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Research Article

Hypoxia-Inducible Factor-1α Promotes Nonhypoxia-Mediated Proliferation in Colon Cancer Cells and Xenografts

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Abstract

Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that directly transactivates genes important for the growth and metabolism of solid tumors. HIF-1α is overexpressed in cancer, and its level of expression is correlated with patient mortality. Increased synthesis or stability of HIF-1α can be induced by hypoxia-dependent or hypoxia-independent factors. Thus, HIF-1α is expressed in both nonhypoxic and hypoxic cancer cells. The role of HIF-1α in nonhypoxia-mediated cancer cell proliferation remains speculative. We have disrupted *HIF-1α* by targeted homologous recombination in HCT116 and RKO human colon cancer cells. Loss of HIF-1α significantly reduced nonhypoxia-mediated cell proliferation *in vitro* and *in vivo*. Paradoxically, loss of HIF-1α expression did not grossly affect the hypoxic compartments within tumor xenografts *in vivo*, although HIF-1α promoted cell proliferation and survival under hypoxia *in vitro*. To further test the role of HIF-1α within tumor compartments, we generated cells with combined disruptions of both *HIF-1α* and *vascular endothelial growth factor* (*VEGF*). In all xenografts, disruption of *VEGF* led to marked expansion of the hypoxic compartments and growth delay. Nonetheless, the presence or absence of HIF-1α did not grossly affect these expanded hypoxic compartments. These data provide compelling evidence that, in a subset of colon cancers, (*a*) HIF-1α is a positive factor for nonhypoxia-mediated cell proliferation *in vitro* and *in vivo* and (*b*) HIF-1α is a positive factor for cell proliferation and survival under hypoxic conditions *in vitro*, but does not grossly contribute to the tumor hypoxic compartments *in vivo*. (Cancer Res 2006; 66(3): 1684-93)

Introduction

Colorectal cancer is the second leading cause of cancer deaths in the United States. Colorectal cancer develops through multiple steps, with the sequential acquisition of genetic alterations in key tumor suppressors and oncogenes (1). These genetic alterations in turn activate genes that support tumor proliferation and survival (2, 3). Thus, a potential therapeutic approach against cancer is targeting the mediators of tumor growth and survival.

Hypoxia-inducible factor-1 (HIF-1) is strongly associated with cancer cell growth and survival (4). HIF-1 is composed of the HIF-1α and HIF-1β subunits. Whereas HIF-1β is constitutively expressed, HIF-1α protein stability and synthesis are regulated by intratumoral hypoxia and genetic alterations (5–12). The HIF-1 complex transactivates hypoxia-responsive genes through binding to the hypoxia response elements (HRE) located on the promoter or enhancer regions of hypoxia-inducible genes (4, 5). Promoter analyses for HRE show that HIF-1α directly transactivates >60 target genes, including those important for two universal characteristics of solid tumors: angiogenesis and glycolysis (4).

HIF-1α is overexpressed in cancer, and its level of expression is correlated with patient mortality (13, 14). However, further clarification of its role in tumorigenesis is required to determine if it is a valid therapeutic target (4). For example, HIF-1α is generally a positive factor in tumor xenograft models (15–21). Yet, depending on the types of cells or tumor microenvironments tested, *HIF-1α*-deficient tumors have also been shown to grow faster than their wild-type counterparts (22, 23).

In cancer cells, genetic alterations in key tumor suppressors or oncogenes increase synthesis or stability of HIF-1α, which in turn results in its expression under nonhypoxic conditions (8, 24–27). The literature has suggested that the genes induced via HIF-1α under nonhypoxic conditions are similar to those under hypoxia (9, 12, 24, 28–31). However, the functional consequences of HIF-1α expression on nonhypoxic cancer cell proliferation, glycolysis, and angiogenesis remain speculative (18, 32). Furthermore, it remains unclear how the induction of HIF-1α by hypoxia-independent factors affects the compartments within a tumor (22, 33).

In this article, we report that, in a subset of colon cancers, (*a*) HIF-1α promotes nonhypoxia-mediated cell proliferation *in vitro* and *in vivo* and (*b*) HIF-1α does not grossly affect the hypoxic compartments within tumor xenografts, although HIF-1α promotes cell proliferation and survival under hypoxic culture conditions.

Materials and Methods

Cell lines. HCT116 and RKO human colon cancer cells were acquired from the American Type Culture Collection (Manassas, VA) and cultured in recommended medium. Both cell lines harbor wild-type alleles of *adenomatous polyposis coli* (*APC*) and *p53* (34, 35). HCT116 cells harbor one mutated allele of *β-catenin* and *K-Ras*, which are wild-type in RKO cells (34, 36). For normoxic culture conditions, cells were incubated at low confluence and 37°C in 5% CO_2 and room air (21% O_2). For hypoxic culture conditions, cells were incubated at low confluence and 37°C in a BBL GasPak 100 anaerobic system in which O_2 was ~0.1% (BD Biosciences, Cockeysville, MD).

Real-time reverse transcription-PCR analyses. Real-time PCR reactions were done in triplicate on reverse transcription–derived cDNA as described previously (37). Crossing point (CP) at which fluorescence

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increases appreciably above the background fluorescence was determined (37). Gene expression relative to *β-actin* was calculated using the formula: ratio = $2^{-[CPGene - CP\beta\text{-actin}]}$ (38).

Strategy for disruption of the human *HIF-1α* and *vascular endothelial growth factor* genes. The endogenous locus, adenoassociated virus (AAV) knockout construct, and resulting targeted locus are shown in Figs. 1*A* and 5*A*. The targeting strategy is as described previously (37, 39). Exons 3 and 4 of *HIF-1α* or exon 2 of *vascular endothelial growth factor* (*VEGF*) were targeted for disruption with an AAV cassette containing the *Neo* resistance gene under the constitutive control of a *SV40* promoter flanked by left and right homology arms ~1 kb long. Successful disruption resulted in a 226-bp deletion (*HIF-1α*) or a 125-bp insertion (*VEGF*), with translation stop codons in all three reading frames. Cells exhibiting neomycin resistance were screened with locus-specific PCR. Once the first allele was successfully targeted, the *Neo* resistance gene was excised using *Cre* recombinase (Microbix Biosystems, Inc., Toronto, Ontario, Canada). The same targeting vector was then used to target the second allele. For locus-specific PCR to confirm homologous integration of the targeting vector, genomic DNA was amplified using primers specific for the targeted exons 3 and 4 (*HIF-1α*) or exon 2 (*VEGF*). Loss of HIF-1α was confirmed by Western blot analyses and loss of VEGF was confirmed by ELISA.

Western blot analyses. Whole-cell protein extracts were prepared from various cell lines, separated by electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies as described previously (37). Antibodies were obtained from BD Transduction Laboratories (San Jose, CA; mouse anti-human HIF-1α), Jackson Immunoresearch Laboratories (West Grove, PA; anti-mouse horseradish peroxidase), and Sigma (St. Louis, MO; α-tubulin). Antibody dilutions were as recommended by the manufacturer.

