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Research Article

Hypoxia-Inducible Carbonic Anhydrase IX and XII Promote Tumor Cell Growth by Counteracting Acidosis through the Regulation of the Intracellular pH

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Abstract

Acidosis of the tumor microenvironment is typical of a malignant phenotype, particularly in hypoxic tumors. All cells express multiple isoforms of carbonic anhydrase (CA), enzymes catalyzing the reversible hydration of carbon dioxide into bicarbonate and protons. Tumor cells express membrane-bound CAIX and CAXII that are controlled via the hypoxia-inducible factor (HIF). Despite the recognition that tumor expression of HIF-1α and CAIX correlates with poor patient survival, the role of CAIX and CAXII in tumor growth is not fully resolved. To understand the advantage that tumor cells derive from expression of both CAIX and CAXII, we set up experiments to either force or invalidate the expression of these enzymes. In hypoxic LS174Tr tumor cells expressing either one or both CA isoforms, we show that (*a*) in response to a "CO_2 load," both CAs contribute to extracellular acidification and (*b*) both contribute to maintain a more alkaline resting intracellular pH (pH_i), an action that preserves ATP levels and cell survival in a range of acidic outside pH (6.0–6.8) and low bicarbonate medium. *In vivo* experiments show that *ca9* silencing alone leads to a 40% reduction in xenograft tumor volume with up-regulation of *ca12* mRNA levels, whereas invalidation of both CAIX and CAXII gives an impressive 85% reduction. Thus, hypoxia-induced CAIX and CAXII are major tumor prosurvival pH_i-regulating enzymes, and their combined targeting shows that they hold potential as anticancer targets. [Cancer Res 2009;69(1):358–68]

Introduction

Adaptation of tumor cells to hypoxia and acidosis is a critical driving force in tumor progression and metastasis (1, 2). Cancer cells produce a large amount of lactic acid (3), which is generated through glucose metabolism and inefficient vascular clearing, resulting in an acidic microenvironment within many solid tumors (4). Extracellular acidosis represents a threat to cell survival by modifying the intracellular pH (pH_i), wherein a 0.1 pH_i variation can disrupt multiple biological functions, including ATP production, protein synthesis, cell proliferation, migration, and apoptosis (5–7). Because numerous intracellular processes require close regulation of pH_i, most mammalian cells, particularly hypoxic tumor cells, have developed key strategies to regulate their pH_i. Activation of the hypoxia-inducible factor-1 (HIF-1) in hypoxia plays a major role in regulating pH homeostasis by enhancing expression of membrane located transporters, exchangers, pumps and ecto-enzymes (8). To survive in an acidic environment, the pH_i-regulating system of tumor cells actively extrudes acids via the growth factor–activated Na^+/H^+ exchanger 1 (NHE-1; refs. 9–12) and the monocarboxylate transporters (MCT1 and MCT4; ref. 13). We showed previously that NHE-1 plays a key role in tumor development particularly for cells producing large amounts of lactic acid (14). In the opposite direction to H^+ extrusion, HCO_3^- influx through Na^+-HCO_3^- cotransporters (NBC) and Cl^-/HCO_3^- exchangers (AE) contributes to cytoplasmic alkalinization (15–17).

Carbonic anhydrases (CA), which catalyze the reversible hydration of cell-generated carbon dioxide into protons and bicarbonate ions, have also been proposed to contribute to cellular alkalinization (18–20). The direction of the reaction is dependent on the form, CO_2 or bicarbonate and protons, that predominates. Mammalian cells express 13 active isoforms of CAs, with a conserved active site and variable levels of activity, and 3 inactive isoforms. They differ in their tissue distribution and cellular localization. The expression of the membrane-associated CAIX and CAXII is tightly controlled by oxygen levels in multiple epithelial tumor types (21–23), and CAIX has a higher extracellular activity than CAXII (23–25). CAIX is highly induced in an HIF-1–dependent manner (26) and is constitutively expressed in von Hippel-Lindau (VHL)–defective cells. CAXII is up-regulated in VHL-defective renal tumors and induced in hypoxia in tumor cells, but its dependence on HIF is not well established (22). Whereas tumor expression of HIF-1α and CAIX correlate with poor patient survival (19, 27), the significance of CAXII, which lacks the extracellular proteoglycan domain of CAIX implicated in cell adhesion (28–30), is less obvious (24). CAIX may be functionally linked to the regulation of the tumor pH, because it contributes to extracellular acidification (31) and forced CAIX expression in three-dimensional cultured aggregates influences pH_i homeostasis (32). However, direct evidence of the conjugated roles of CAIX and CAXII in pH_i regulation in cell lines and in tumor growth is still missing.

In this study, we show that the hypoxia-inducible CAIX and CAXII proteins promote cell survival and growth through pH_i maintenance. We conclude that CAIX and CAXII constitute a robust pH_i-regulating system able to confer a tumor growth and survival advantage on cells exposed to a hypoxic and acidic microenvironment. Finally, as very often hypothesized, but not

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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shown (23, 24), we validate here that CAIX and CAXII constitute two new anticancer therapeutic targets.

Materials and Methods

Cell Culture and Hypoxic Exposure

Chinese hamster lung CCL39 fibroblasts (American Type Culture Collection) and the CCL39-derived mutant PS120 cells, lacking the amiloride-sensitive Na^+/H^+ exchanger (6), were maintained in DMEM (Sigma) supplemented with 7.5% FCS in a humidified atmosphere of 5% CO_2, 95% air, or 100% air at 37°C. The colon adenocarcinoma cell line LS174Tr expressing the tetracycline (Tet) repressor was provided by Dr. van de Wetering (33) and maintained in DMEM supplemented with 10% FCS. Other human tumor cell lines were likewise cultured. Incubation in hypoxia at 1% O_2 was carried out at 37°C in 95% humidity and 5% CO_2/94% N_2 in a sealed anaerobic workstation (Ruskinn).

Plasmids

Full-length human *ca9* cDNA was obtained from hypoxic HeLa mRNA extracts by PCR using the following specific primers: forward 5′-CGGGGTACCGCCGCCACCATGGCTCCCCTGTGCCCC-3′ and reverse 5′-GCTCTAGACTAGGCTCCAGTCTCGGC-3′. *ca9* cDNA was ligated into the pTREX-A (pcDNA4/TO/myc-His A; Invitrogen) vector (p*ca9*) between the *Kpn*I and *Xba*I sites. The short hairpin RNA (shRNA)–*ca9* (sh*ca9*) was obtained with oligonucleotide sequences forward 5′-AGTTAAGCCTAAAT-CAGAA-3′ and reverse 5′TTCTGATTTAGGCTTAACT-3′ and inserted into the pTER vector. The shRNA–*hif-1α* (sh*hif-1α*) was obtained with oligonucleotide sequences forward 5′-CTGATGACCAGCAACTTGA-3′ and reverse 5′-TCAAGTTGCTGGTCATCAG-3′ and inserted into the pTER vector. Lentivirus particles for two independent sequences (1 and 2) of pLKO.1-Puro Vector shRNA targeting *ca12* (*ca12*$^-$) and nontarget shRNA (*ctl*) were from Sigma (TRCN0000116249, TRCN0000116251, and SHC002V).

Stable Transgenic Cells

CCL39, PS120, and LS174Tr cells were transfected with p*ca9*, whereas only LS174Tr cells were transfected with sh*ca9* or sh*hif-1α*, using Polyfectamine (HiPerFect Transfection Reagent, Qiagen) according to the manufacturer's instructions. Isolated clones were maintained under zeocin (500 μg/mL, Invitrogen). Tet (10 μg/mL) induces CAIX expression or *ca9* or *hif-1α* silencing. LS-sh*ca9* cells were also transduced with lentiviral particles containing shRNA-*ca12* (*ca12*$^-$; Sigma) or nontarget shRNA (*ctl*) according to the manufacturer's instructions and named, respectively, LS-sh*ca9/ca12*$^-$ and LS-sh*ca9/ctl*.

RNA Extraction and Relative and Absolute Real-Time Quantitative PCR

Total RNA was extracted from cells using the RNA extraction kit (Qiagen) according to the manufacturer's instructions. Total RNA (2 μg) was added to a 20 μL reverse transcription–PCR reaction using the Omniscript kit (Qiagen). The relative expression level of *ca9* and *ca12* was quantified by real-time quantitative PCR (qPCR), as reported previously (26). The absolute quantification of *ca9* and *ca12* mRNA was obtained by absolute real-time quantification. A standard curve was prepared using dilutions of the pTREX-*ca9* and pTREX-*ca12* vectors from 30 to 3×10^8 copies in triplicate. The cycle number of *ca9* or *ca12* amplification of each extract was compared with the standard curve obtained respectively with pTREX-*ca9* or pTREX-*ca12* vectors.

Immunoblotting

Cells were lysed in SDS sample buffer. Proteins (40 μg) were separated on 7.5% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blotted with the M75 antibody to CAIX (Bayer), Hsp90 (Abcam), a polyclonal antibody to recombinant CAXII (Sigma), and a polyclonal antibody to HIF-1α prepared and validated in our laboratory (34). Immunoreactive bands were detected with a horseradish peroxidase (HRP) anti-mouse or anti-rabbit antibody (Promega) by enhanced chemiluminescence (Amersham Biosciences).

