogenesis are responsible for chronic oxygen deprivation in rapidly growing tumors. Therefore, tumor survival and expansion depend on the development of resistance to hypoxia. Resistance of normal and tumor cells to hypoxia has been extensively studied. Our current understanding of this process assigns a central role to the hypoxia-inducible factor 1 (HIF-1; ref. 1).

Hypoxia inhibits tumor growth. However, cellular adaptations required for survival under hypoxia give rise to tumors with progressively increasing malignant potential. The hypoxia-induced overexpression and activation of HIF-1 results in metabolic reprogramming of the tumor cells, which is characterized by increased glucose uptake, stimulation of aerobic glycolysis, shifting of pyruvate metabolism from the Krebs cycle to lactate production, and inhibition of mitochondrial oxidative phosphorylation (2). These changes favor the generation of NADPH, which is required for various biosynthetic processes (3). They are also characteristically observed in undifferentiated cells including normal and cancer stem cells. The metabolic shifts induced by hypoxia, therefore, promote the acquisition of stem cell properties by the tumor cells (4, 5).

In addition to the HIF-1 activation pathway, other pathways may also contribute to the adaptation of tumor cells to hypoxia. These include the unfolded protein response (UPR) pathway and the LKB1/AMPK pathway. Of these, UPR activates the protein kinase PERK, which phosphorylates and inactivates eIF2, and induces the expression of the transcription factor ATF4 (6). The LKB1/AMPK pathway on the other hand, inhibits mTOR and activates NF-κB and Notch1. The latter interacts with and functionally activates HIF-1 (7). Finally, it was recently shown that hypoxia alters the expression of various microRNAs (8), which may also contribute to hypoxia adaptation, by

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regulating the expression of proteins involved in HIF-1–dependent and -independent hypoxia-activated pathways. Here, we present evidence for a novel pathway that is activated by oxygen deprivation and depends on the protein kinase Akt2 and microRNA (miR) 21.

Akt, also known as PKB, defines a family of PH domain–containing serine threonine kinases, Akt1, Akt2, and Akt3, all of which are regulated by phosphorylation via a phosphoinositide-3-kinase (PI3K)-dependent process. Individual Akt isoforms may be overexpressed or selectively activated in tumor cells. Thus, in ovarian tumors, Akt2 is selectively overexpressed because of amplification (9), whereas in other tumor types, Akt1, Akt2, or Akt3 may be overexpressed because of selective transcriptional activation or mutation (10, 11). Similarly, activating mutations of Akt may target selectively one isoform over another (10) and Akt inhibitors may exhibit isoform specificity (12). On the basis of these considerations, we initiated studies to address potential signaling differences between isoforms (13).

Experiments reported in this article show that hypoxia signals, transduced selectively via Akt2, induce miR-21, which targets PTEN, PDCD4, and Sprouty 1 (Spry1), and that the coordinated downregulation of these proteins by miR-21 is both necessary and sufficient for the enhanced survival of Akt2-expressing cells during hypoxia. Through the downregulation of PTEN, Akt2 functions as a master regulator of all Akt isoforms. The hypoxia-activated, Akt2-dependent pathway operates in cultured cells, in mammary adenocarcinomas developing in MMTV-PyMT transgenic mice, as well as in human ovarian carcinomas. These findings shed light into the tumor-promoting consequences of selective Akt2 activation in human cancer.

Materials and Methods

Cell culture, retroviral and lentiviral infections

Spontaneously immortalized mouse lung fibroblasts from $Akt1^{fl/fl}/Akt2^{-/-}/Akt3^{-/-}$ mice were transduced with myc-tagged wild-type Akt1, Akt2, and Akt3. Ablation of endogenous *Akt1* by Cre gave rise to *Akt*-null cells (TKO) or cells expressing a single Akt isoform at a time (13). Cancer cell lines were transduced with shAkt1 or shAkt2 constructs in the lentiviral vector pLKO.1 (14).

Hypoxia, TUNEL assay, and cell-cycle analysis

Cells were cultured in 0.2% O_2, 5% CO_2 (hypoxia). Live cells were counted using a standard hemocytometer. Apoptosis was monitored with the TUNEL Assay Kit (11684795910; Roche). Cell-cycle distribution was monitored by EtBr staining and flow cytometry (15).

Real-time reverse transcriptase PCR

Real-time reverse transcriptase (RT) PCR was carried out to determine the expression levels of miR-21. RNA was isolated using the mirVana miRNA Isolation Kit (AM1561; Ambion). miR-21 expression levels were normalized to the levels of RNU44 or U6 small nuclear RNA (internal controls). Real-time RT-PCR was also carried out to determine the expression of HIF-1α, PTEN, and AKT2 in RNAs from ovarian adenocarcinomas with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control.

Immunoprecipitation and immunoblotting

Immunoprecipitation from total cell lysates was carried out using either anti-myc-tag or anti-HA antibody beads (E6779; Sigma) under continuous agitation overnight at 4°C. SDS-PAGE–resolved total cell lysates or immunoprecipitates were transferred to polyvinylidene difluoride (PVDF) membranes and probed with the indicated antibodies.

Identification of transcription factor–binding motifs, luciferase reporter constructs, and luciferase assay

Three selection criteria were used sequentially to identify transcription factor binding motifs in the *miR-21* promoter (16): (i) the Lever algorithm (17); (ii) the phylogenetic conservation of the binding motifs identified with the use of the Lever algorithm. Conservation was evaluated with the PhylCRM algorithm (17); and (iii) nucleosome occupancy in the conserved binding sites, which was visually evaluated with the UCSC genome browser track. The top transcription factor–binding motifs identified in the *miR-21* promoter with this methodology were 2 cAMP responsive element–binding protein (CREB) motifs and 1 NF-κB–binding motif. (See Supplementary Materials and Methods for more details).

The *miR-21* 5′-flanking region was amplified by PCR from genomic DNA isolated from IMR-90 cells. Ligation of the amplified DNA to pGL3 (Promega) gave rise to miR-21pro4,6-Luc, which was sequence verified. Point mutations were introduced in the 2 CREB sites and the NF-κB site with the QuikChangeII Site-Directed Mutagenesis Kit (200524-5; Stratagene). Luciferase assays were carried out using cells transfected with wild-type or mutant constructs in combination with a receptor tyrosine kinase (RTK)-*Renilla* luciferase construct. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (TM040; Promega). Firefly luminescence units were normalized on the basis of the *Renilla* luminescence units in each sample.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out as described previously (18). Briefly, chromatin fragments derived from Akt1- and Akt2-expressing lung fibroblasts were immunoprecipitated with 8 μg of anti-CREB (ab32515; Abcam Inc.), 6 μg of anti-NF-κB (p65; ab7970; Abcam Inc.), 5 μg of anti-CBP (ab32646; Abcam Inc.), and 8 μg of anti-Histone H3K9Ac (ab10812; Abcam Inc.). DNA extraction was carried out using the Qiagen Purification Kit (Qiagen). The samples were analyzed by real-time PCR using the primer pairs listed in the Supplementary Data section.

MMTV-PyMT–induced mammary adenocarcinomas in mice

The establishment and characterization of homozygous MMTV PyMT transgenic mice in the wild-type, $Akt1^{-/-}$, and $Akt2^{-/-}$ genetic backgrounds was described previously (19).