ELISA for VEGF. Equal numbers of cells were plated overnight. The VEGF protein level in the cultured medium was analyzed using the Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's protocol.

Measurements of cell proliferation, apoptotic index, and clonogenic survival. Cells were trypsinized, counted, and plated. For cell proliferation studies, cells were harvested daily for 5 days and stained with trypan blue, and viable (nonblue) cells counted on a hemocytometer. For calculations of apoptotic index, cells were harvested at days 3 to 5 and stained with Hoechst 33258, and nuclear morphology was scored (37). Apoptotic index was calculated as the number of pyknotic nuclei divided by the total number of cells counted per ×100 power field. At least 10 fields were photographed and counted. For colony formation assays, cells were allowed to grow undisturbed for 2 weeks and stained with crystal violet.

Analyses of DNA content and 5-bromodeoxyuridine incorporation. For analyses of cellular DNA content, cells were harvested, permeated with 70% ice-cold ethanol, and stained with 50 μg/mL propidium iodide in PBS containing 0.2% Tween 20 and 2.5 μg/mL RNase. DNA content in 10,000 cells was measured on a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ) and data were analyzed using Modfit Lt software (Verity Software House, Topsham, ME). For analyses of cellular incorporation of the halogenated DNA precursor 5-bromodeoxyuridine (BrdUrd), cells were labeled with 20 μg/mL BrdUrd, fixed with 70% ice-cold ethanol, and stained with FITC-conjugated anti-BrdUrd antibody and propidium iodide using the BrdUrd cell detection kit (BD PharMingen, San Jose, CA). The intensity and distribution of cell-associated BrdUrd-FITC fluorescence were detected in 10,000 cells using a FACSCalibur cytometer.

Glycolysis assays. The parental and knockout cell lines were harvested at standardized concentrations and lysed by repeated freeze-thaw cycles. Intracellular ATP concentrations were measured using the ATP Assay kit (Biomedical Research Service Center at State University of New York, Buffalo, NY). In the presence of ATP, the enzyme luciferase catalyzes the oxidation of luciferin with concomitant emission of yellow green light. Measurements were made on a luminometer and compared with a standard curve of ATP concentrations. Cellular lactate or lactate dehydrogenase (LDH) concentrations were measured using the Lactate or LDH Assay kits (Biomedical Research Service Center). The assay is based on the reduction of the tetrazolium salt INT in a NADH-coupled enzymatic reaction to formazan, which exhibits an absorption maximum at 492 nm. Measurements were made on a spectrophotometer and compared with a standard curve of lactate or LDH. For each experiment, relative fold change in ATP or lactate was calculated by dividing each sample concentration by the concentration in HCT116 cells.

***In vivo* tumorigenesis.** Cells were grown in standard conditions and harvested, and 5.0×10^6 cells were implanted s.c. into the flanks of 6-week-old female athymic *nu/nu* mice (Charles River Laboratories, Wilmington, MA). Tumor sizes in two dimensions were measured with calipers, and volumes were calculated with the formula: $(L \times W^2) \times 0.5$, where L is length and W is width. To examine tumor blood flow, mice were given the fluorescent perfusion marker Hoechst 33342 (Sigma) 15 mg/kg per tail vein injection 3 minutes before sacrifice. To examine tumor cell proliferation, mice were given the DNA synthesis marker BrdUrd (Sigma) 1,500 mg/kg i.p. 2 hours before sacrifice (40). I.p. injection of BrdUrd results in its uptake by proliferating cells, and bound BrdUrd can be detected in xenografts using antibody to BrdUrd. To examine intratumor hypoxia, mice were given the hypoxia marker pimonidazole 60 mg/kg i.p. 2 hours before sacrifice. Pimonidazole binds to the thiol-containing proteins specifically in hypoxic cells (41). I.p. injection of pimonidazole results in its uptake by hypoxic tumor cells, and bound pimonidazole can be detected in xenografts using antibody to pimonidazole (41). Mice were euthanized once overwhelmed by tumor burden or at the end of the study as defined by animal care guidelines. Tumor xenografts were quickly removed and frozen in liquid nitrogen or fixed in formalin. Mice were housed in barrier environments, with food and water provided *ad libitum* as approved by the University of Michigan Animal Care and Use Committee.

Immunohistochemistry. Tumor cryosections (10 μm thick) were cut with a Leica CM1900 cryostat. Formalin-fixed xenografts were paraffin embedded, sectioned, and stained with H&E by the University of Michigan Tissue Core Facility. Vasculature was stained using rat anti-mouse CD31 antibody (BD PharMingen; ref. 42). Hoechst 33342 was visualized under a fluorescent microscope (42). Bound pimonidazole was detected using the Hypoxyprobe-1 Plus kit (Chemicon International, Inc., Temecula, CA). Incorporated BrdUrd was detected using the BrdUrd *In situ* Detection kit (BD PharMingen). All antibody dilutions were according to the manufacturer's directions.

Statistics. *In vitro* studies were done in triplicate and repeated twice. *In vivo* studies were repeated once. Results are expressed as mean ± SD of all experiments. Statistical analyses of data were done using Student's paired t test and Ps < 0.05 were considered significant.

Results

Targeted disruption of *HIF-1α* in HCT116 and RKO cells. Somatic cell knockout technology was used to generate HCT116 and RKO human colorectal cancer cell lines that were absent for HIF-1α expression. Exons 3 and 4 of *HIF-1α* were targeted for disruption as outlined in Fig. 1*A*. Homozygous disruption of *HIF-1α* was identified by genomic locus-specific PCR (Fig. 1*B*). Loss of HIF-1α protein was confirmed by Western blot analysis using HIF-1α antibody (Fig. 1*C*). The resulting cells, hereafter called $HCT116^{HIF\text{-}1\alpha -/-}$ or $RKO^{HIF\text{-}1\alpha -/-}$ cells, are essentially identical with their respective parental HCT116 and RKO cells, with the exception of ablated HIF-1α expression. To control for clonal variation with passage, parental cells were passed in parallel with knockout cells and two independent clones of $HIF\text{-}1\alpha^{-/-}$ cells were selected for further analyses.