Immunofluorescence

Cells at sparse density were grown on glass coverslips and fixed with 3% paraformaldehyde for 30 min followed by saturation for 30 min in PBS–2% gelatin and PBS–1% bovine serum albumin. Cells were then incubated for 1 h with the CAIX antibody without permeabilization, followed by incubation for 1 h with an anti-mouse Alexa 594–conjugated IgG antibody (Invitrogen). Cells were mounted onto slides with citifluor and analyzed with a Leica microscope (objective, 100×).

In vitro Determination of CA Activity

Cells incubated in normoxia (PS120-p*ca9* cells) or hypoxia (to induce CAIX and CAXII expression in LS174Tr cells) were placed on ice in normoxia, scrapped into ice-cold PBS, to obtain intact membrane-bound CAIX and CAXII. The cell suspension was immediately centrifuged and resuspended in a bicarbonate-free medium (Sigma) buffered at outside pH (pH_o) 7.4 with 30 mmol/L HEPES. A 0.1 volume of this cell suspension was added to a 3 mmol/L HEPES-buffered solution (HBS) adjusted to pH_o 8.2 before rapid addition to a CO_2-saturated nonbuffered solution and pH determined over time (microelectrode, Schott Instrument) to monitor the rapid hydration of CO_2 to carbonic acid. For inhibition of the total CA activity, 100 μmol/L acetazolamide (ACTZ; Sigma) was added to the cell suspension in normoxia 15 min before the experiment.

Resting pH_i Measurement

[^{14}C]Benzoic acid. The pH_i was measured using the technique of distribution of the weak acid [7-^{14}C]benzoic acid (Amersham Biosciences) in intracellular and extracellular spaces for exponentially growing cells (35). Diisothiocyanatostilbene-2′,2-disulfonic acid (DIDS; 1 mmol/L, Sigma) was used as an inhibitor of HCO_3^- transporters.

BCECF-AM probe. Exponentially growing cells seeded on glass coverslips were incubated for 25 min in bicarbonate-free HBS (pH_o 7.4), MES-buffered solution (MBS; pH_o 6.6), or bicarbonate-buffered solution (BBS; adjusted to pH_o 7.4). The pH-sensitive fluorescent dye BCECF-AM 1 μmol/L (Sigma) was then added for 5 min at room temperature. Cells were then quickly washed with the appropriate HBS, MBS, or BBS solution and transferred to a laminar flow cell chamber perfused with the same solution. Ratiometric measurement of the fluorescence of 50 randomly selected individual cells per coverslip was performed in a workstation (Acquacosmos). The pH_i was estimated by an *in situ* two-point calibration (pH_o 6.6–7.6) with perfusion of a high K^+ buffer solution containing 130 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L glucose in 20 mmol/L HEPES-Tris (or MES-Tris), and 25 μmol/L nigericin to allow pH_i to equilibrate with the external pH_o.

DNA Synthesis

Cells (5,000) were plated on 96-well dishes before transfer to a CO_2-free incubator for 24 h in HCO_3^-–free DMEM buffered with 30 mmol/L MES or HEPES adjusted to different pH_o (6.2–7.4), supplemented with 10% dialyzed serum, hypoxanthine (0.1 mmol/L), and UTP (0.1 mmol/L). DNA synthesis was measured using an ELISA colorimetric kit (Roche Diagnostics) based on a 2-h incorporation of BrdUrd (20 μmol/L).

ATP Determination

Cells were seeded in DMEM and grown at different pH_o, as described for DNA synthesis. ATP levels were measured using a Cell Titer Glo kit (Promega) according to the manufacture's instructions. The relative luminescence unit was normalized to the quantity of protein.

Clonogenicity Assay

Cells (1,000) were seeded onto 60-mm dishes. Once attached, the medium was replaced by HCO_3^-–free DMEM buffered at pH_o 6.4 (30 mmol/L MES) or at pH_o 7.4 (30 mmol/L HEPES), supplemented with 10% dialyzed serum, hypoxanthine (0.1 mmol/L), and UTP (0.1 mmol/L) for growth in the absence of CO_2/HCO_3^- and transferred to a CO_2-free atmosphere for 24 h. Dishes were then returned to 5% CO_2 in a regular media for 7 d before staining with Giemsa (Fluka).

Cell Proliferation in Three Dimensions

To grow spheroids, 1,600 cells were seeded in drops in 20 μL of HCO_3^-–free DMEM buffered with 30 mmol/L HEPES adjusted to pH_o 7.4 supplemented with 10% FCS. After 12 d, spheroids were collected and cells

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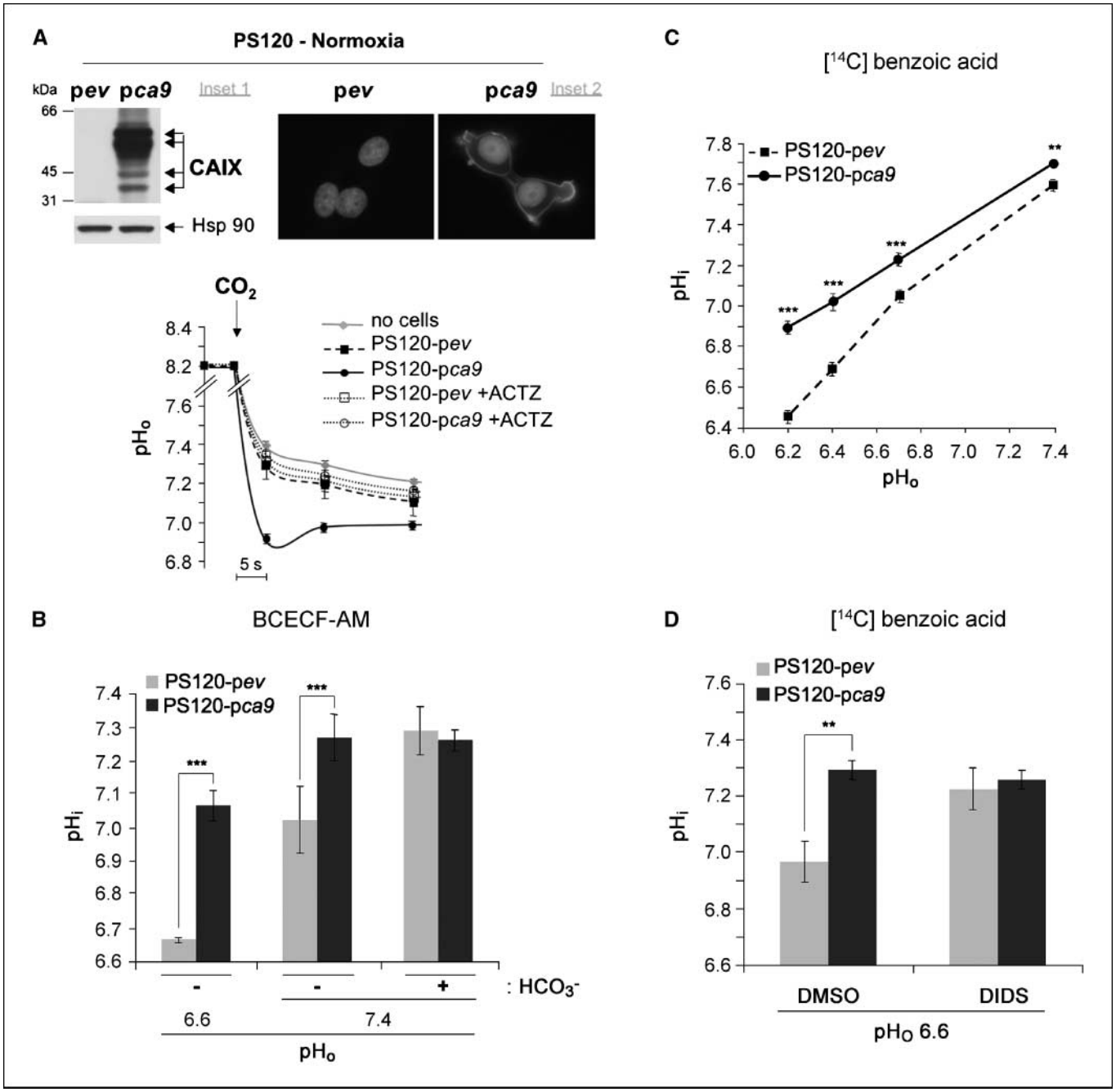