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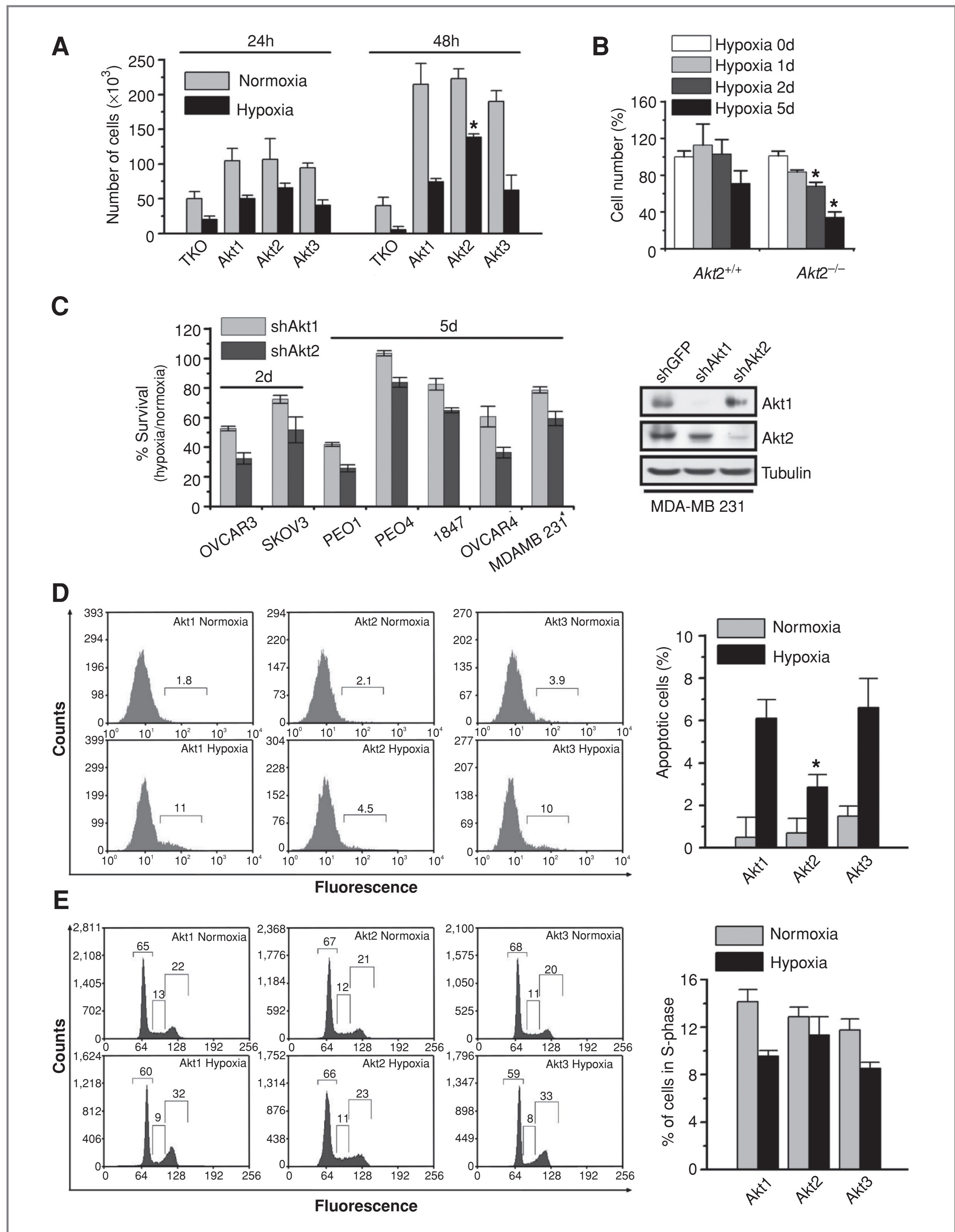

Figure 1. Akt2-expressing cells are more resistant to hypoxia than Akt1- or Akt3-expressing cells. A, numbers of Akt1-, Akt2-, or Akt3-expressing cells surviving hypoxia. Data are expressed as mean ± SD. *, statistically significant difference between Akt2- and Akt1- or Akt3-expressing cells in hypoxia (*, $P < 0.05$). B, numbers of Akt2$^{-/-}$ and Akt2$^{+/+}$ MEFs surviving hypoxia. Data are expressed as mean ± SD. *, statistically significant difference

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RNA isolation was carried out using the mirVana PARIS Kit (Ambion). Protein extraction was carried out using the radioimmunoprecipitation assay (RIPA) lysis buffer (Pierce). For *in situ* hybridization, a miRCURY LNA Detection probe 5′-end labeled with 56-FAM for mmu-miR-21 (39103-04; Exiqon) was used as previously described (13). For immunohistochemistry, sections were deparaffinized and following antigen unmasking, they were probed for HIF-1α.

Human ovarian cancer samples

RNAs from 31 ovarian adenocarcinomas were purchased from Origene Technologies Inc. and Biochain Inc. and they were used for real-time PCR analyses.

Results

Akt2-expressing cells are more resistant to hypoxia than Akt1- or Akt3-expressing cells

To determine the role of individual Akt isoforms in the cellular response to hypoxia, immortalized triple *Akt* knockout lung fibroblasts (TKO), and their derivatives expressing equal levels of Akt1, Akt2, or Akt3 (Supplementary Fig. S1), were exposed to low oxygen (0.2%) and 24 or 48 hours later, the abundance of live cells in each culture was determined by direct counting. The results showed that Akt2-expressing cells survive under hypoxia significantly better than the TKO or the Akt1- and the Akt3-expressing cells (Fig. 1A). The same experiment repeated in primary mouse embryonic fibroblasts (MEF) revealed that *Akt2*$^{-/-}$ cells are more sensitive to hypoxia than wild type cells (Fig. 1B). Comparison of the ratio of live cells in hypoxic versus normoxic cultures of shAkt1- and shAkt2-transduced cells in a set of 7 human ovarian or mammary carcinoma cell lines revealed that shAkt2-transduced cells are more sensitive to hypoxia than shAkt1-transduced cells (Fig. 1C).

Measuring DNA fragmentation in Akt1-, Akt2-, and Akt3-expressing cells with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay (Fig. 1D) and monitoring the cleavage of caspase-3 in wild-type and *Akt2*$^{-/-}$ MEFs (Supplementary Fig. S2) revealed that Akt2 protects cells from hypoxia-induced apoptosis. Parallel experiments revealed a significant drop in the percentage of S-phase cells, in Akt1- and Akt3-expressing cultures, upon exposure to hypoxia. In contrast, the changes in the Akt2-expressing cultures were minimal (Fig. 1E and Supplementary Fig. S3). Similar data were obtained when Akt1, Akt2, and Akt3 were replaced by their constitutively active mutants MyrAkt1, MyrAkt2, and MyrAkt3 (Supplementary Figs. S4 and S5). In agreement with the preceding data, the percentage of S-phase cells during hypoxia was significantly lower in *Akt2*$^{-/-}$ than in wild-type MEFs (Supplementary Fig. S6). These data collectively suggest that the Akt1- and Akt3-expressing cells exposed to hypoxia undergo apoptosis as they progress through S-phase or they undergo apoptosis with a parallel G_1/S-phase block. In contrast, Akt2-expressing cells are protected from both hypoxia-induced cell death and hypoxia-induced G_1/S arrest.