To further confirm the disruption of HIF-1α, we measured the expression of four known HIF-1α target genes, the glucose transporter *GLUT-1* and the glycolysis enzymes *phosphofructokinase, aldolase*, and *LDH* (4). Consistent with the literature, hypoxia induced the expression of these genes in both parental HCT116 and RKO cells (Fig. 1*D*), whereas hypoxic induction of these genes was diminished in the $HIF\text{-}1\alpha^{-/-}$ cells. Under normoxic conditions (21% O_2), *phosphofructokinase* and *LDH* showed decreased basal expression in $HCT116^{HIF\text{-}1\alpha -/-}$ cells. These latter findings support

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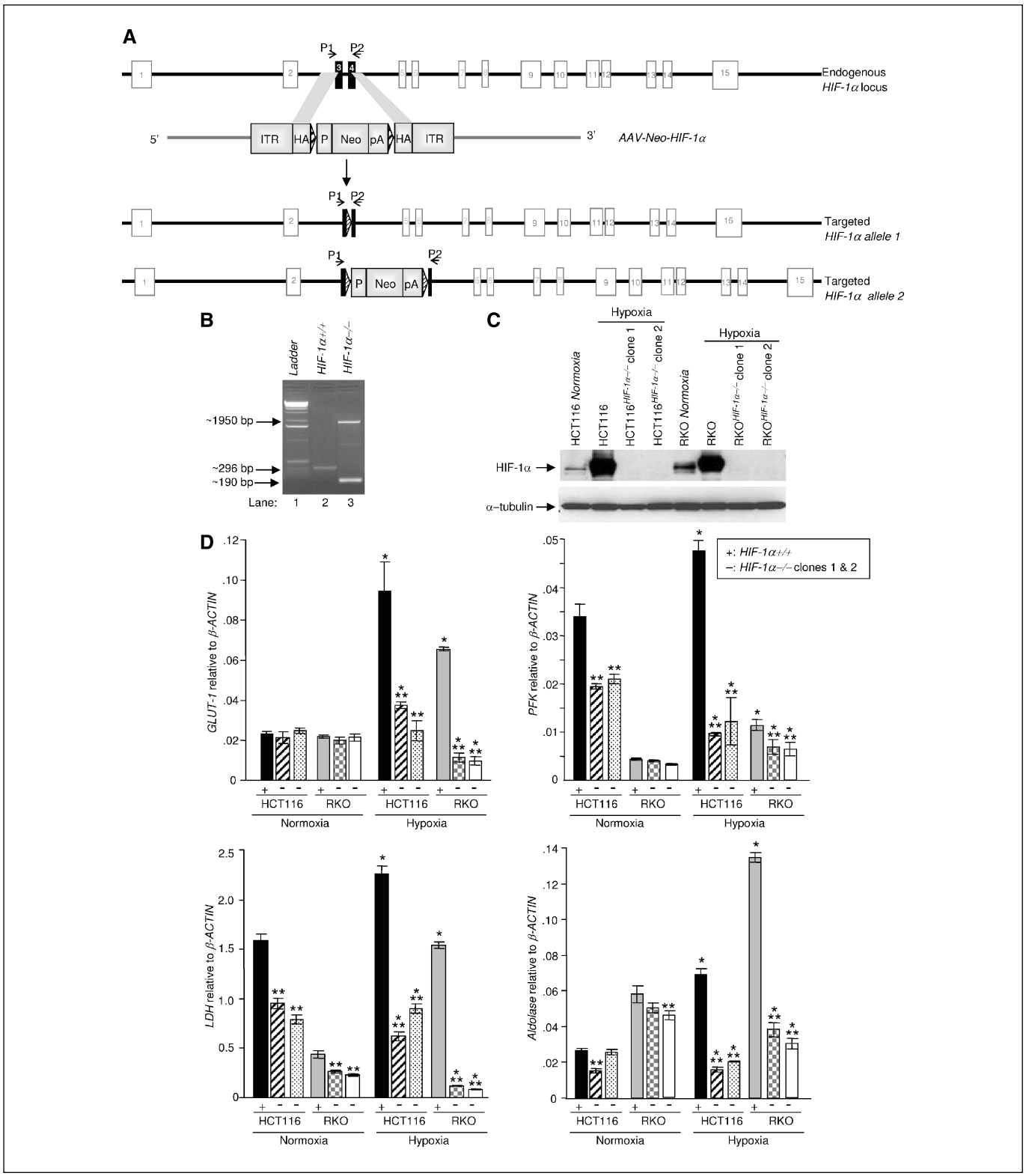

Figure 1. Disruption of *HIF-1α* in colon cancer cells. *A,* disruption of *HIF-1α*. Endogenous *HIF-1α* locus, AAV knockout construct, and resulting targeted locus. *Numbered boxes,* exons; *black boxes,* targeted exons 3 and 4; *ITR,* inverted terminal repeats; *HA,* homology arm; *P,* SV40 promoter; *Neo,* neomycin resistance gene; *pA,* polyadenylate tail; *striped triangles,* loxP sites. *B,* confirmation of disruption of *HIF-1α* by locus-specific PCR. Primers P1 and P2, which amplify the targeted region in exons 3 and 4, are shown on the endogenous and targeted *HIF-1α locus* diagrams. *Lane 2,* endogenous locus contains a ~296-bp fragment; *lane 3,* after successful targeting of the first allele and treatment with *Cre* recombinase, the amplification product is a smaller ~190-bp fragment. With successful targeting of the second allele, the amplification product is a ~1,950-bp fragment encompassing the *Neo* gene. *C,* confirmation of loss of HIF-1α by Western blot analysis. *D,* expression of HIF-1α target genes. *GLUT-1, phosphofructokinase, LDH*, and *aldolase* relative to *β-actin* by real-time reverse transcription-PCR are shown in noted cell lines and conditions ($n = 9$). *, $P < 0.01$, comparing the same cell line in hypoxia versus normoxia; **, $P < 0.01$, comparing *HIF-1α*$^{-/-}$ with *HIF-1α*$^{+/+}$ clones in the same culture conditions.