Figure 1. Forced expression of catalytically active CAIX induced extracellular acidification and cytoplasmic alkalinization. *A, top,* stable expression of human CAIX in nonneoplastic PS120 fibroblasts lacking the Na^+/H^+ exchanger (NHE-1). *Inset 1,* immunoblot to CAIX of lysates of control, empty plasmid vector (PS120-p*ev*), or human CAIX stable expressing cells (PS120-p*ca9*). The Hsp90 protein level was used as a loading control. *Inset 2,* immunofluorescence to CAIX in PS120-*pev* and PS120-p*ca9* cells. *Bottom,* rate of extracellular acidification in response to a CO_2 load without cells (*no cells*) or with samples of intact normoxic PS120-p*ev* or PS120-p*ca9* cells. The rate of acidification reflects an estimation of the CA activity at the cell surface evaluated with or without a specific inhibitor of CAs (100 μmol/L ACTZ). *B,* CAIX expression increases the resting pH_i. pH_i of PS120-p*ev* and PS120-p*ca9* cells was determined with pH-sensitive fluorescent dye BCECF-AM. Histograms indicate the mean basal pH_i for cells incubated for 30 min in either a 25 mmol/L HCO_3^--buffered solution (+HCO_3^-) or HCO_3^-–free MES or HBS (-HCO_3^-) adjusted to a pH_o of 6.6 or 7.4, respectively. Just before their transfer to a laminar flow cell chamber, BCECF-AM was added to the cells for 5 min. Cells were then perfused with the respective solutions, and a three-point calibration curve was created with high KCl/nigericin solutions to measure pH_i as a function of the fluorescence ratio. Significant differences based on the Student's *t* test (***, $P < 0.001$; $n = 200$ cells for each PS120-p*ev* and PS120-p*ca9* cells at pH_o 6.6, $n = 3000$ cells for pH_o 7.4 with or without HCO_3^-). In the absence of HCO_3^-, PS120-p*ca9* cells had a statistically higher resting pH_i than PS120-p*ev* cells perfused at either a pH_o of 6.6 or 7.4. *C,* CAIX expression increases pH_i over a range of pH_o. The pH_i was determined in PS120-p*ev* and PS120-p*ca9* cells as a function of pH_o with [^{14}C]benzoic acid. Exponentially growing cells of two independent clones for PS120-p*ca9* and PS120-p*ev* cells were equilibrated for 15 min in a nominally HCO_3^-–free solution with pH_o varying from 6.2 to 7.4. Cells were then shifted for 15 min to the same solution containing [^{14}C]benzoic acid at the specific activity of 1 μCi/mL. pH_i was calculated as described under Materials and Methods. Determinations were done in quadruplicate for each condition, and the experiment was repeated at least thrice. Significant differences are based on the Student's *t* test (**, $P < 0.01$; ***, $P < 0.005$). *D,* DIDS, an inhibitor of HCO_3^- transporters, abolishes the differential in CAIX-induced alkalinization. The steady-state pH_i of PS120-p*ev* and PS120-p*ca9* cells was measured with [^{14}C]benzoic acid. Exponentially growing cells were incubated for 15 min in a nominally HCO_3^-–free MBS at a pH_o of 6.6 without (*DMSO*) or with 1 mmol/L DIDS. Cells were then shifted for 15 min to the same medium containing [^{14}C]benzoic acid at a specific activity of 1 μCi/mL. pH_i was calculated as described under Materials and Methods. The mean of quadruplicate determinations is given. Significant differences are based on the Student's *t* test (**, $P < 0.01$).

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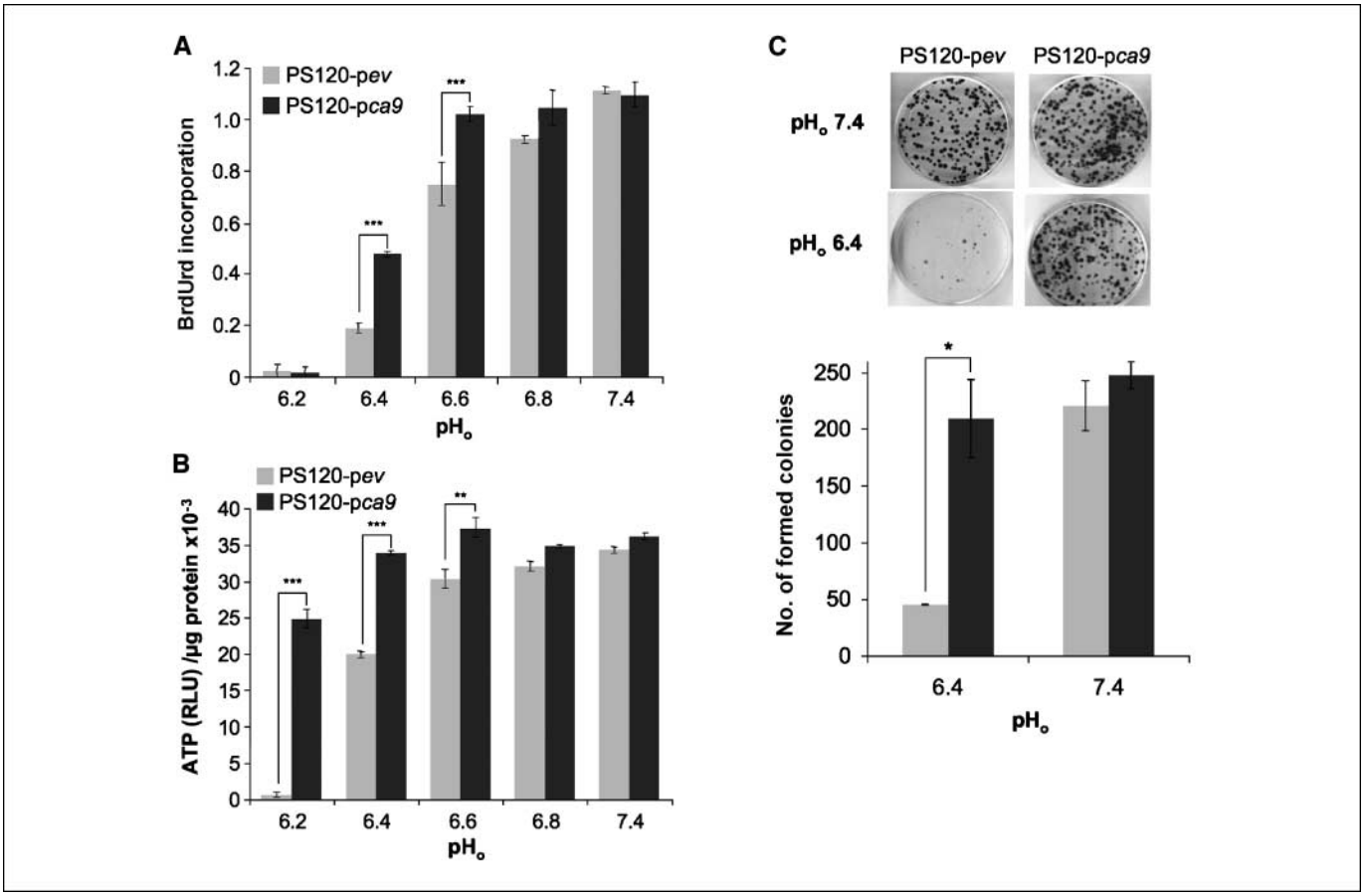

Figure 2. CAIX expression promotes survival and growth of PS120 fibroblasts impaired in NHE-1 *in vitro* in an acidic microenvironment. *A,* proliferation of PS120-p*ev* and PS120-p*ca9* cells (two independent clones for each type) was measured after a 24-h incubation in normoxia in a CO_2/HCO_3^-–free microenvironment for media buffered with either 30 mmol/L MES for a pH_o of 6.2, 6.4, 6.6, and 6.8 or with 30 mmol/L HEPES for a pH_o of 7.4 and supplemented with 100 μmol/L hypoxanthine/100 μmol/L uridine/10% dialyzed FCS. A cell proliferation ELISA colorimetric kit, which measures BrdUrd incorporation into newly synthesized DNA, was used. Data are expressed as an average of three independent experiments performed in triplicate using two independent clones. Significant differences are based on Student's *t* test (***, $P < 0.005$). *B,* quantification of ATP levels in PS120-p*ev* and PS120-p*ca9* cells (two independent clones for each type) grown in normoxia in a CO_2/HCO_3^-–free environment with varying pH_o from 6.2 to 7.4 for 24 h using a luciferin/luciferase-based assay, with results expressed as relative luminescence units. Determinations were done in triplicate, and the entire experiment was done thrice using two independent clones. Significant differences are based on the Student's *t* test (***, $P < 0.005$). *C,* viability assay of PS120-p*ev* and PS120-p*ca9* cells in normoxia as function of pH_o. *Top,* cells (1,000) were seeded in 60-mm dishes. Once attached, cells were incubated in HCO_3^-–free media adjusted to pH_o 6.4 or 7.4 for 24 h in a CO_2-free atmosphere. Fresh medium containing HCO_3^- (40 mmol/L) at pH_o 7.4 was then added to the dishes that were maintained for 10 d in a 5% CO_2 incubator before staining for visualization of the colonies. *Bottom,* the colony number per plate was counted by eye for two independent PS120-p*ca9* and PS120-p*ev* clones. The experiments were repeated thrice using two independent clones. Student's *t* test (*, $P < 0.05$).

were dissociated in Accutase (Life Technologies) to determine the number of individualized living cells.

Nude Mice Tumorigenicity and Immunohistochemistry

Cells (1×10^6) suspended in 500 μL of serum-free DMEM supplemented with insulin-transferrin-selenium (Life Technologies) were s.c. injected into the back of 4-wk-old male athymic mice (Harlan). Animal studies were conducted according to Centre National de la Recherche Scientifique institutional guidelines. Food and water were given *ad libitum*. Doxycycline (Dox; 750 μg/mL; Sigma) was given in the drinking water to induce silencing of *hif-1α* or *ca9*. Five mice were used for each experimental condition. The tumor volume was determined using the formula: $(4\pi / 3) \times L / 2 \times W / 2 \times H / 2$ (L, length; W, width; H, height). When the tumor volume reached ~1,500 mm^3, mice were injected i.p. with hypoxyprobe (60 mg/kg; Chemicon) 4 h before sacrifice. Tumors were collected for RNA, protein, and immunohistochemical analysis, as described (34). Sections were incubated with antibodies to hypoxyprobe or CAXII for 1.5 h followed by incubation with anti-mouse or anti-rabbit IgG-HRP antibodies. Analysis was performed with a Leica microscope (objective, 20×).