The differential sensitivity of Akt1-, Akt2-, and Akt3-expressing cells to hypoxia is due to the differential induction of miR-21

Our earlier studies had shown that microRNAs are differentially regulated by Akt1, Akt2, and Akt3 (13). In this report, we focus on miR-21, a microRNA that plays a critical role in a variety of human cancers and is upregulated by hypoxia (20, 21). Using real-time RT-PCR, we showed that hypoxia induced miR-21 in Akt2- but not in Akt1- or Akt3-expressing cells (Fig. 2A, top). Also, hypoxia induced miR-21 in wild-type but not in *Akt2*$^{-/-}$ primary MEFs (Fig. 2A, bottom), confirming that Akt2 is required for the induction of miR-21 by hypoxia.

Transfection of Akt1-, Akt2-, or Akt3-expressing cells with anti-miR-21 reversed the resistance of Akt2-expressing cells to hypoxia (Fig. 2B, top). Moreover, transfection of pre-miR-21 in *Akt2*$^{-/-}$ MEFs rendered them as resistant to hypoxia as the wild-type cells (Fig. 2B, bottom). Transfection with pre-miR-21 rendered Akt1- and Akt3-expressing cells as resistant to hypoxia as the Akt2-expressing cells (Supplementary Fig. S7, left), whereas anti-miR-21 rendered wild-type MEFs as sensitive to hypoxia as the Akt2$^{-/-}$ MEFs (Supplementary Fig. S8). Collectively, these experiments confirmed that the induction of miR-21 is both necessary and sufficient for the growth advantage of Akt2-expressing cells upon exposure to hypoxia.

To explore the miR-21 mechanism of action, we transfected Akt1-, Akt2-, and Akt3-expressing cells with anti-miR-21 or pre-mi-R21 and examined the rate of apoptosis and the cell-cycle distribution before and after exposure to hypoxia. The results showed that anti-miR-21 interferes with the antiapoptotic phenotype and with the cell-cycle phenotype of oxygen-deprived Akt2-expressing cells (Fig. 2C and D and Supplementary Fig. S9) and that pre-miR-21 reproduces the Akt2 phenotype in Akt1- and Akt3-expressing cells (Supplementary Figs. S10 and S11).

miR-21 regulates PTEN expression, by regulating PTEN via miR-21, Akt2 functions as a master regulator of Akt during hypoxia

miR-21 is an oncogenic microRNA that is known to play an important role in the induction and progression of many human tumors. One of its targets, the D3 phosphoinositide phosphatase PTEN (22), is selectively downregulated in Akt2-expressing cells upon oxygen deprivation and its downregulation correlates with the activation of Akt2 (Fig. 3A). In agreement with these observations, probing lysates of

(*Continued*)
between Akt2$^{-/-}$ and Akt2$^{+/+}$ MEFs in hypoxia (*, $P < 0.05$). C, ratios of live tumor cells in hypoxia versus normoxia. Data are expressed as mean ratios ± SD × 100 (left). Lysates of shAkt1- and shAkt2-tranduced MDA-MB 231 cells probed for Akt1, Akt2, and tubulin (right). D, TUNEL assay carried out on cells cultured in hypoxia or normoxia (left). Mean percentage of apoptotic cells ± SD. Cumulative data of 4 experiments (right). *, statistically significant difference between Akt2- and Akt1- or Akt3-expressing cells in hypoxia (*, $P < 0.05$). E, Cell-cycle distribution 24 hours after exposure to hypoxia (left). Data show the mean percentage of cells in S-phase ± SD. Cumulative data of 8 experiments (right). GFP, green fluorescent protein.

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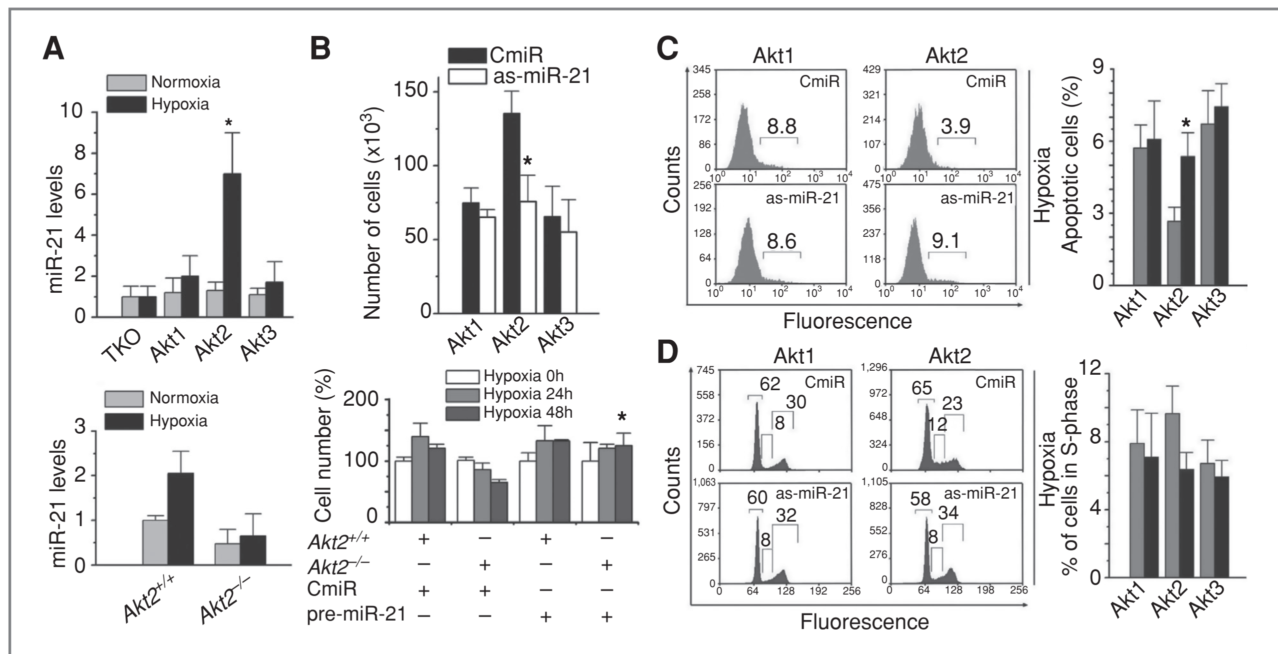

Figure 2. The resistance of Akt2-expressing cells to hypoxia depends on miR-21. A, miR-21 levels in TKO lung fibroblasts and their derivatives expressing individual Akt isoforms (top), and in MEFs of the indicated genotypes (bottom), were measured by real-time RT-PCR. Values are expressed as mean ± SD. *, statistically significant differences (*, $P < 0.05$). B, effect of anti-miR-21 on the number of live cells in cultures exposed to hypoxia for 48 hours (top). Effect of pre-miR-21 on the number of MEFs of the indicated genotypes exposed to hypoxia (bottom). Data are expressed as mean ± SD. *, statistically significant differences (*, $P < 0.05$). CmiR, control miR. C, Numbers of apoptotic Akt1-, Akt2-, and Akt3-expressing cells transduced with anti-miR-21 and cultured in hypoxia for 24 hours. Representative experiment (left) and cumulative data from 3 experiments (right) expressed as mean ± SD. *, statistically significant difference between control and as-miR-21–transfected Akt2-expressing cells (*, $P < 0.05$). D, cell-cycle distribution before and after exposure to hypoxia. Representative experiment (left) and cumulative data (right) expressed as mean ± SD.

primary $Akt2^{+/+}$ and $Akt2^{-/-}$ MEFs with phospho-Akt and Akt phosphosubstrate antibodies, as well as with phosphoantibodies against the indicated Akt substrates, revealed that the activity of Akt upon exposure to hypoxia was significantly lower in $Akt2^{-/-}$ MEFs (Fig. 3B). Transfection of Akt1- and Akt2-expressing cells with anti-miR-21 and $Akt2^{+/+}$ and $Akt2^{-/-}$ MEFs with pre-miR-21 confirmed that miR-21 is indeed the mediator of the effects of Akt2 on PTEN expression, upon oxygen deprivation (Fig. 3C).