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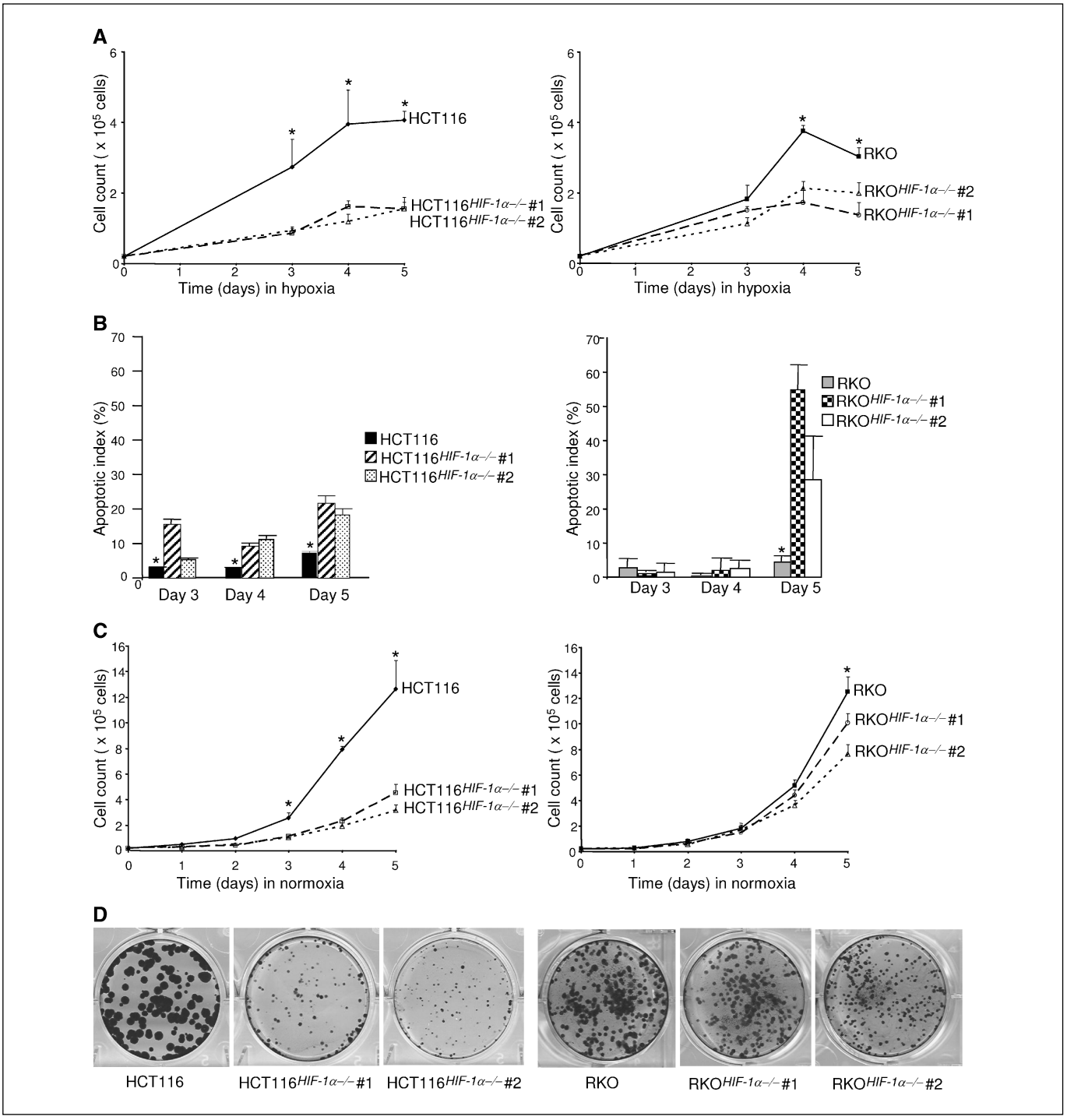

Figure 2. Cell proliferation, apoptosis, and clonogenic survival in parental and *HIF-1α*$^{-/-}$ cells. *A,* cell proliferation under hypoxia. Cells were incubated under hypoxic conditions and counted daily from days 3 to 5 ($n = 9$). *B,* apoptotic index as calculated by the number of pyknotic nuclei divided by the total number of cells. Cells were seeded and examined by Hoechst 33258 staining after 3, 4, and 5 days in hypoxia ($n = 30$). *C,* cell proliferation under normoxia. Cells were incubated under normoxic conditions and counted daily for 5 days ($n = 9$). *, $P < 0.01$, comparing parental with *HIF-1α*$^{-/-}$ cells in the same condition. *D,* clonogenic survival assays. Cells were incubated for 2 weeks under standard normoxic conditions and stained with crystal violet.

the notion that HIF-1α is expressed and may be functional in HCT116 cells under normoxia (9, 12, 24, 28–31).

Disruption of *HIF-1α* impairs cell proliferation and survival under hypoxic culture. Loss of HIF-1α led to significant decreases in cell proliferation and survival under hypoxia. Both HCT116$^{HIF\text{-}1\alpha-/-}$ and RKO$^{HIF\text{-}1\alpha-/-}$ cells had at least 2-fold decreases in cell numbers compared with their parental cells when grown under hypoxia (Fig. 2*A*). Both HCT116$^{HIF\text{-}1\alpha-/-}$ and RKO$^{HIF\text{-}1\alpha-/-}$ cells underwent marked apoptosis under hypoxia when compared with their parental cells (Fig. 2*B*). These data reaffirm that HIF-1α promotes proliferation and survival in human colon cancer cells under hypoxic culture.

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Disruption of *HIF-1α* impairs cell proliferation in normoxic culture. Under normoxic conditions, faint levels of HIF-1α protein were observed in parental HCT116 and RKO cells (Fig. 1*C*). Loss of HIF-1α resulted in a 3-fold decrease in HCT116 cell proliferation (Fig. 2*C*). In contrast, loss of HIF-1α resulted in only a modest decrease in RKO cell proliferation (Fig. 2*C*). We found no significant differences in apoptosis between parental and *HIF-1α*$^{-/-}$ cell lines under normoxic conditions (data not shown). Standard colony formation assays confirmed that under normoxic conditions, HCT116$^{HIF\text{-}1\alpha-/-}$ cells formed significantly smaller colonies compared with HCT116 cells (Fig. 2*D*). The decreases in colony formation in RKO$^{HIF\text{-}1\alpha-/-}$ cells were more subtle compared with RKO cells (Fig. 2*D*). These results show, for the first time, that HIF-1α is required for colon cancer cell proliferation in normoxic culture.

Disruption of *HIF-1α* impairs DNA synthesis and aerobic glycolysis in HCT116 but not RKO cells. We next examined the effects of HIF-1α on G_1-S cell cycle progression. Hypoxia induced significant G_1-S arrest in all cell lines tested as shown by the increases in G_1-S ratios (Fig. 3*A*). Loss of HIF-1α did not significantly alter the G_1-S ratios in HCT116 cells grown under hypoxia, which was consistent with reports of HIF-1α-independent mechanisms in hypoxia-induced cell cycle arrest (43). In contrast, loss of HIF-1α induced significant G_1-S arrest in RKO cells (Fig. 3*A*). Thus, cell cycle progression under hypoxic conditions can be HIF-1α dependent or HIF-1α independent.

Under normoxic culture conditions, loss of HIF-1α did not alter the G_1-S ratio (Fig. 3*A*), consistent with our proliferation data in RKO cells (Fig. 2*C*). However, these findings were inconsistent with our observations that loss of HIF-1α significantly decreased normoxic proliferation in HCT116 cells (Fig. 2*C*). To more directly examine DNA synthesis, we measured BrdUrd incorporation in *HIF-1α*$^{-/-}$ cells compared with parental cells. We found decreased incorporation of BrdUrd in HCT116$^{HIF\text{-}1\alpha-/-}$ cells compared with HCT116 cells in normoxia (Fig. 3*B*). In contrast, incorporation of BrdUrd was equivalent in RKO$^{HIF\text{-}1\alpha-/-}$ cells compared with RKO cells (Fig. 3*B*). Thus, loss of HIF-1α impaired HCT116 cell proliferation under normoxia through decreased S-phase activity.