Statistical Analysis

The Student's *t* test was used wherein *P* values of <0.05 were considered significant.

Results

Forced expression of catalytically active CAIX-induced extracellular acidification and cytoplasmic alkalinization. Nonneoplastic Chinese hamster CCL39 lung fibroblasts and CCL39-derived PS120 mutant cells defective in the Na^+/H^+exchanger, which do not express endogenous CAIX or CAXII in normoxia or in hypoxia, were selected for expression of human CAIX and examined for their contribution to pH_i with (CCL39 cells) or without (PS120 cells) interference from pH_i regulation by NHE-1. Stable expression of human CAIX in normoxia was found to be plasma membrane located in PS120 (Fig. 1*A*) and CCL39 cells (Supplementary Fig. S1). The level of expression of transfected human CAIX in these fibroblasts in

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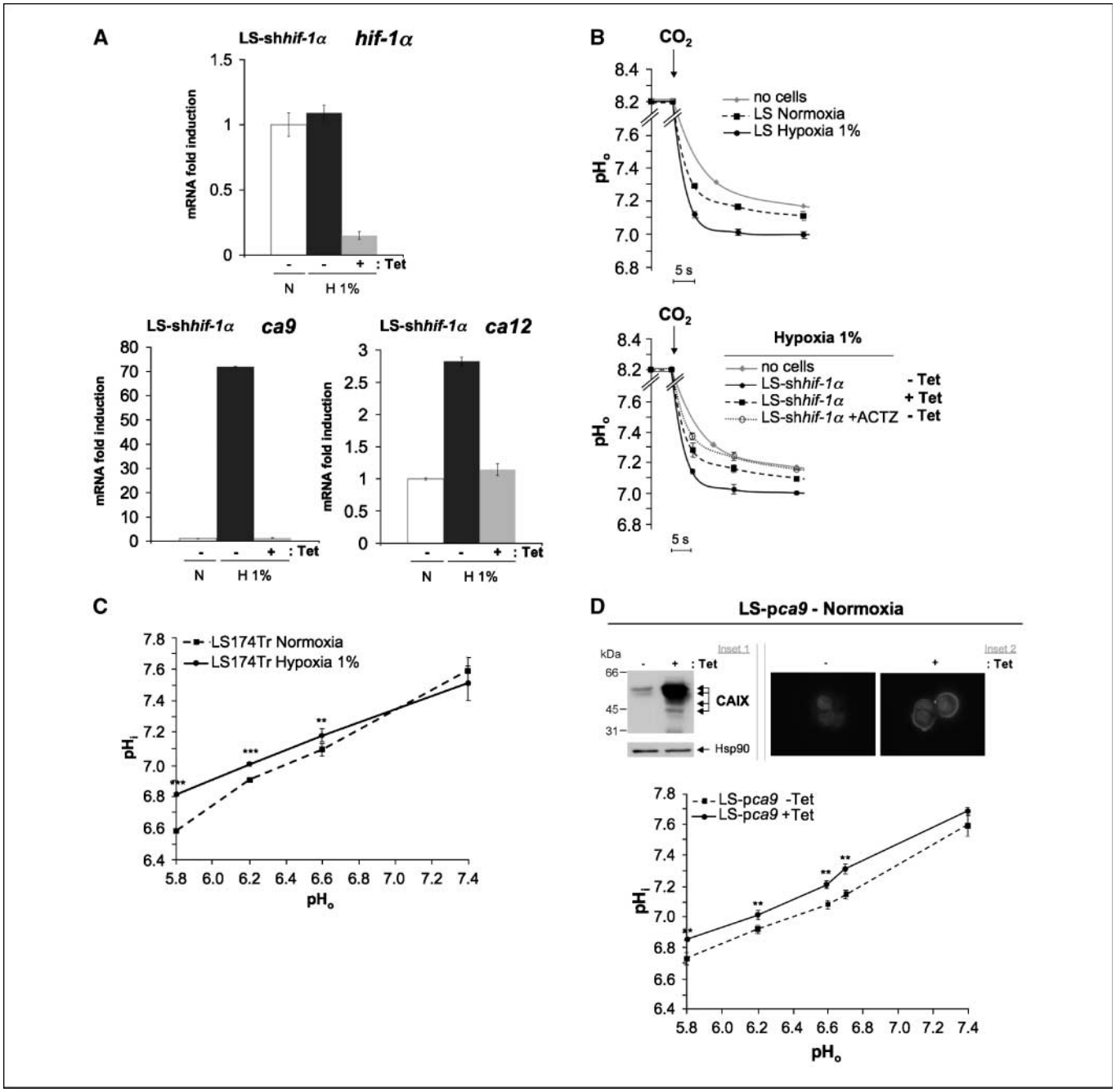

Figure 3. The hypoxia-induced CAIX and CAXII activity contributes to cytoplasmic alkalinization in an acidic microenvironment. *A,* the genes *ca9* and *ca12* are HIF-1–dependent in human colon adenocarcinoma LS174Tr cells. Expression of the mRNA of *hif-1α* (*top*), *ca9*, and *ca12* (*bottom*) was determined by real-time qPCR in the stable LS-sh*hif-1α* clone, in which Tet induces shRNA of *hif-1α*. Cells were incubated without (−*Tet*) or with (+*Tet*) Tet for 4 d and maintained in either normoxia 21% O_2 (*N*) or hypoxia 1% O_2 (*H 1%*) for 48 h. Each difference in gene expression was estimated within the limits of a 95% confidence interval. The results are representative of at least three separate experiments. *B,* hypoxia increases the extracellular CA activity, as reflected in an increase in the rate of extracellular acidification compared with normoxic conditions. *Top,* LS174Tr cells were incubated either in normoxia or hypoxia 1% O_2 for 48 h. The rate of extracellular acidification in response to a CO_2 load was measured without cells (*no cells*) or with intact LS174Tr cells. *Bottom,* The LS-sh*hif-1α* clone was incubated without (−*Tet*) or with (+*Tet*) Tet to allow for *hif-1α* silencing for 4 d before incubation in hypoxia 1% O_2 for 48 h without (−*Tet*) or with (+*Tet*) Tet. The rate of acidification in response to a CO_2 load for intact hypoxic LS-sh*hif-1α* cells silenced (+*Tet*) or not (−*Tet*) for *hif-1α* was measured. ACTZ (+*ACTZ*) at 100 μmol/L was added to a suspension of hypoxic LS-sh*hif-1α* −Tet cells for 15 min before CO_2 addition. *C,* determination of resting pH_i with [^{14}C]benzoic acid as a function of pH_o in LS174Tr cells exposed to either normoxia or hypoxia 1% O_2 for 48 h. Exponentially growing LS174Tr cells incubated in normoxia or hypoxia were equilibrated for 15 min in a nominally HCO_3^-–free HBS or MBS with a pH_o varying from 5.8 to 7.4. Cells were incubated for 15 min in the same appropriate equilibration solution containing [^{14}C]benzoic acid at a specific activity of 1 μCi/mL. The pH_i was calculated as described under Materials and Methods. The experiment was repeated at least thrice, and each point represents the average of quadruplicates for each experiment (**, $P < 0.01$; ***, $P < 0.005$). *D,* stable Tet-inducible expression of CAIX in LS174Tr cells in normoxia maintains a higher pH_i in an acidic and HCO_3^-–free media. *Top, inset 1,* immunoblot of inducible expression of CAIX in normoxia in LS174Tr cells (LS-p*ca9*) without (−*Tet*) or with (+*Tet*) Tet for 4 d. Total extracts were analyzed by immunoblotting with antibodies against CAIX and Hsp90. The latter was used as a loading control. *Inset 2,* immunofluorescence of inducible expression of CAIX expression (LS-p*ca9*) in LS174Tr cells in the absence (−*Tet*) or presence (+*Tet*) of Tet for 4 d. *Bottom,* determination of resting pH_i with [^{14}C]benzoic acid in as a function of pH_o in Tet-inductible LS-p*ca9* clone expressing CAIX in normoxia under treatment for 4 d with (+*Tet*) or without (−*Tet*) Tet. Determinations were done in quadruplicate for each condition, and each experiment was repeated at least thrice. Results represent the average of quadruplicates for each experiment (**, $P < 0.01$; ***, $P < 0.005$).