By removing the D3 phosphate from D3 phosphorylated phosphoinositides, PTEN inhibits the activation of not only Akt2 but also Akt1 and Akt3. We therefore reasoned that if Akt2 regulates the levels of PTEN, it should function as a master regulator of all Akt isoforms in oxygen-deprived cells. This was confirmed by monitoring Akt phosphorylation in immortalized triple *Akt* knockout fibroblasts reconstituted with myc-Akt1 and HA-Akt2, or myc-Akt1 and HA-Akt2 before and after exposure to hypoxia. The results linked the expression of Akt2 with the phosphorylation of both Akt1 and Akt2 (Fig. 3D and Supplementary Fig. S12).

By inducing miR-21 during hypoxia, Akt2 also inhibits the expression of PDCD4 and Spry1 and the activation of ERK

In addition to PTEN, miR-21 also targets the proapoptotic regulator of protein translation PDCD4 (23) and the adaptor protein Spry1, which negatively regulates the activation of extracellular signal–regulated kinase (ERK; ref. 24). Probing Western blot analyses of lysates of Akt1-, Akt2-, and Akt3-expressing lung fibroblasts and wild-type and $Akt2^{-/-}$ primary MEFs confirmed that Akt2 selectively inhibits the expression of not only PTEN but also PDCD4 and Spry1 in oxygen-deprived cells (Fig. 4A and B). Transfection of Akt1- and Akt2-expressing cells with anti-miR-21 (Fig. 4C) and wild-type and $Akt2^{-/-}$ cells with pre-miR-21 (Fig. 4D) or anti-miR-21 (Supplementary Fig. S13) confirmed that the Akt2-mediated downregulation of PTEN, PDCD4, and Spry1 upon exposure to hypoxia is mediated by miR-21.

The combined downregulation of PTEN, PDCD4, and Spry1 in oxygen-deprived Akt2-expressing cells is sufficient to induce resistance of these cells to hypoxia

The preceding data raised the question whether the downregulation of PTEN, PDCD4, and Spry1 by miR-21 in oxygen-deprived Akt2-expressing cells is sufficient to induce resistance to hypoxia. This question was addressed by transfecting Akt1-expressing cells with siRNAs for PTEN, PDCD4, and Spry1 singly or in combination and by comparing their resistance with the resistance of control siRNA–transfected Akt2-expressing cells. The siRNAs selected for the experiment induced only partial downregulation of these proteins, similar to that observed upon exposure of Akt2-expressing cells to hypoxia (Fig. 4E). The results confirmed that the combined downregulation of all 3 proteins renders Akt1-expressing cells

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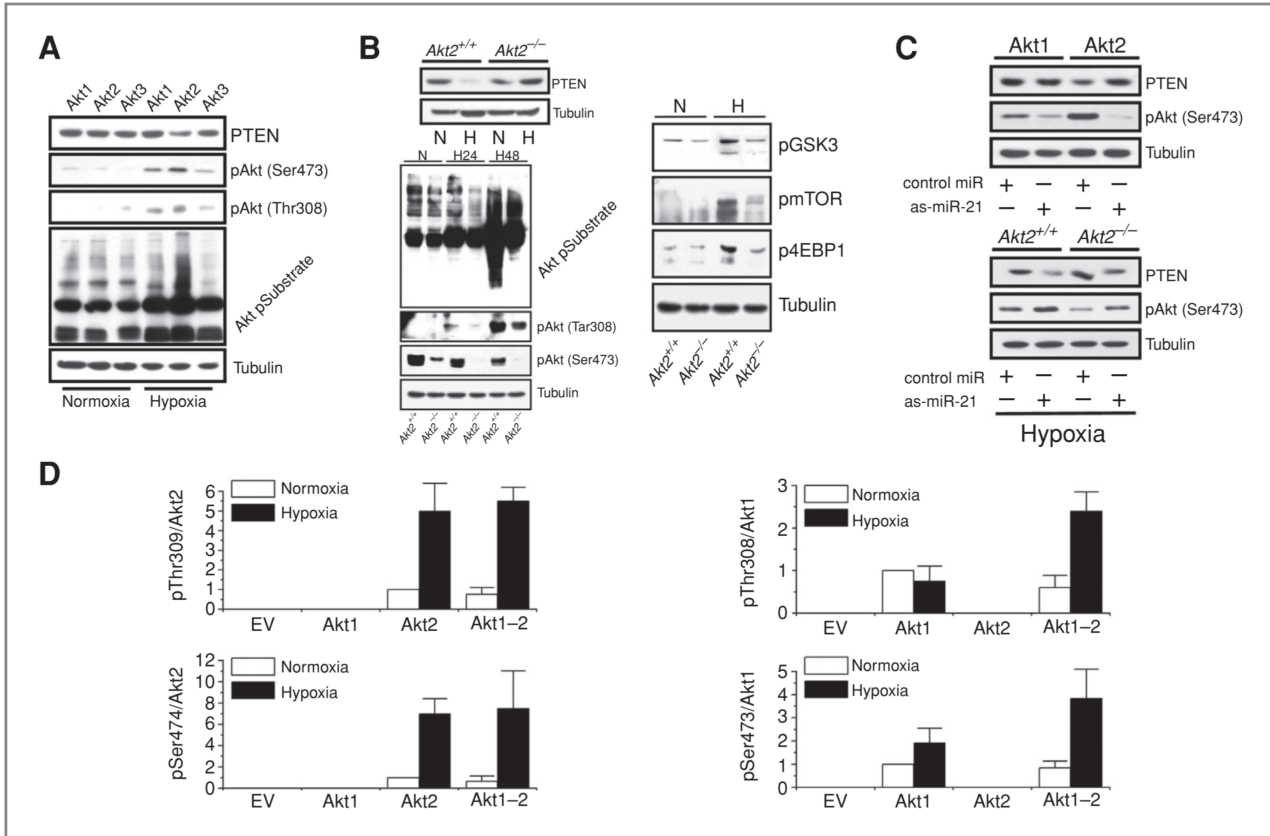

Figure 3. The Akt2/miR-21/PTEN pathway is a master regulator of all Akt isoforms in cells growing in hypoxia. A, PTEN protein levels and Akt activity in immortalized TKO lung fibroblasts and their derivatives expressing Akt1, Akt2, or Akt3 before and after oxygen deprivation. B, PTEN protein levels and Akt activity in Akt2$^{-/-}$ and Akt2$^{+/+}$ MEFs before and after oxygen deprivation. C, anti-miR-21 inhibits the downregulation of PTEN by hypoxia in Akt2-expressing lung fibroblasts (top). Pre-miR-21 promotes the downregulation of PTEN by hypoxia in both Akt2$^{+/+}$ and Akt2$^{-/-}$ MEFs (bottom). Lysates of the transfected cells cultured in hypoxia for 24 hours probed with the indicated antibodies. D, Akt2 promotes the activation of both Akt1 and Akt2 in cells exposed to hypoxia. TKO lung fibroblasts were transduced with myc-Akt1, HA-Akt2, or the combination of Akt1 and Akt2 (Akt1–2) or the empty vector MigR1 (EV) were grown in normoxia or hypoxia and harvested 24 hours later. Probing Akt2 (HA) immunoprecipitates with the anti-phospho-Thr308 and the anti-phospho-Ser473 antibodies showed that phosphorylation of Akt2 at both sites in Akt2 and Akt1–2-transduced cells grown in hypoxia is equal (left). Probing Akt1 (myc) immunoprecipitates showed that its phosphorylation at both sites is higher in Akt1–2- than in Akt1-transduced cells grown in hypoxia (right). N, normoxia; H, hypoxia.

as resistant to hypoxia as the Akt2-expressing cells (Fig. 4F), suggesting that the Akt2-induced resistance to hypoxia is mediated by the downregulation of PTEN, PDCD4, and Spry1 via miR-21.