Because HIF-1α is well described to mediate anaerobic glycolysis (4), we hypothesized that HIF-1α may mediate aerobic glycolysis. Cancer cells may maintain high rates of aerobic glycolysis and produce increased levels of lactate even in the presence of oxygen, a phenomenon first described by Dr. Otto Warburg (44). These

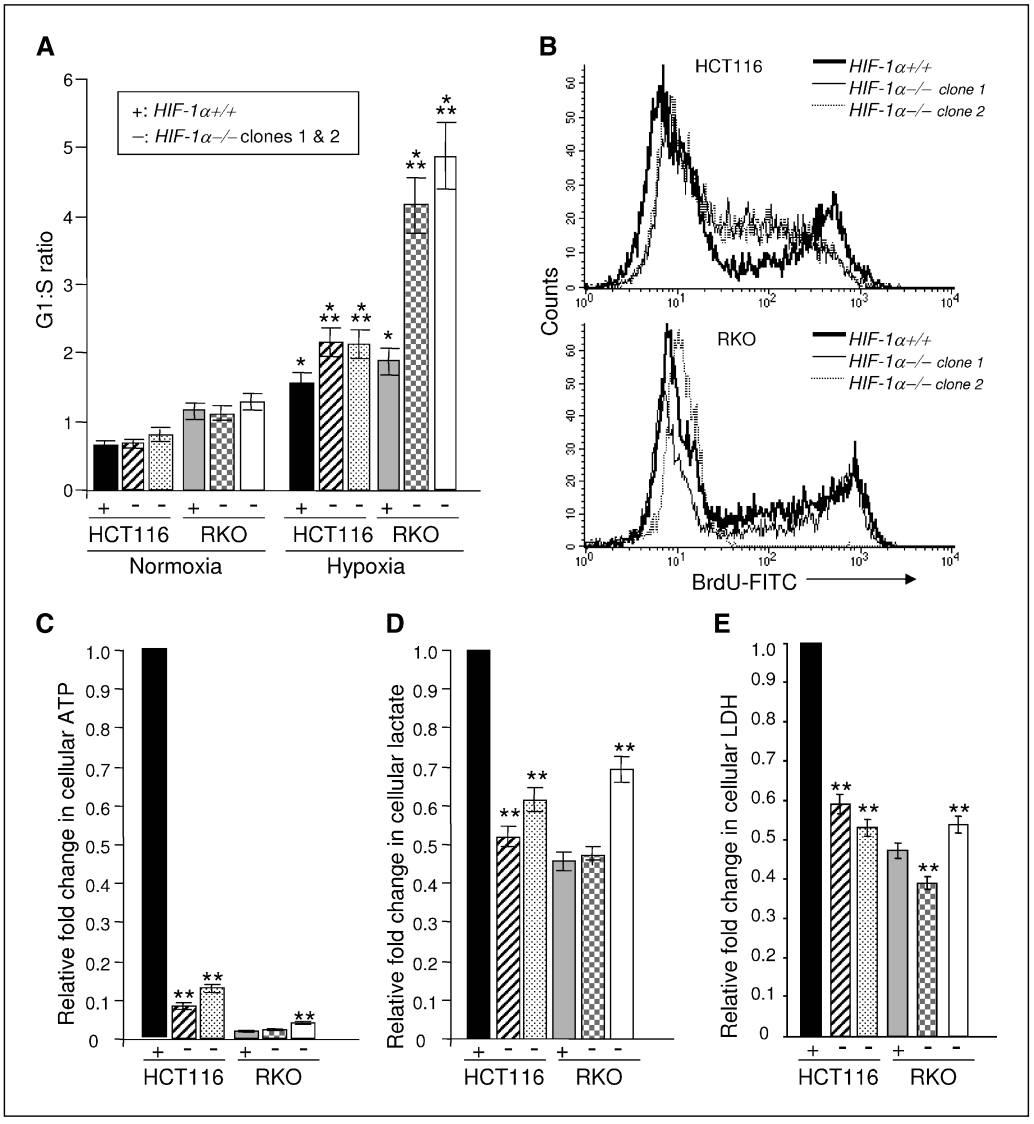

Figure 3. DNA content, DNA synthesis, and glycolysis in *HIF-1α*$^{+/+}$ and *HIF-1α*$^{-/-}$ cells. *A,* G_1-S ratios in cells under normoxia and hypoxia. DNA content and cell cycle analyses were done on 10,000 cells, G_1-S ratios were calculated, and averages were graphed ($n = 3$). *, $P < 0.01$, comparing the same cell line in hypoxia versus normoxia; **, $P < 0.01$, comparing *HIF-1α*$^{-/-}$ with *HIF-1α*$^{+/+}$ clones under the same culture conditions. *B,* BrdUrd incorporation in cells under normoxia. Cell-associated BrdUrd-FITC fluorescence intensity and distribution are plotted for each cell line. There is a shift in BrdUrd uptake in both HCT116$^{HIF\text{-}1\alpha-/-}$ clones. *C,* change in intracellular ATP levels. Intracellular [ATP] was measured and graphed relative to intracellular [ATP] in HCT116 cells ($n = 9$). *D,* change in intracellular lactate levels. Intracellular [lactate] was measured and graphed relative to intracellular [lactate] in HCT116 cells ($n = 9$). *E,* change in intracellular LDH levels. Intracellular [LDH] was measured and graphed relative to intracellular [LDH] in HCT116 cells ($n = 9$).

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glycolytic enzymes in turn may have a role in the cell cycle (45). Recent reports have indicated that HIF-1α may be activated by aerobic glycolysis (8, 46).

We examined glycolysis under normoxic culture conditions by measuring ATP, lactate, and LDH. We found that loss of HIF-1α induced significant decreases in intracellular ATP, lactate, and LDH in HCT116 but not RKO cells (Fig. 3*C-E*). These findings provide direct evidence that HIF-1α mediates aerobic glycolysis in HCT116 but not RKO cells. Taken together, these series of *in vitro* experiments reaffirm the notion that HIF-1α mediates cell proliferation and survival under hypoxia. Extending on the literature, these *in vitro* studies directly show that HIF-1α is essential for aerobic glycolysis and associated proliferation in HCT116 cells.

Disruption of *HIF-1α* impairs HCT116 but not RKO xenograft growth. To determine how disruption of *HIF-1α* affected tumors, we implanted parental and *HIF-1α*$^{-/-}$ cells into the flanks of athymic nude mice and examined the ensuing xenografts. HCT116$^{HIF\text{-}1\alpha-/-}$ xenografts exhibited marked delays in growth compared with HCT116 xenografts (Fig. 4*A*). After 18 days of growth, HCT116$^{HIF\text{-}1\alpha-/-}$ xenograft volumes were up to 8-fold less than HCT116 xenograft volumes. In contrast, no significant differences in volumes were noted between RKO$^{HIF\text{-}1\alpha-/-}$ and parental RKO xenografts (Fig. 4*B*). These findings were surprising and suggested that the role of HIF-1α in normoxic cell proliferation *in vitro* may be more predictive of its functional significance *in vivo*.