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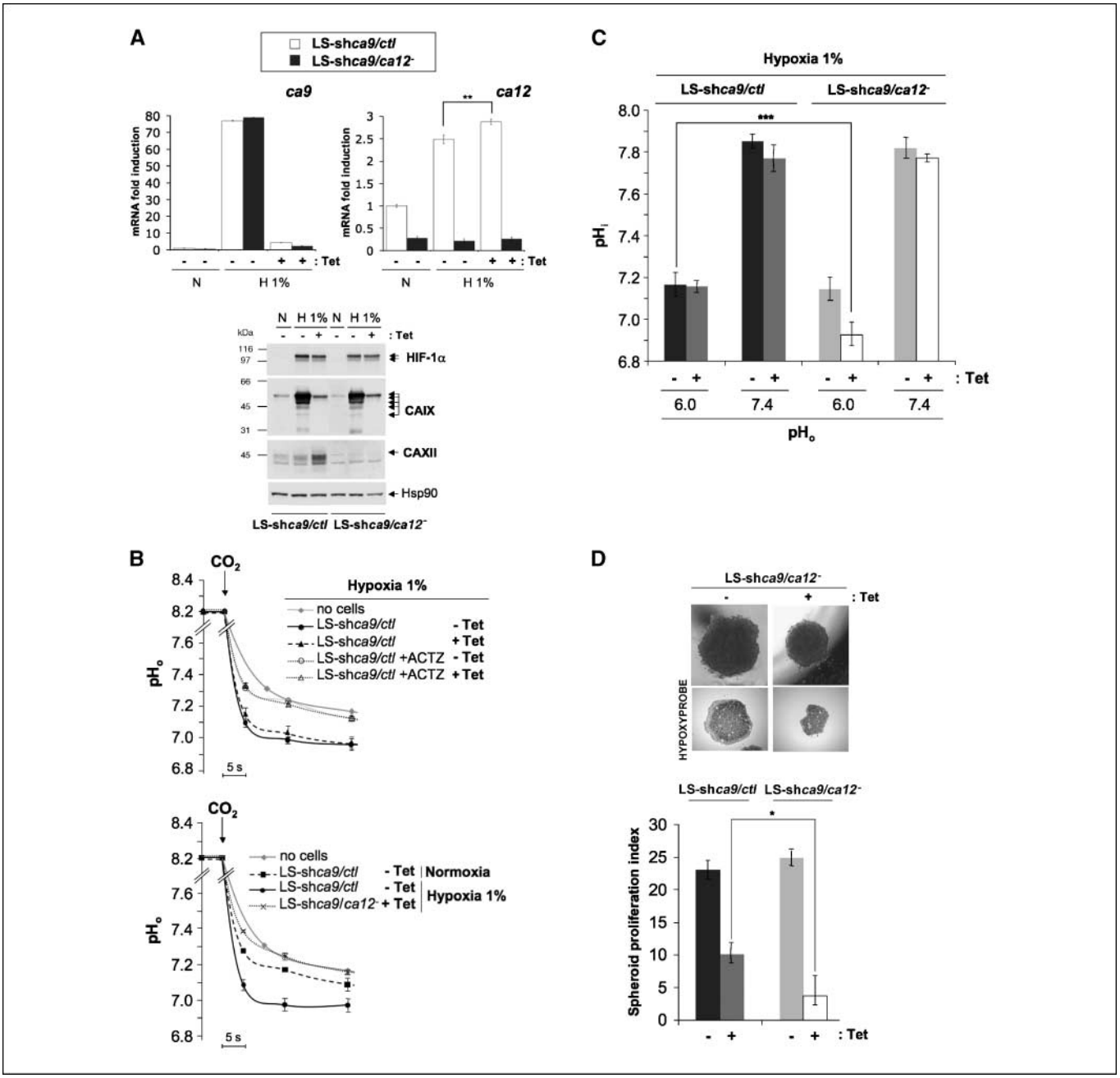

Figure 4. Combined invalidation of CAIX and CAXII reduces pH_i and spheroids growth. *A,* silencing of *ca9, ca12*, and *ca9/ca12* in LS174 cells. *Top left,* expression of the mRNA levels of *ca9* determined by real-time qPCR. Stable LS-sh*ca9/ctl* and LS-sh*ca9/ca12*$^-$ clones were incubated with (−*Tet*) or without (+*Tet*) Tet for 4 d before incubation in either normoxia (*N*) or hypoxia 1% O_2 (*H 1%*) for 48 h. *Top right,* expression of the mRNA levels of *ca12* determined by real-time qPCR under the conditions described below. LS-sh*ca9/ctl* and LS-sh*ca9/ca12*$^-$ clones were incubated with (−*Tet*) or without (+*Tet*) Tet for 4 d before incubation in either normoxia (*N*) or hypoxia 1% O_2 (*H 1%*) for 48 h. The results are representative of at least three separate experiments (**, $P < 0.01$). *Bottom,* total extracts were analyzed by immunoblotting with antibodies against HIF-1α, CAIX, CAXII, and Hsp90. The latter was used as a loading control. *B, top,* rate of acidification of a medium set at pH 8.2 in response to the addition of CO_2 incubated without cells (*no cells*) or with intact LS-sh*ca9/ctl* cells previously incubated for 4 d with (−*Tet*) or without (+*Tet*) Tet and exposed in hypoxia 1% O_2 for 48 h. Control LS-sh*ca9/ctl* −Tet and *ca9* silenced LS-sh*ca9/ctl* +Tet cells were also treated with 100 μmol/L ACTZ 15 min before addition of CO_2. *Bottom,* rate of acidification in response to a CO_2 load of intact LS-sh*ca9/ctl* and LS-sh*ca9/ca12*$^-$ cells incubated without (−*Tet*) or with (+*Tet*) Tet for 4 d and then exposed to either normoxia (*N*) or hypoxia 1% O_2 (*H 1%*) for 48 h. *C,* combined *ca9* and *ca12* invalidation (LS-sh*ca9/ca12*$^-$ +Tet) diminishes resting pH_i in an acidic (pH_o 6.0), MES-buffered, nominally HCO_3^-–free solution. Stable expressing LS-sh*ca9/ca12*$^-$ cells were constitutively invalidated for *ca12*, whereas exponentially growing Tet-inducible LS174Tr (LS-sh*ca9/ctl* and LS-sh*ca9/ca12*$^-$) clones were invalidated for *ca9* after a 4-d treatment with Tet. Cells were then incubated in hypoxic incubation 1% O_2 for 48 h. Dishes were then returned to normoxia for resting pH_i determination in a 20 mmol/L MES-buffered, nominally HCO_3^-–free saline/glucose solution adjusted to pH_o 6.0 or in a 20 mmol/L HEPES-buffered, nominally HCO_3^-–free saline/glucose solution adjusted to pH_o 7.4 (15 min incubation to reach the equilibrium). For pH_i determination, cells were shifted for 15 min to the same medium containing [^{14}C]benzoic acid at a specific activity 1 μCi/mL. The pH_i was calculated as described under Materials and Methods. The experiment was done twice. Each point represents the average of quadruplicates for each experiment (**, $P < 0.01$). *D,* silencing of *ca9* and *ca12* diminished proliferation of three-dimensional spheroids. *Top,* an inducible LS174Tr clone invalidated for *ca12* (LS-sh*ca9/ca12*$^-$ −Tet) or both *ca9* and *ca12* (LS-sh*ca9/ca12*$^-$ +Tet) with a 4-d Tet treatment (+*Tet*) were cultured as spheroids in the absence (−*Tet*) or presence (+*Tet*) of Tet for 12 d. Hypoxyprobe (pimonidazole) was added to the extracellular media for staining of hypoxic zones 4 h before formaldehyde fixation. *Bottom,* spheroids were subjected to Accutase dissociation, and individualized live cells were counted using Trypan blue. The spheroid proliferation index was calculated as the ratio of the cell number counted at day 12 to the cell number at day 0. Data represent the average of three independent experiments.

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normoxia was comparable with the expression of CAIX in tumor cells after 48 h in hypoxia 1% O_2 (data not shown). The CA activity associated with the plasma membrane was determined by the rapid acidification of a minimally buffered medium in response to addition of a CO_2-saturated solution. In the presence of cell suspensions, the rate and magnitude of acidification was higher for CAIX-expressing PS120 cells (Fig. 1*A*) and CAIX-expressing CCL39 cells (Supplementary Fig. S1) than for control cells (p*ev*). The CA inhibitor ACTZ reduced the activity of CAIX-expressing cells to the spontaneous basal level obtained with the control cells with or without ACTZ. In addition, PS120-p*ca9* cells incubated in normoxia or hypoxia (48 h, 1% O_2) showed the