The induction of miR-21 in Akt2-expressing cells upon exposure to hypoxia is due to the selective activation and binding of CREB/CBP and NF-κB to the miR-21 enhancer and the selective regional acetylation of histone H3 at K9

Analysis of a 5-kb DNA sequence upstream of the *miR-21* transcriptional start site identified 1 NF-κB- and 2 CREB-binding sites (Fig. 5A). To determine the functional significance of these sites, we generated a reporter construct placing the luciferase gene at the 3′ of this sequence. Reporter assays using the wild-type and mutant constructs in which the CREB- and NF-κB–binding sites were inactivated by point mutations, singly or in combination, revealed that the luciferase gene is induced by hypoxia only in Akt2-expressing cells transfected with the wild-type construct. All mutant constructs were defective suggesting that both CREB and NF-κB binding are required for the full activation of the promoter by hypoxia. However, the mutation of all the CREB- and NF-κB–binding sites simultaneously had a more robust inhibitory effect on promoter activation (Fig. 5B).

The preceding data suggested that Akt1 and Akt2 may regulate differentially the activation and DNA binding of CREB and NF-κB. ChIP provided support to the hypothesis by showing that the binding of CREB and NF-κB to their respective binding sites in cells exposed to hypoxia were Akt2 dependent (Fig. 5C).

CREB undergoes phosphorylation by PKA, PKC, and perhaps Akt at Ser133 (25). Probing cell lysates harvested before and after exposure to hypoxia for phospho-CREB (Ser133) revealed that hypoxia promotes phosphorylation of CREB most efficiently in Akt2-expressing cells. Given that phosphorylation at this site regulates CREB binding to CBP (26), we hypothesized that, in oxygen-deprived cells, Akt2 may regulate the binding of

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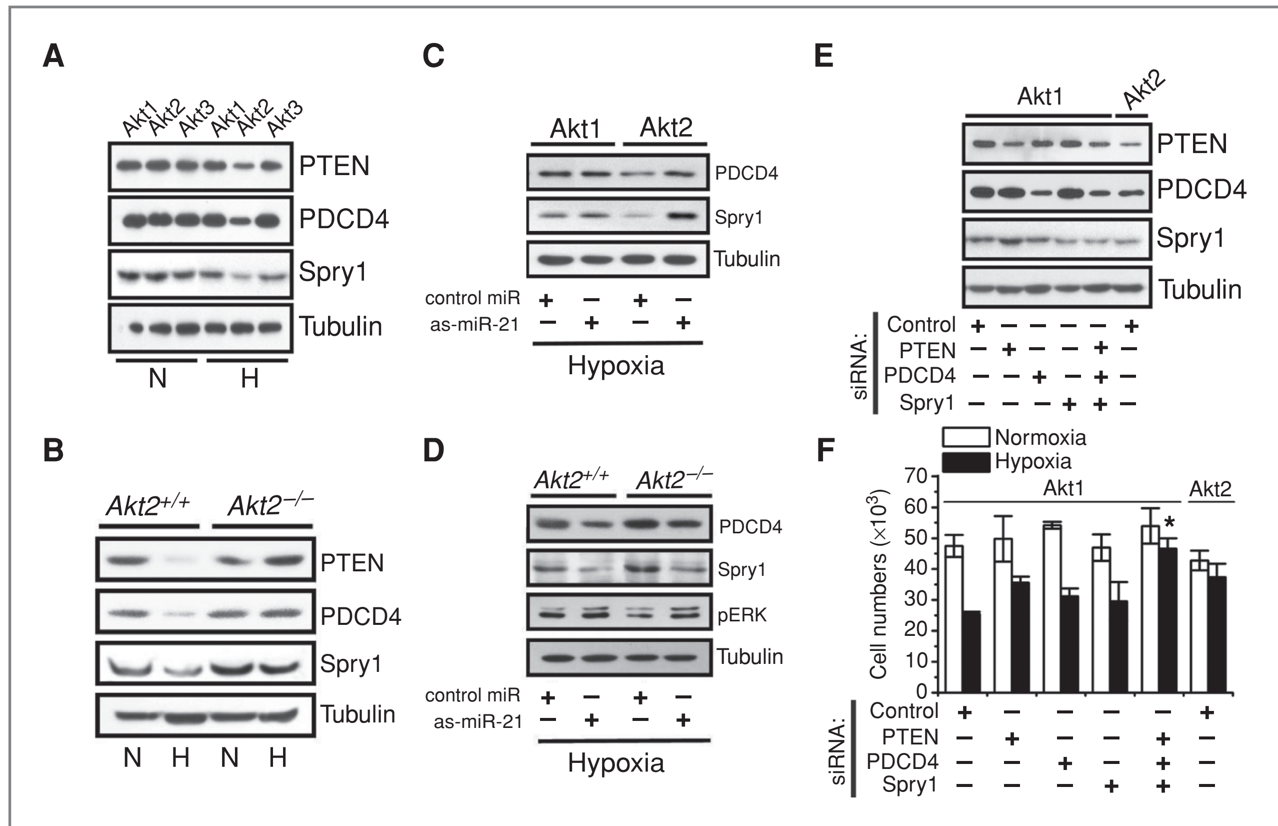

Figure 4. Akt2 downregulates PDCD4 and Spry1 via miR-21 in hypoxia. The combined downregulation of PTEN, PDCD4, and Spry1 is sufficient to induce resistance to hypoxia. A, Akt2 promotes the downregulation of PTEN, PDCD4, and Spry1 in cells exposed to hypoxia. Lysates of cells cultured in normoxia or hypoxia for 24 hours probed with the indicated antibodies. B, Akt2 ablation interferes with the downregulation of PTEN, PDCD4, and Spry1 in MEFs exposed to hypoxia. Lysates of MEFs of the indicated genotypes probed with the indicated antibodies. C, anti-miR-21 inhibits the downregulation of PDCD4 and Spry1 in Akt2-expressing cells. Lysates of cells transfected with anti-miR-21 and cultured in hypoxia were probed with the indicated antibodies. D, pre-miR-21 promotes the downregulation of PDCD4 and Spry1 and the activation of ERK in $Akt2^{-/-}$ MEFs exposed to hypoxia. MEFs transfected with pre-miR-21 were cultured in hypoxia. Cell lysates harvested 24 hours later probed with the indicated antibodies. E, knockdown of PTEN, PDCD4, and Spry1. Akt1- or Akt2-expressing lung fibroblasts were transfected with the indicated siRNAs. Cell lysates harvested 48 hours after the transfection were probed with the indicated antibodies. F, the cells in (E) were grown in normoxia or hypoxia. Cell numbers, after 24 hours in hypoxia, expressed as mean ± SD (combined results from 2 experiments carried out in quadruplicate). *, statistically significant difference in cell numbers between hypoxia-treated Akt1-expressing cells transfected with all 3 siRNAs or siRNA control (*, $P < 0.05$).