Disruption of *HIF-1α* impairs proliferation in the nonhypoxic compartments of HCT116 xenografts. It is generally accepted that a tumor is composed of three compartments: the nonhypoxic compartment composed of proliferating cells, the hypoxic compartment composed of quiescent cells, and the anoxic compartment composed of necrotic cells (33, 47). To distinguish the role of HIF-1α in these tumor compartments, we determined tumor microvessel density (MVD), blood flow, functional hypoxia, and cellular proliferation. MVD was determined by staining with antibodies to the endothelial cell marker CD31. Tumor blood flow was determined by the diffusion of i.v. given Hoechst 33342. Functional hypoxia was determined by tumor uptake of the hypoxia marker pimonidazole. Cell proliferation was determined by cell incorporation of the DNA synthesis marker BrdUrd.

Consistent with a previous report (16), the presence or absence of HIF-1α did not significantly affect MVD as measured by CD31 staining or tumor blood flow as measured by Hoechst 33342 uptake (Fig. 4*C*, *rows 1* and *2*). As such, the areas of functional hypoxia, as determined by pimonidazole staining, were equivalent in *HIF-1α*$^{+/+}$ and *HIF-1α*$^{-/-}$ xenografts (Fig. 4*C, row 4*). Consistent with our *in vitro* data that hypoxia induced G_1-S arrest, the pimonidazole-positive cells did not incorporate BrdUrd, which indicated that they were concomitantly quiescent (*asterisks* in Fig. 4*C, row 4* versus *row 5*). These findings allude to the possibility that HIF-1α expression may not maintain or expand the hypoxic compartment within tumors *in vivo*.

We next determined whether HIF-1α expression affected the nonhypoxic compartment of xenografts. The nonhypoxic compartments were mapped according to two variables: the active incorporation of BrdUrd and the concomitant lack of pimonidazole incorporation (33, 41, 47). Compared with parental HCT116 xenografts, HCT116$^{HIF\text{-}1\alpha-/-}$ xenografts had decreased proliferation within the nonhypoxic compartments as shown by significantly diminished BrdUrd uptake in the pimonidazole-negative regions (Fig. 4*C, row 5*). In contrast, there was equivalent BrdUrd incorporation and proliferation within the nonhypoxic compartments of RKO and RKO$^{HIF\text{-}1\alpha-/-}$ xenografts (Fig. 4*C, row 5*). Thus, HIF-1α promotes proliferation in the nonhypoxic compartments of HCT116 but not RKO xenografts.

Altogether, these series of *in vivo* experiments show that in a subset of colon cancers, HIF-1α is essential for cell proliferation within the nonhypoxic compartments of tumors. Surprisingly, HIF-1α may not be essential for maintaining the hypoxic compartment of tumors *in vivo*. This observation is particularly perplexing, given the fact that HIF-1α was essential for cell proliferation and survival under hypoxic conditions *in vitro*. A potential explanation is that the regions of hypoxia within a tumor are small. Thus, perturbations in these small regions of hypoxia may not significantly affect tumor volume. If this were the case, then expanding the hypoxic compartments within the xenografts may unveil a more significant contribution from HIF-1α.

Disruption of *HIF-1α* and *VEGF* in HCT116 and RKO xenografts. To determine whether expansion of the tumor hypoxic compartments would alter the growth of HIF-1α-deficient xenografts, we ablated *VEGF* gene expression. As VEGF is a dominant tumor angiogenic factor (48, 49), we reasoned that loss of VEGF would effectively inhibit tumor angiogenesis, thus converting tumor nonhypoxic compartments to hypoxic and anoxic compartments. We proceeded to disrupt the *VEGF* gene by targeted homologous recombination (Fig. 5*A*). Disruption of VEGF was confirmed by genomic locus-specific PCR (data not shown). Loss of VEGF was confirmed by ELISA and loss of HIF-1α was reconfirmed by Western blot analyses (Fig. 5*B* and *C*). *In vitro*, presence or absence of VEGF did not alter cell proliferation (data not shown).

Disruption of *HIF-1α* does not alter the hypoxic compartments within *VEGF*-disrupted xenografts. As expected, HCT116$^{VEGF-/-}$ and RKO$^{VEGF-/-}$ xenograft volumes were decreased by 5- to 10-fold compared with their respective parental HCT116 and RKO xenografts (Fig. 5*D* and *E*). The growth of *VEGF*-disrupted xenografts were suppressed due to expansion of the tumor hypoxic compartments, which is consistent with the extensive literature (50). Indeed, the *VEGF*-disrupted xenografts exhibited up to 90% decreases in tumor MVD and blood flow.[7] This was consistent with the marked increase in tumor regions with pimonidazole uptake (Fig. 4*D* compared with Fig. 4*C*, *row 4*). Thus, as expected, disruption of *VEGF* led to significant expansion of the tumor hypoxic compartments and growth delay.

HCT116$^{VEGF-/-HIF\text{-}1\alpha-/-}$ xenografts were significantly smaller than HCT116$^{VEGF-/-}$, HCT116$^{HIF\text{-}1\alpha-/-}$, and HCT116 xenografts (Fig. 5*D*). However, the tumor hypoxic compartments were equivalent between HCT116$^{VEGF-/-HIF\text{-}1\alpha-/-}$ and HCT116$^{VEGF-/-}$ xenografts (Fig. 4*D*). Thus, HIF-1α did not affect the hypoxic compartments, and again, loss of HIF-1α selectively compromised the nonhypoxic compartment in HCT116 xenografts. As such, the negative effects of *HIF-1α* disruption on nonhypoxia-mediated proliferation were additive with the growth suppressive effects of *VEGF* disruption. Hence, in HCT116 xenografts, the effects of HIF-1α and VEGF are independent and additive. These data provide a mechanism and preliminary "proof-of-principle" that inhibition of HIF-1α is additive with inhibition of VEGF. Furthermore, these data support the conclusion that HIF-1α is essential for the nonhypoxic rather than the hypoxic compartments in HCT116 xenografts.

[7] D.T. Dang and L.H. Dang, unpublished data.