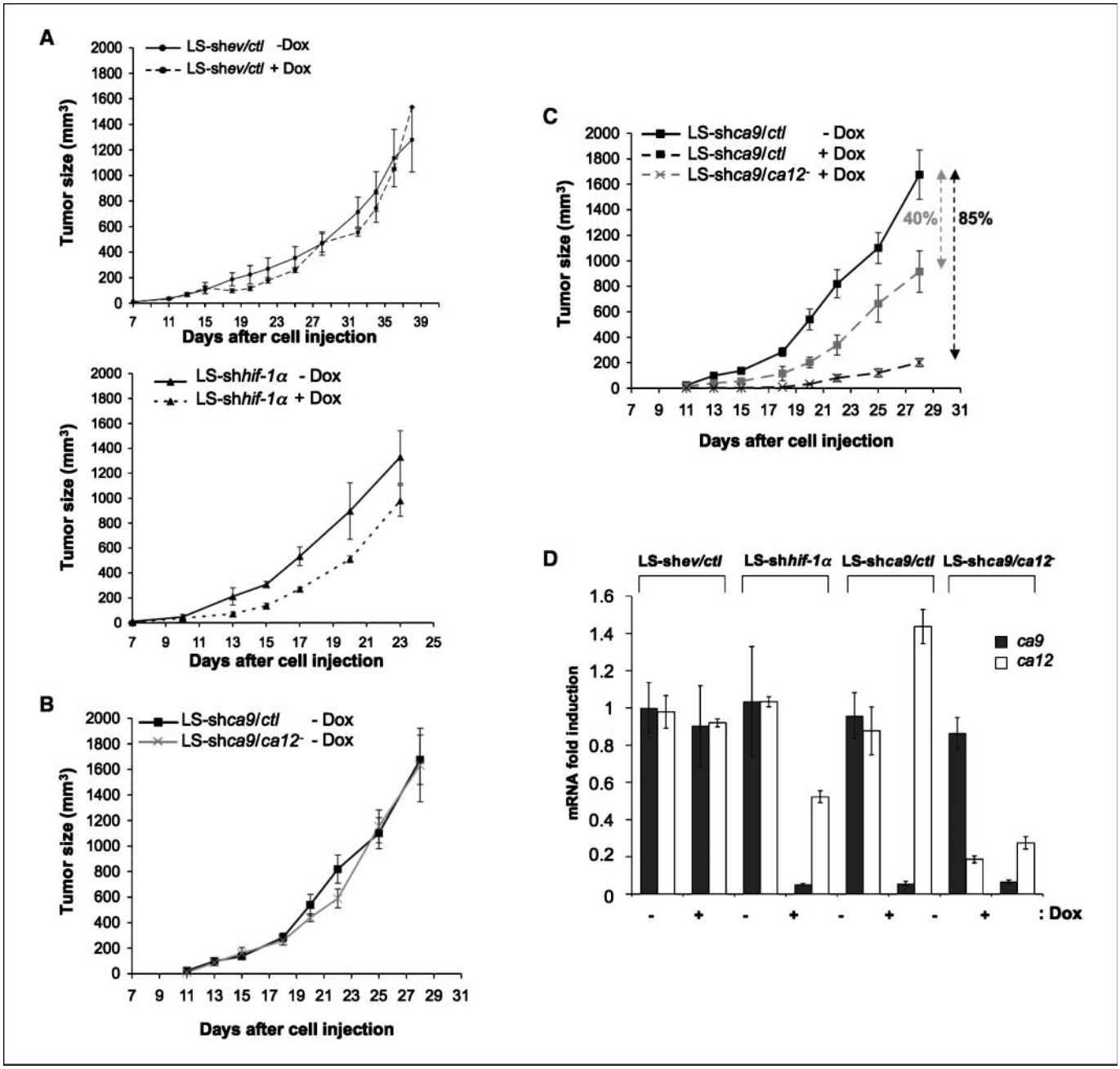

Figure 5. Inducible invalidation of CAIX and CAXII reduces the rate of xenograft tumor growth. Characterization of the growth properties of CAIX-deficient, CAXII-deficient, and CAIX/CAXII-deficient xenograft tumors. At 4 d before injection of LS-sh*ev/ctl*, LS-sh*hif-1α*, LS-sh*ca9/ctl*, and LS-sh*ca9/ca12*$^-$ cells, cells were incubated without (−*Tet*) or with (+*Tet*) Tet to silence *hif-1α*, *ca9* or both *ca9* and *ca12,* respectively. For the respective conditions, mice received Dox or not, a semisynthetic Tet, in the drinking water 4 d before cell injection. *In vivo* xenograft assays were performed by injecting s.c. into the back of athymic nude mice (1×10^6) viable and individual tumor cells LS-sh*ev/ctl* −Tet, LS-sh*ev/ctl* +Tet, LS-sh*hif-1α* −Tet, LS-sh*hif-1α* +Tet, LS-sh*ca9/ctl* −Tet, LS-sh*ca9/ctl* +Tet, LS-sh*ca9/ca12*$^-$ −Tet, and LS-sh*ca9/ca12*$^-$ +Tet. Xenograft growth was determined by measuring the tumor volume. *A, top,* Dox in the drinking water does not affect tumor growth of control LS-sh*ev/ctl* cells; *bottom,* invalidation of *hif-1α* (LS-sh*hif-1α* +Tet) showed a slight reduction in tumor growth compared with tumor growth of control cells (LS-sh*hif-1α* +Tet). *B,* cells defective for *ca12* (LS-sh*ca9/ca12*$^-$ −Tet) formed xenograft tumors at the same rate and of the same size as control cells not silenced for *ca12* (LS-sh*ca9/ctl* −Tet). *C,* cells silenced for *ca9* (LS-sh*ca9/ctl* +Tet) or for both *ca9* and *ca12* (LS-sh*ca9/ca12*$^-$ +Tet) grew slower and gave a smaller tumor volume than control (LS-sh*ca9/ctl* −Tet) cells. Five mice were studied per condition. *In vivo* experiments were repeated twice. *D,* level of mRNA expression of *ca9* and *ca12* in the xenograft tumors of different transgenic cells, as determined by real-time qPCR. Results represent an average of four tumors for each condition.

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same level of activity (data not shown). The resting pH_i of PS120-p*ca9* cells, with the BCECF-AM dye, showed it to be more alkaline compared with control PS120-p*ev* cells when incubated in a nominally HCO_3^-/CO_2-free solution set at a pH_o of 6.6 to 7.4 (Fig. 1*B*). The difference was more pronounced for a pH_o of 6.6 (0.4 units) compared with 7.4 (0.15 units). In the presence of 25 mmol/L bicarbonate, no difference in pH_i was observed between CAIX-expressing cells and control cells. The intracellular alkalinization associated with CAIX expression was confirmed in PS120 (Fig. 1*C*) and CCL39 (Supplementary Fig. S2) cells with another technique that uses [^{14}C]benzoic acid, indicating that CAIX expression protects cells against cytoplasmic acidification. In the presence of endogenous NHE-1 (CCL39 cells), CAIX was able to restore a more alkaline pH_i in acidic environments. This was more marked in fibroblasts impaired in NHE-1 because CAIX was able to compensate for the lack of NHE-1 in maintaining pH_i in acidic environments. The implication of bicarbonate transport in changes in pH_i is shown by its suppression in the presence of the bicarbonate transport inhibitor DIDS (Fig. 1*D*). This shows that the function of CAIX as a pH_i regulator is revealed only in the absence of added extracellular bicarbonate.

CAIX-mediated cytoplasmic alkalinization and cell survival in an acidic environment. To examine if hypoxia-induced CAs protect cells from extracellular acidosis, we assessed the effect of forced expression of CAIX in PS120 and CCL39 cells on proliferation, ATP level, and cell survival. Cell proliferation determined by BrdUrd incorporation into DNA was significantly increased in normoxic CAIX-expressing cells at low pH_o (Fig. 2*A* and Supplementary Fig. S3), as was the production of ATP (Fig. 2*B* and Supplementary Fig. S4) after 24 h in the absence of CO_2/HCO_3^-. Cell colony formation was not significantly different when comparing CAIX-expressing and control cells incubated at a pH_o of 7.4; however, it was substantially diminished in non–CAIX-expressing cells at a low pH_o of 6.4 (Fig. 2*C* and Supplementary Fig. S5). Stable PS120 clones expressing human CAXII were also obtained and showed similar characteristics to those expressing CAIX: extracellular acidification, conserved pH_i regulation, and increase in cell survival in acidic conditions (data not shown). However, the CAXII activity was slightly lower compared with that of CAIX. These results suggest that CAIX and CAXII, by maintaining a more alkaline resting pH_i, sustain ATP levels, promoting cell survival in a bicarbonate-free acidic microenvironment.

Hypoxia-induced CAIX and CAXII activity contributes to cytoplasmic alkalinization in an acidic microenvironment. A number of human tumor cell lines showed an increase in the number of copies of *ca9* and *ca12* mRNA and CAIX and CAXII protein expression in response to hypoxia 1% O_2, including RCC4, HeLa, A375Tr, A549, and LS174Tr cells (Supplementary Table S1; Supplementary Fig. S6). To further investigate the contribution of both CAIX and CAXII, we chose to use the Tet-inducible LS174Tr human colorectal adenocarcinoma cells to silence *hif-1α* with shRNA. In LS-sh*hif-1α* cells, comparable levels of expression of *hif-1α* mRNA were detected in normoxia and hypoxia, confirming absence of transcriptional regulation of *hif-1α* by oxygen. In contrast, incubation of hypoxic LS-sh*hif-1α* cells with Tet resulted in a substantial decrease in the mRNA level of *hif-1α* (Fig. 3*A, top*). Expression of *ca9* and *ca12* was significantly decreased in hypoxic LS-sh*hif-1α* cells when *hif-1α* was silenced (+Tet; Fig. 3*A, bottom*). The LS174Tr cells endogenously express both membrane-bound and catalytically active CAs in a HIF-1–dependent manner (Fig. 3*B*). Hypoxic induction was associated with significantly enhanced alkalinization of the resting pH_i of cells when exposed to a low pH_o in the absence of extracellular bicarbonate (Fig. 3*C*). To study the contribution of CAIX to pH_i regulation, hypoxia-induced CAIX expression was mimicked in LS174Tr cells expressing a basal level of endogenous CAXII in normoxia (Fig. 3*D*; Supplementary Table S1; Supplementary Fig. S7). Forced Tet-inducible expression of CAIX leads to enhanced alkalinization of the resting pH_i in an acidic and bicarbonate-free environment (Fig. 3*D* and Supplementary Fig. S7). Thus, CAIX expression plays a key role in maintaining the resting pH_i in an acidic and bicarbonate-limiting environment in LS174Tr cells.