CBP to CREB on the *miR-21* promoter. ChIP, using a CBP-specific antibody, confirmed the hypothesis (Fig. 5E, left). CBP is a histone H3K9 acetyltransferase (27). Therefore, its binding to the *miR-21* promoter may induce H3K9 acetylation. ChIP indeed confirmed that hypoxia promotes H3K9 acetylation in the vicinity of the first CREB-binding site, preferentially in Akt2-expressing cells (Fig. 5E, right).

HIF-1α–positive mammary adenocarcinomas arising in MMTV-PyMT/$Akt2^{-/-}$ mice express lower levels of miR-21 and higher levels of the miR-21 targets than those arising in MMTV-PyMT/Akt wild-type and MMTV-PyMT/$Akt1^{-/-}$ mice

To determine whether the Akt2/miR-21 axis is active during the progression of hypoxic tumors, we examined the expression of miR-21 and its targets in mammary adenocarcinomas in HIF-1α–expressing MMTV-PyMT/$Akt^{+/+}$, MMTV-PyMT/$Akt1^{-/-}$, and MMTV-Pymt/$Akt2^{-/-}$ mice. The tumors analyzed expressed robust levels of HIF-1α, a hypoxic marker. HIF-1α–positive tumors arising in $Akt2^{-/-}$ mice were shown to express lower levels of miR-21 than tumors arising in wild-type and $Akt1^{-/-}$ mice. Western blotting showed that the expression of miR-21 targets, PTEN, PDCD4, and Spry1, was higher in tumors arising in $Akt2^{-/-}$ than in tumors arising in wild-type and $Akt1^{-/-}$ mice (Fig. 6A and Supplementary Fig. S14). Immunohistochemistry studies revealed similar numbers of focal areas of HIF-1α expression (hypoxia) in tumors arising in wild type, $Akt1^{-/-}$, and $Akt2^{-/-}$ mice. However, the hypoxic areas expressing high levels of miR-21 were significantly reduced in the $Akt2^{-/-}$ tumors (Fig. 6B).

The expression of miR-21 exhibits a positive correlation, whereas the expression of PTEN exhibits a negative correlation with the expression of Akt2 in HIF-1α–positive ovarian carcinomas

Thirty-one human ovarian carcinomas were classified into hypoxic (high HIF-1α) and normoxic (low HIF-1α) groups. The hypoxic tumors were subdivided into high and

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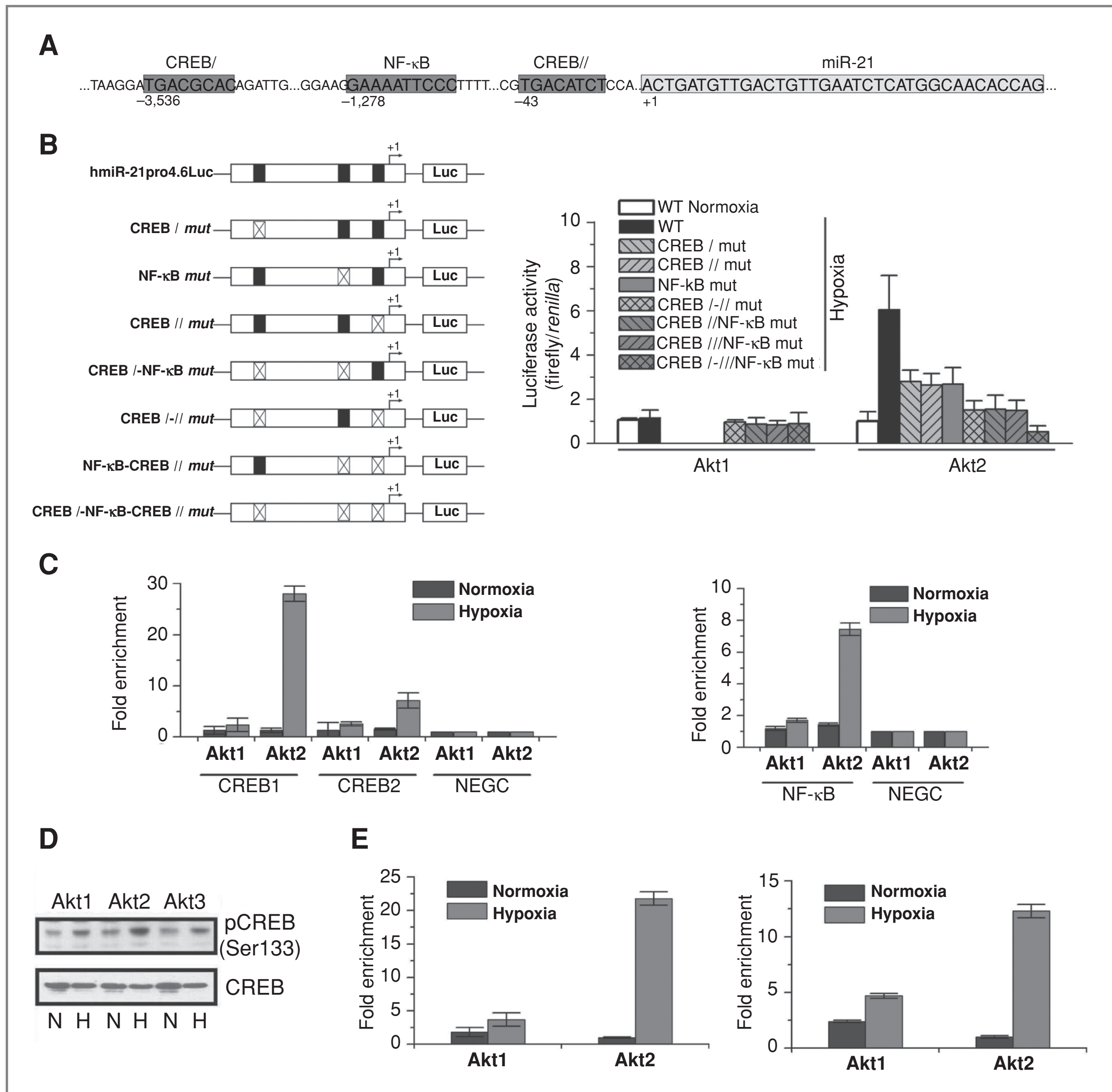

Figure 5. Selective miR-21 promoter binding of CREB, CBP, and NF-κB and selective histone H3K9 acetylation in the miR-21 promoter in Akt2-expressing cells exposed to hypoxia. A, schematic diagram of the promoter of miR-21. Red boxes: binding sites for CREB and NF-κB; Green box: miR-21 gene. B, miR-21 promoter activity in Akt1- and Akt2-expressing cells cultured in hypoxia for 24 hours. Schematic diagram of the wild-type reporter gene vector and its point mutants (left) and luciferase assay (right). Relative luciferase activity was set to 1 in Akt1-expressing cells growing in normoxia. Data are expressed as mean ± SD. C, ChIP for CREB and NF-κB in Akt1- and Akt2-expressing cells growing under normoxic or hypoxic conditions. The binding of CREB (left) and NF-κB (right) was measured by real-time PCR. Data are expressed as mean ± SD. D, CREB phosphorylation in Akt1-, Akt2-, or Akt3-expressing cells upon oxygen deprivation. E, ChIP for CBP and H3K9 acetylation in Akt1- and Akt2-expressing cells. The fold enrichment of CBP binding (left) and H3K9Ac (right) to the promoter of miR-21 was measured by real-time PCR. Data are expressed as mean ± SD.

low Akt2-expressing subgroups. Analysis of the subgroups revealed a positive correlation between Akt2 and miR-21 expression and a negative correlation between Akt2 and PTEN (Fig. 6C and Supplementary Figs. S15 and S16). In addition, it revealed a negative correlation between PTEN and miR-21 (Supplementary Fig. S17). These data indicate that Akt2 functions as the critical regulator of the miR-21–mediated resistance of human ovarian carcinoma cells to hypoxia *in vivo*.