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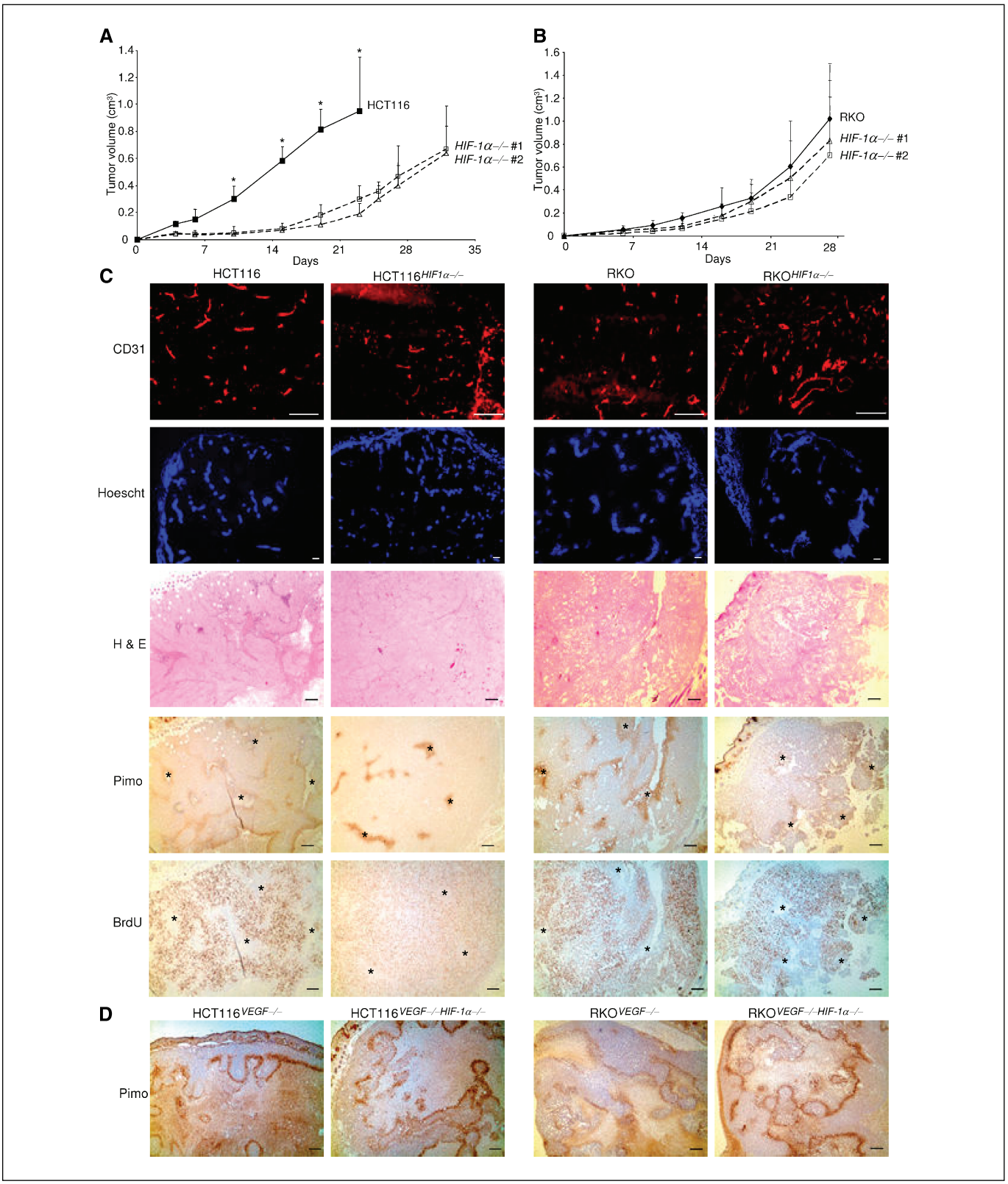

Figure 4. *In vivo* tumorigenesis in *HIF-1α*-disrupted xenografts. *A* and *B,* xenograft growth after implantation of 5.0 × 10^6 HCT116 and HCT116$^{HIF\text{-}1\alpha\,-/-}$ cells (*A*; *n* = 20) or RKO and RKO$^{HIF\text{-}1\alpha\,-/-}$ cells s.c. (*B*; *n* = 20). *, $P < 0.01$, comparing parental with both clones of *HIF-1α*$^{-/-}$ xenografts at the same time point. *C,* immunohistochemistry of HCT116, HCT116$^{HIF\text{-}1\alpha\,-/-}$, RKO, and RKO$^{HIF\text{-}1\alpha\,-/-}$ xenografts. *Row 1,* CD31 staining for MVD (*red*); *row 2,* Hoechst 33342 perfusion as a measure of tumor blood flow (*blue*); *row 3,* H&E staining; *row 4,* pimonidazole (*Pimo*) staining for hypoxic regions (*brown*); *row 5,* BrdUrd incorporation in proliferating cells (*brown*). The sections in *rows 3* to *5* are serial sections. *Asterisks,* examples of concordant regions of pimonidazole staining (hypoxia) that do not stain with BrdUrd (no proliferation). Bar, 200 μm. *D,* pimonidazole staining for functional hypoxia in *VEGF*$^{-/-}$ and *VEGF*$^{-/-}$*HIF-1α*$^{-/-}$ knockout xenografts. *Brown areas,* functional hypoxia. Bar, 200 μm.

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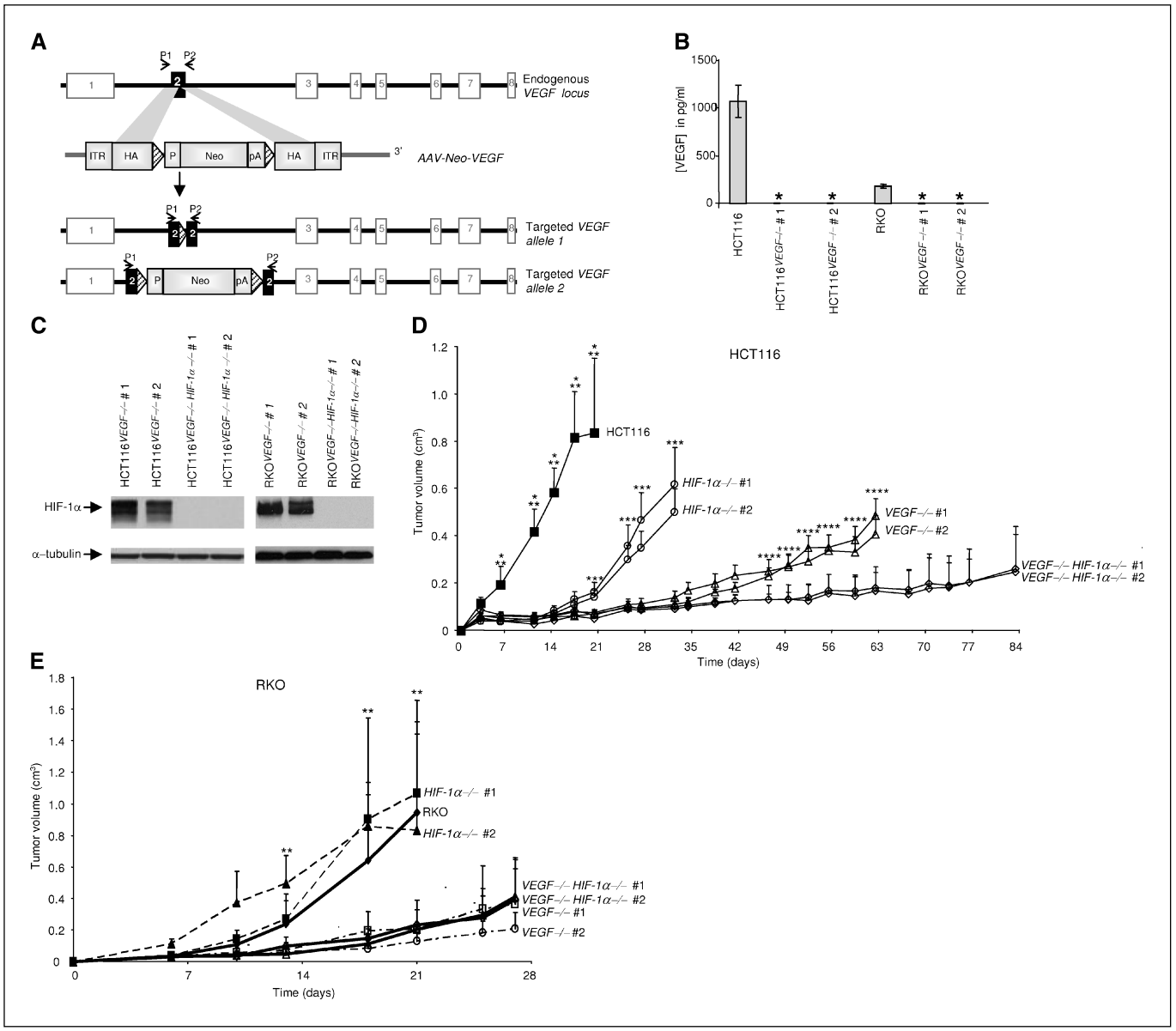