Combined invalidation of CAIX and CAXII reduces pH_i and spheroid growth. LS174Tr cells were selected for Tet-inducible silencing of *ca9* combined (LS-sh*ca9/ca12*$^-$) or not (LS-sh*ca9/ctl*) with constitutive silencing of *ca12*. Tet addition to sh*ca9* cells (LS-sh*ca9/ctl* and LS-sh*ca9/ca12*$^-$) resulted in 95% invalidation of *ca9* mRNA (Fig. 4*A, top*) and protein (Fig. 4*A, bottom*), whereas 90% of the *ca12* mRNA (Fig. 4*A, top*) and protein (Fig. 4*A, bottom*) were silenced in LS-sh*ca9/ca12*$^-$ cells. Note that an increase in hypoxia-inducible expression of the mRNA and protein levels of CAXII was observed when *ca9* was silenced. The copy number of *ca9* mRNA is 18-fold lower than that of *ca12* in LS-sh*ca9/ctl* in normoxia (Supplementary Table S2), whereas in hypoxia, the number of copies of *ca9* mRNA was only twice higher than that of *ca12*. Moreover, when *ca9* is suppressed in hypoxia, the number of *ca12* mRNA copies was almost similar to *ca9* induced in hypoxia. In addition, when *ca9* was silenced in hypoxia, the overall hypoxia-induced CA activity was unchanged whereas ACTZ showed a marked reduction in acidification (Fig. 4*B, top*). When *ca12* was silenced in hypoxia, no reduction in extracellular acidification occurred (data not shown); however, combined silencing of *ca9* and *ca12* in hypoxia (LS-sh*ca9/ca12*$^-$ +Tet) reduced the CA activity to the basal level (Fig. 4*B, bottom*).

We then determined the relative contributions of CAIX and CAXII in hypoxia regarding pH_i regulation. At a neutral pH_o of 7.4, silencing of *ca9, ca12*, or both together did not affect the resting pH_i (Fig. 4*C*). When cells were exposed to an acidic pH_o of 6.0, only combined invalidation of *ca9* and *ca12* resulted in a significantly lower resting pH_i (0.2 units), whereas silencing of either isoform alone had no effect (Fig. 4*C*); results were obtained with two independent sequences targeting *ca12* (data not shown for the second sequence). We then assessed the importance of this pH_i regulating system in hypoxia on cells grown as three-dimensional spheroids. In a bicarbonate-limiting environment, a hypoxic gradient is established (hypoxyprobe labeling; Fig. 4*D, top*) and, thus, lactic acid is produced. When *ca9* was silenced (LS-sh*ca9/ctl* +Tet) the proliferation index of hypoxic spheroids diminished compared with control cells (LS-sh*ca9/ctl* −Tet) and diminished further when both isoforms were silenced (LS-*shca9/ca12*$^-$ +Tet; Fig. 4*D, bottom*). These results indicate that both CAIX and CAXII play an important role in the regulation of pH_i recapitulating the protective effect of hypoxia in promoting cell survival in an acidic environment.

Inducible invalidation of CAIX and CAXII reduces the rate of xenograft tumor growth. To investigate the *in vivo* functional consequence of CAIX and CAXII expression on tumor growth, athymic mice were s.c. injected with Tet-inducible LS-sh*hif-1α*, LS-sh*ca9/ctl*, or LS-*shca9/ca12*$^-$ cells. Dox had no significant effect on tumor growth of control cells (LS-sh*ev/ctl*; Fig. 5*A, top*), whereas Dox silencing of *hif-1α* showed a substantial decrease in the size of tumors (Fig. 5*A, bottom*). The constitutive silencing of *ca12* in an

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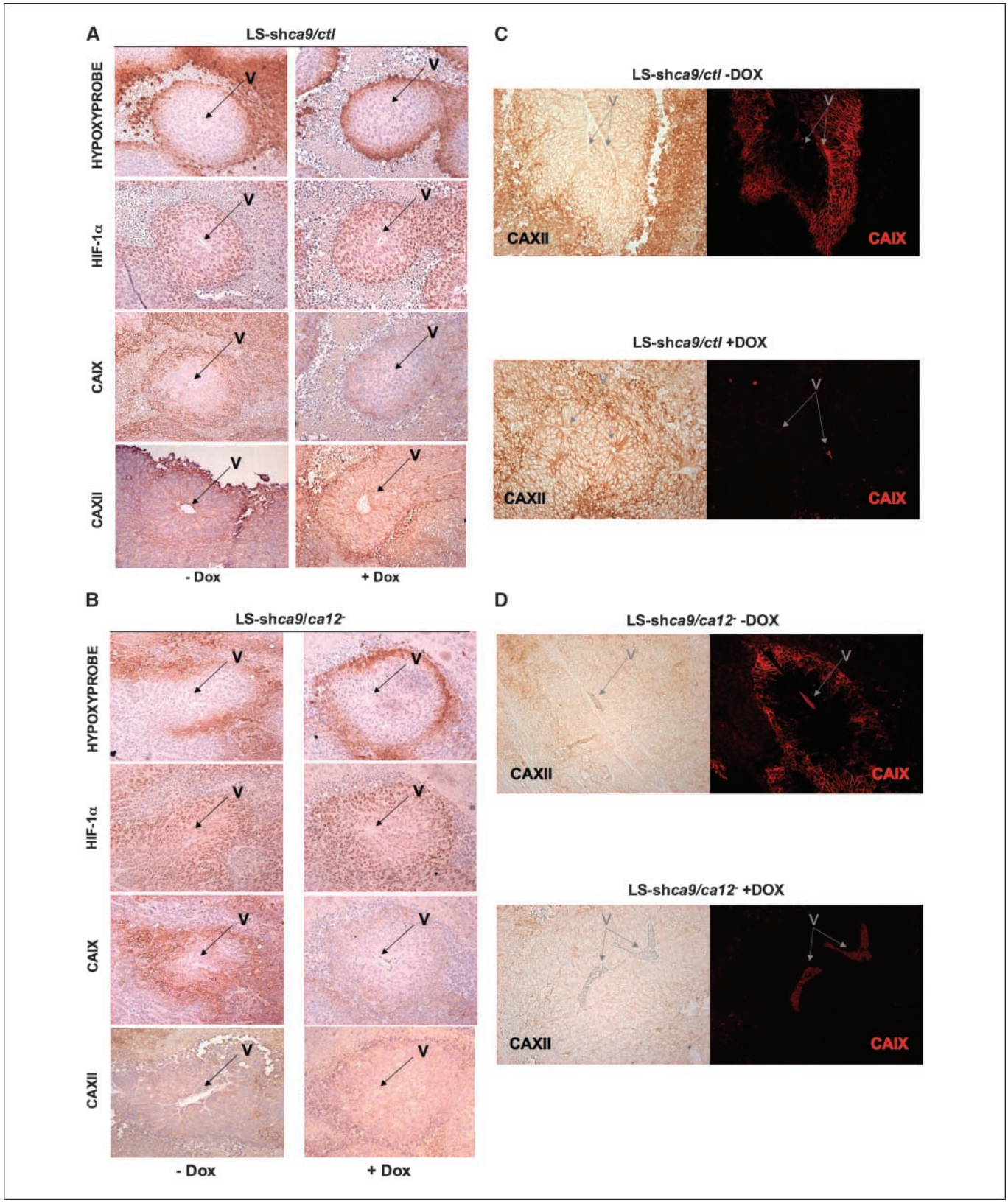

Figure 6. Immunohistologic confirmation of the expression of HIF-1α, CAIX, or CAXII in hypoxic regions of the corresponding tumor xenografts. Mice were injected with *ca9*-deficient LS-sh*ca9/ctl* cells (*A*) or *ca9*- and *ca12*-deficient LS-sh*ca9/ca12*$^{-}$ cells (*B*) and drinking water supplemented without (−*Dox*) or with (+*Dox*) Dox was provided to mice. Serial sections of tumors of the same size were stained for hypoxic regions with antibodies to hypoxyprobe (pimonidazole), for nuclear staining of HIF-1α, or for membrane staining of CAIX or CAXII. Costaining of tumor sections was done first using the immunofluorescence protocol for CAIX followed by immunohistochemistry for CAXII in the same tumor section of LS-sh*ca9/ctl* cells (*C*) and LS-sh*ca9/ca12*$^{-}$ (*D*). Magnification, 20×. *V*, major blood vessel of the tumor lobe analyzed in serial sections.