Analysis of the normoxic (low HIF-1α) tumors revealed universally low expression of Akt2, low expression of miR-21, and high expression of PTEN. Our data (Supplementary Fig. S18) showed that HIF-1α is induced by hypoxia to similar

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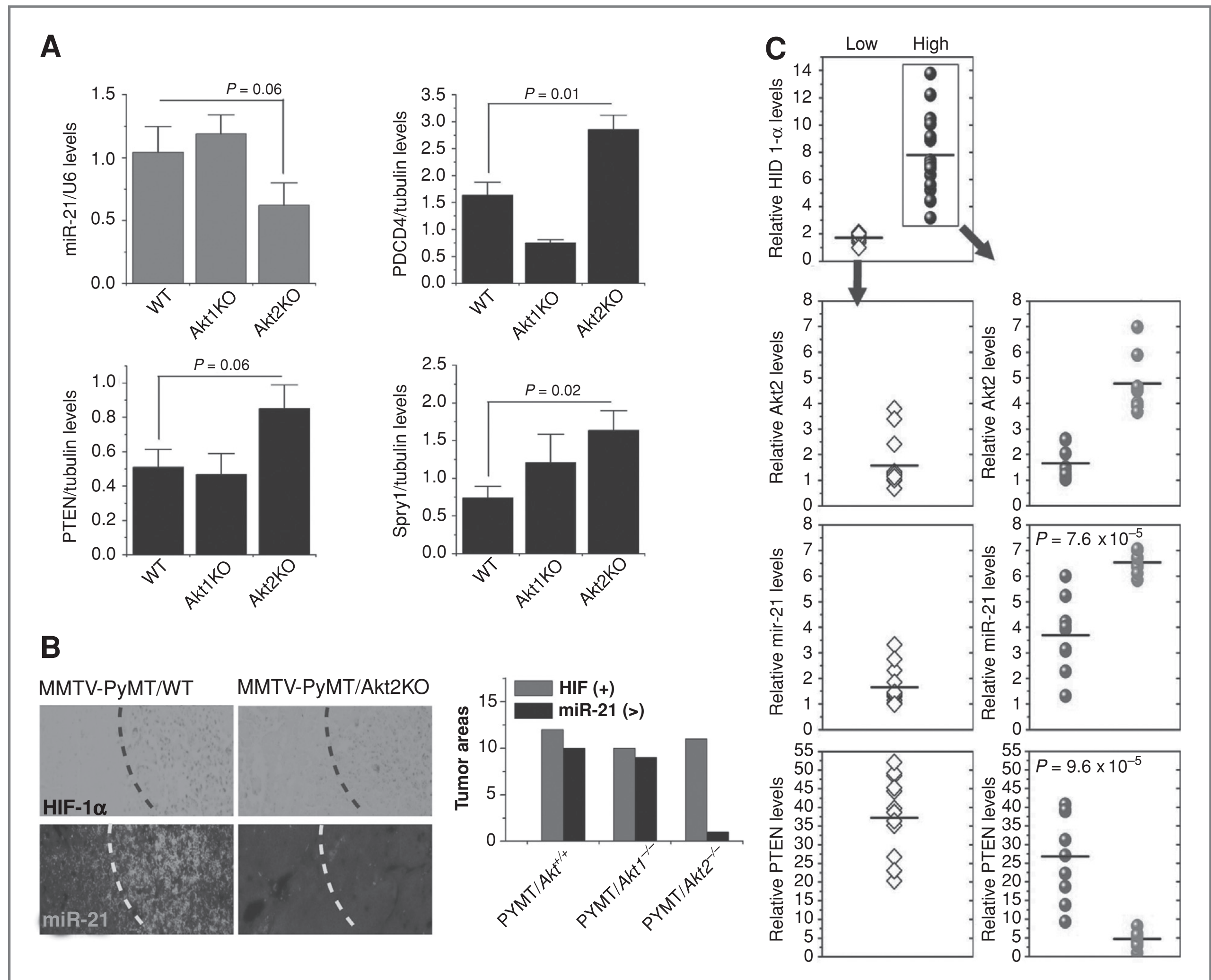

Figure 6. The Akt2/miR-21 pathway is active in hypoxic tumors. A, expression of miR-21 and its targets in mammary adenocarcinomas in HIF-1α–expressing MMTV-PyMT/Akt$^{+/+}$, MMTV-PyMT/Akt1$^{-/-}$, and MMTV-Pymt/Akt2$^{-/-}$ mice. Relative miR-21 levels were measured by real-time RT-PCR, with U6 small nuclear RNA as the internal control. Data are expressed as mean ± SD ($n = 4$–5 animals per group). PTEN, PDCD4, and Spry1 levels were analyzed by Western blotting. α-Tubulin was used as a loading control. The intensity of the bands was quantified using Scion Image software. Data are expressed as mean ± SD ($n = 4$–5 animals per group). B, immunohistochemistry for HIF-1α and *in situ* hybridization for miR-21 in tumors arising in MMTV-PyMT/Akt$^{+/+}$ and MMTV-Pymt/Akt2$^{-/-}$ mice (left). The great majority of hypoxic (HIF-1α^{+}) focal areas in tumors arising in Akt2$^{-/-}$ mice express low levels of miR-21 (right). C, expression of Akt2, miR-21, and PTEN in human ovarian carcinomas. Samples classified into 2 groups expressing high and low HIF-1α were analyzed for Akt2, miR-21, and PTEN levels. Dark gray dots represent tumors with low levels of Akt2 and light gray dots represent tumors with high levels of Akt2. WT, wild type; KO, knockout.

levels in cells expressing Akt1, Akt2, or Akt3, suggesting that the 3 Akt isoforms do not differ in their ability to transduce hypoxia signals that regulate HIF-1α. Moreover, there is no evidence to date for the regulation of Akt by HIF-1. We conclude that the low Akt2 levels in low HIF-1α tumors suggest the selective survival of Akt2-expressing cells in hypoxic tumors and support the concept that Akt2 plays a major role in the adaptation to hypoxia.

Discussion

Exposure to hypoxia may induce cell-cycle arrest and/or cell death. Alternatively, it may give rise to a metabolic switch that protects cells from the resulting energy stress. In tumors exposed to hypoxia, this switch is characterized by metabolic changes that enhance the growth, survival, invasiveness, and metastatic potential of the tumor cells (2). In addition, hypoxia induces angiogenesis and a proinflammatory microenvironment that promotes oncogenesis (28). Central to the hypoxia-induced switch is the transcription factor HIF-1α. However, other pathways may also contribute to the hypoxia response and may synergize with the HIF pathway.

Earlier studies provided evidence that Akt contributes to the cellular response to hypoxia and is required for hypoxia-stimulated angiogenesis. The activation of Akt in oxygen-deprived cells may be due to mTOR inhibition, which

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eliminates mTOR-induced feedback pathways that inhibit Akt activation by RTK and other Akt activation signals (29). Upon activation, Akt promotes the induction of HIF-1α, which is thought to be responsible for Akt-mediated protection from hypoxia (30). However, the protection from hypoxia depends primarily on Akt2 and not on Akt1 or Akt3, despite the fact that the induction of HIF-1α is similar in all the Akt-expressing cells, independent of which Akt isoform they express. These data suggest that Akt2 protects from hypoxia via a novel HIF-1α–independent mechanism.