Figure 5. Disruption of *VEGF* and *in vivo* tumorigenesis. *A,* disruption of *VEGF*. Endogenous *VEGF* locus, AAV knockout construct, and resulting targeted locus. *Numbered boxes,* exons; *black boxes,* targeted exon 2; *ITR,* inverted terminal repeats; *HA,* homology arm; *P,* SV40 promoter; *Neo,* neomycin-resistance gene; *pA,* polyadenylate tail; *striped triangles,* loxP sites. *B,* confirmation of loss of VEGF by ELISA. *C,* confirmation of loss of HIF-1α by Western blot analysis with anti-human HIF-1α and anti-human α-tubulin antibodies (for loading). *D* and *E,* xenograft growth after implantation of 5.0×10^6 HCT116, HCT116$^{HIF\text{-}1\alpha\,-/-}$, HCT116$^{VEGF\,-/-}$, and HCT116$^{VEGF\,-/-\,HIF\text{-}1\alpha\,-/-}$ cells (*D*; $n = 20$) or RKO, RKO$^{HIF\text{-}1\alpha\,-/-}$, RKO$^{VEGF\,-/-}$, and RKO$^{VEGF\,-/-\,HIF\text{-}1\alpha\,-/-}$ cells (*E*; $n = 20$). *, $P < 0.01$, comparing parental cells with both clones of *HIF-1α*$^{-/-}$ xenografts at the same time point; **, $P < 0.01$, comparing parental cells with both clones of *VEGF*$^{-/-}$ xenografts at the same time point; ***, $P < 0.01$, comparing *HIF-1α*$^{-/-}$ xenografts with *VEGF*$^{-/-}$*HIF-1α*$^{-/-}$ xenografts at the same time point; ****, $P < 0.01$, comparing *VEGF*$^{-/-}$ xenografts with *VEGF*$^{-/-}$*HIF-1α*$^{-/-}$ xenografts at the same time point.

Despite the expanded hypoxic compartments within RKO$^{VEGF-/-}$ compared with RKO xenografts (Fig. 4*D* versus Fig. 4*C, row 4*), the presence or absence of HIF-1α did not alter tumor pimonidazole uptake or volume (Figs. 4*D* and 5*E* respectively, RKO$^{VEGF-/-HIF\text{-}1\alpha-/-}$ versus RKO$^{VEGF-/-}$). These findings support the conclusion that HIF-1α may not be essential for the gross maintenance of tumor hypoxic compartments.

Discussion

As with previous studies that report the effects of *HIF-1α* using established cell lines, our data should be interpreted within the tumor-specific genetic alterations of the cells studied. It is also important to note that although our findings within the *in vivo* nonhypoxic compartments are consistent with our *in vitro* findings under normoxia, it remains unknown how *in vivo* and *in vitro* oxygen tensions may correlate. With these caveats in mind, several novel conclusions can be drawn from this study.

First, HIF-1α is a positive regulator of cell proliferation in HCT116 cells grown in normoxic culture *in vitro*. Furthermore, HIF-1α mediates the Warburg effect in HCT116 cells. To our knowledge, these are the first data to directly demonstrate the role of HIF-1α on aerobic glycolysis and cell proliferation in a

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human colon cancer cell line. These findings are consistent with the literature that HIF-1α is a positive growth factor that is regulated by oncogenic mutations.

Second, HIF-1α is a positive regulator of cell proliferation within the nonhypoxic compartments of HCT116 xenografts *in vivo*. Furthermore, in these tumors, disruption of *HIF-1α* is additive with disruption of *VEGF* in suppressing growth. To our knowledge, these are the first data to demonstrate the role of HIF-1α in the proliferative compartment of certain tumors. Furthermore, the biological effects of HIF-1α and VEGF can be independent and additive.

Third, although HIF-1α is absolutely essential for cell proliferation and survival under hypoxia *in vitro*, it does not grossly contribute to the bulk of the hypoxic compartments in this subset of xenografts. That HIF-1α is essential for cell proliferation and survival under hypoxia *in vitro* is consistent with previous reports. The finding that HIF-1α does not grossly contribute to the hypoxic compartment *in vivo* would suggest that either compensatory or cooperating mechanisms are activated in these tumors. Thus, other factors may need to be disrupted to appreciate the global effects of HIF-1α within the hypoxic compartments of xenografts.

A potential clinical implication from these data is that therapy with anti-HIF-1α agents may be effective against tumors in which HIF-1α contributes to nonhypoxic cell proliferation. Moreover, in this subset of tumors, anti-HIF-1α therapy may be additive with antiangiogenic therapy. Certainly, further investigations are warranted and ongoing. The mechanisms by which HIF-1α regulates aerobic glycolysis and cell proliferation are not fully delineated. The cell-specific mechanisms that render a cell dependent or independent on HIF-1α remain to be determined. The mechanisms by which cells compensate in the absence of HIF-1α are not yet identified. Nevertheless, these are the first data to directly show that HIF-1α regulates nonhypoxia-mediated proliferation in colon cancer cells and xenografts.

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