Oxygen deprivation promotes the induction of miR-21 via an Akt2-dependent process and miR-21 inhibits the expression of the D3 phosphoinositide phosphatase PTEN, the proapoptotic inhibitor of protein translation PDCD4, and the inhibitor of ERK activation Spry1. More important, the downregulation of PTEN, PDCD4, and Spry1 is necessary and sufficient for the protection from hypoxia via Akt2. We conclude that Akt2 inhibits hypoxia-induced cell death and cell-cycle arrest via a novel pathway (Fig. 7) that depends on the induction of miR-21 and downregulation of PTEN, PDCD4, and Spry1, independently of HIF-1α. Although Akt2 regulates the expression of all three proteins, it may regulate PDCD4 also functionally. PDCD4 inhibits protein translation by binding eIF4A (31) and its binding to eIF4A is inhibited by p70S6K-mediated phosphorylation (32). Given that p70S6K is activated by mTOR, an Akt-regulated kinase, Akt2 may indeed regulate not only the expression but also the functional activity of PDCD4.

The inhibition of PTEN by the Akt2/miR-21 pathway suggests that Akt2 may have an expanded role in Akt signaling. PTEN downregulation by miR-21 allows the accumulation of PI3K-generated PIP3 at the plasma membrane and promotes the activation of Akt. Given that all Akt isoforms are activated via the same PI3K-dependent mechanism, we confirmed that Akt2 promotes the activation of Akt1 and functions as a master regulator of Akt activity in oxygen-deprived cells.

The induction of miR-21 by hypoxia-generated signals transduced by Akt2 depends on NF-κB and CREB binding to the miR-21 promoter. Akt2 also preferentially phosphorylates CREB at Ser133 (Fig. 5D). Phosphorylation at this site promotes the binding of CBP/p300 and the acetylation of histone H3 at K9, which may be at least partially responsible for the induction of miR-21. A recent report indeed showed that inhibition of histone deacetylation promotes miR-21 expression (33). We conclude that selectivity in histone modification by signals transduced by Akt1, Akt2, and Akt3 may be at least partially responsible for the differential effects of the 3 Akt isoforms on the global regulation of gene expression. Whereas our data in this report showed that the induction of miR-21 by hypoxia depends on NF-κB and CREB, another recent study showed that the induction miR-21 by interleukin (IL) 6 depends on the activation of STAT3 (34). These findings suggest that critical factors responsible for the induction of miR-21 may be signal specific.

HIF-1 promotes metabolic reprogramming, which supports the survival and proliferation of cancer cells. Specifically, HIF-1 stimulates glucose uptake and aerobic glycolysis and inhibits the activity of the Krebs cycle and oxidative phosphorylation in the mitochondria (2). Akt2 also promotes glucose uptake and aerobic glycolysis (35, 36), and like HIF-1, it synergizes with c-myc (37, 38). Thus, the metabolic activities of Akt2 and HIF may overlap and hypoxia-activated Akt2 may synergize with HIF-1 to reprogram cell metabolism. Given that the metabolic shifts caused by HIF-1 and Akt2 are also shared by stem cells, the acquisition of stem cell properties by the tumor cells, which is promoted by hypoxia (4, 5), may be due to the combined effects of HIF-1 induction and Akt2 activation.

HIF-1α promotes epithelial-mesenchymal transition (EMT) and enhances tumor cell invasiveness and metastasis (39). Given that our earlier studies had shown that upon activation by growth factors, Akt2 selectively promotes EMT (13), we propose that hypoxia signals transduced by Akt2 may synergize with HIF-1 to induce EMT during hypoxia. Several molecular mechanisms may contribute to this synergy. First, HIF-1 and Akt2 may contribute to the induction of Zeb1 and Zeb2 by different mechanisms. HIF-1 promotes Zeb1/Zeb2 transcription (40), whereas Akt2 promotes Zeb1/Zeb2 expression posttranscriptionally by inhibiting the expression of the miR-200 microRNA family (13). HIF-1 and Akt2 also act in concert to regulate Twist and its downstream targets. HIF-1 induces Twist (39), whereas Akt2 induces the Twist target miR-10b (13), known to activate a signal cascade that promotes tumor cell invasiveness and metastasis (41). Finally, Akt2 may activate Notch1 and may enhance the activation of HIF-1 during hypoxia. Both these effects are mediated by NF-κB (7, 42–44) which is preferentially activated by Akt2 during oxygen deprivation.

Data presented in this report also showed that the Akt2/miR-21 pathway may be functional in naturally occurring tumors *in vivo*. Hypoxic MMTV-PyMT–induced mammary adenocarcinomas arising in *Akt2*$^{-/-}$ mice express low levels of miR-21 and high levels of PTEN. The low Akt activity in

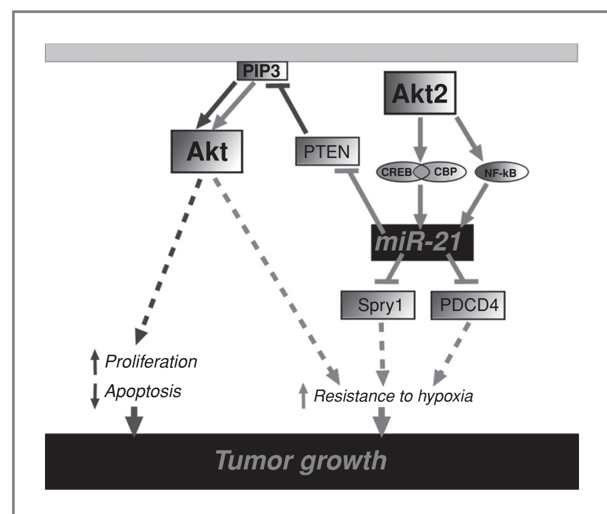

Figure 7. Proposed model. In normoxia, Akt promotes cell survival and proliferation (black line). Differential activation of NF-κB and CREB/CBP by hypoxia, via Akt2, activates an miR-21–dependent pathway (gray line) which confers resistance to hypoxia.

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these tumors would interfere with their adaptation to hypoxia. Hypoxic human ovarian carcinomas with high Akt2 levels express higher levels of miR-21 and lower levels of PTEN than hypoxic ovarian carcinomas with low Akt2. Normoxic carcinomas, on the other hand, express low levels of Akt2 and miR-21 and high levels of PTEN. This observation suggests that HIF-1 may regulate or may be regulated by Akt2. Alternatively, cells expressing high levels of Akt2 may be progressively selected upon exposure to hypoxia. Given that existing evidence does not support the idea of cross-regulation between HIF-1 and Akt2, we favor the hypothesis that hypoxia promotes the selection of cell clones expressing high Akt2 levels. This selection may be one of the mechanisms by which tumor cells adapt to the hypoxic environment.

In summary, evidence presented in this report identifies a novel miR-21–dependent pathway that is under the control of the Akt2 kinase and contributes to the adaptation of tumor cells to hypoxia (Fig. 7). These findings, combined with the results of our earlier studies on the role of Akt2 in the induction of EMT, shed light on the mechanisms by which Akt2 selectively enhances oncogenicity, and they should be taken into account when we target Akt therapeutically in various types of human cancer.

Disclosure of Potential Conflicts of Interest

C. Polytarchou is a fellow of the Leukemia and Lymphoma Society Inc. No potential conflicts of interest were disclosed.

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