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endogenous *ca9* background (LS-sh*ca9/ca12*$^-$ −Dox) showed similar tumor growth to that of the control cells (LS-sh*ca9/ctl* −Dox; Fig. 5*B*). However, the silencing of *ca9*, in the presence of endogenous *ca12* (LS-sh*ca9/ctl* +Dox), gave a slight but significant reduction in tumor growth (40%) compared with control cells (Fig. 5*C*). Invalidation of both isoforms (LS-sh*ca9/ca12*$^-$ +Dox) resulted in a spectacular decrease in tumor size (85%) as a result of slower cell proliferation (Fig. 5*C*). Examination of the mRNA levels of *ca9* and *ca12* in the tumors confirmed almost complete invalidation of *ca9* in the invalidated cell lines when Dox was added (95% for LS-sh*hif-1α*, 94% for LS-*shca9/ctl*, and 92% for LS-sh*ca9/ca12*$^-$*)*. Whereas the expression of the *ca12* mRNA was diminished by a half in *hif-1α* invalidated cells (Figs. 4*A* and 5*D*), its expression increased 1.5-fold when *ca9* (LS-sh*ca9/ctl* +Dox) was silenced (Fig. 5*D*). In the LS-sh*ca9/ca12*$^-$ cell line, the level of *ca12* mRNA was diminished (80%) whereas that of *ca9* in the absence of Dox was not significantly different. These results indicate that the respective tumor types retain their invalidated phenotype and that the expression of *ca12* responds to the level of expression of *ca9*, although the reverse is not the case.

Immunohistochemical and immunofluorescence analysis of tumor sections from mice injected with LS-*ca9/ctl* (Fig. 6*A* and *C*) and LS-*ca9/ca12*$^-$ cells (Fig. 6*B* and *D*) showed diminished levels of CAIX in cells silenced for *ca9* and for *ca9* plus *ca12* in Dox-treated mice, despite the expression of HIF-1α in hypoxic zones (hypoxyprobe staining). CAXII expression is seen in all cells of the tumor (slightly increased in the perinecrotic area) in contrast to the expression of CAIX, which correlates with the hypoxic and perinecrotic regions. It is important to note the increase in CAXII expression when CAIX is silenced *in vivo*. Comparison of the immunohistochemistry of tumor sections of control LS-sh*ev/ctl* cells (Supplementary Fig. S8*A*) and *hif-1α* silenced LS-sh*hif-1α* cells (Supplementary Fig. S8*B*) revealed a significant decrease in HIF-1α, CAIX, and CAXII in *hif-1α* silenced cells. For tumor histology, to insure that the reduced labeling observed for CAIX in *ca9/ca12*$^-$ silenced tumors was indeed due to invalidation and not small-sized tumors with minimal hypoxic zones, mice were maintained for a longer time and sacrificed 27 days later than control mice when the tumors were the same size as for control nonsilenced cells. Thus, these results show that invalidation of both CAIX and CAXII brings about a dramatic decrease in tumor xenograft cell growth.

Discussion

Investigation into the implication of hypoxia-inducible CAs in the regulation of pH has concerned only CAIX and has been restricted to monolayer or three-dimensional cell cultures (31, 32). During the preparation of this manuscript, a study showed that ectopically expressed CAIX in human bladder carcinoma RT112 cells was able to spatially coordinate pH_i, but only when cells are cultured as three-dimensional spheroids (32). In agreement with this report, CAIX expression in our study had no effect on pH_i regulation in isolated cells in a neutral and bicarbonate-buffered medium (25 mmol/L); however, when cells were exposed to a nominally bicarbonate-free and acidic milieu, CAIX effected on the resting pH_i. Here, we conducted *in vitro* studies in the absence of extracellular bicarbonate in order not to saturate bicarbonate transporters at the cell surface. As we showed previously (6, 35), the presence of a high bicarbonate level (25 mmol/L) totally blunts the effect of NHE-1 on pH_i regulation. Thus, we reasoned that, to investigate the putative contribution of membrane-bound CAIX and CAXII in pH_i regulation, it is necessary to operate in nominally bicarbonate-free solutions exposed only to ambient CO_2.

We also examined CAIX regulation of pH_i in fibroblasts impaired in NHE-1 expression (PS120 cells) to ensure the absence of interference by this major player in pH_i homeostasis (12, 36–38). Nonetheless, the effect of CAIX on pH_i was also detected in CCL39 fibroblasts and in a human colon adenocarcinoma cell line LS174Tr expressing endogenous NHE-1. Previous studies showed that NHE–1–deficient cells fail to grow in a range of acidic pH_o (6.2–6.8) due to their inability to reach the permissive pH_i values required for DNA synthesis and ATP production (6, 7, 39). Forced CAIX expression in this pH_i regulation–deficient cell system was able to restore viability of PS120 cells when exposed to a range of acidic pH_o (6.2–6.8). This shows the role of a pH_i-threshold value for growth confirming the role of CAIX in pH_i control.

It is postulated that the mechanism by which membrane-bound CAs regulate pH_i occurs through the efficient uptake of HCO_3^- locally formed in the "mouth" of CAs through Cl^-/HCO_3^- exchangers and/or Na^+/HCO_3^- cotransporters, forming tight functional complexes (40). Although the "metabolon" is an interesting concept, this notion has been challenged (41–43). Interaction of the catalytic domain of CAIX with bicarbonate transporters has also been reported and was shown to increase the AE exchanger activity (21, 44). Future investigation is under way to evaluate the key bicarbonate transporters coupled to CAIX and CAXII in pH_i regulation in hypoxic tumor cells.

In our study, invalidation of CAIX leads to partial compensation by up-regulation of CAXII. This may explain the maintenance of the catalytic activity of *ca9* invalidated cells in hypoxia and suggest that a threshold level of activity is required for cell pH homeostasis. In the recent study of Swietach and colleagues (32), overexpression of CAIX down-regulated cytosolic CAII. This result suggests that different tissues with different expression patterns of CAs may bring into play different CA isoforms that would "communicate" in a yet unresolved network when confronted with an acidic stress.

Hypoxic induction may not be the only mechanism by which CAs regulate pH homeostasis in tumors. Signaling through the epidermal growth factor pathway by phosphorylation of a cytoplasmic tyrosine residue of CAIX may either activate CAIX or enhance its expression by increasing translation of HIF-1α (30). In addition, phosphorylation activates phosphatidylinositol 3-kinase, resulting in phosphorylation of Akt and cell survival. The possible implication of these CAs as signaling molecules, independent of their function as pH-regulating enzymes, is another important point that is under investigation and may explain reduced xenograft growth of *ca9* silenced cells despite maintenance of CA catalytic activity.

It has been proposed that an acidic microenvironment promotes metastasis associated with poor patient survival (4, 45). However, acidosis may not always favor metastasis (4, 46). When renal carcinoma cells were treated with ACTZ, their capacity to invade was diminished (47) but the invasion of carcinoma cells was not influenced by CAIX in another study (48). Here, we not only show that CAIX and CAXII promote survival in an acidic environment in two cell culture systems but extend it in *in vivo* studies. The combined silencing of CAIX and CAXII gave a dramatic decrease in the rate of growth of xenograft tumors that was greater than the invalidation of HIF-1α, which may reflect the pleotropic action of HIF-1 on prosurvival and prodeath genes. This may suggest that the interest being paid to HIF inhibitors as anticancer treatments

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(49, 50) might be better directed to the inhibition of downstream HIF target gene products, as we recently proposed (1) and, in particular to CAIX together with CAXII, as novel and potentially efficient drug approaches.

The present study highlights the role of CAIX and CAXII expression in pH_i regulation, a key event controlling cell viability, and in *in vivo* tumor growth in a hostile acidic and hypoxic microenvironment. The results herein call for the development of specific "CA-antagonist" antibodies or cell impermeable drugs specifically targeting the membrane-associated and hypoxia-inducible CAs, taking into consideration the CA isoform profile of a given tumor type. Such inhibitors are being actively investigated at the molecular and cellular levels (23, 24) and may hold promise as effective anticancer treatments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

1. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 2006;441:437–43.
2. Brahimi-Horn MC, Chiche J, Pouyssegur J. Hypoxia signalling controls metabolic demand. Curr Opin Cell Biol 2007;19:223–9.
3. Gullino PM, Clark SH, Grantham FH. The interstitial fluid of solid tumors. Cancer Res 1964;24:780–96.
4. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004;4:891–9.
5. Roos A, Boron WF. Intracelluar pH. Physiol Rev 1981; 61:296–434.
6. Pouyssegur J, Sardet C, Franchi A, L'Allemain G, Paris S. A specific mutation abolishing Na+/H+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. Proc Natl Acad Sci U S A 1984;81:4833–7.
7. Chambard JC, Pouyssegur J. Intracellular pH controls growth factor-induced ribosomal protein S6 phosphorylation and protein synthesis in the G0-G1 transition of fibroblasts. Exp Cell Res 1986;164:282–94.
8. Brahimi-Horn MC, Pouyssegur J. Oxygen, a source of life and stress. FEBS Lett 2007;581:3582–91.
9. Sardet C, Franchi A, Pouyssegur J. Molecular cloning, primary structure, and expression of the human growth factor-activatable Na+/H+ antiporter. Cell 1989;56: 271–80.
10. Wakabayashi S, Shigekawa M, Pouyssegur J. Molecular physiology of vertebrate Na+/H+ exchangers. Physiol Rev 1997;77:51–74.
11. Cardone RA, Casavola V, Reshkin SJ. The role of disturbed pH dynamics and the Na+/H+ exchanger in metastasis. Nat Rev Cancer 2005;5:786–95.
12. Shimoda LA, Fallon M, Pisarcik S, Wang J, Semenza GL. HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial myocytes. Am J Physiol Lung Cell Mol Physiol 2006;291:L941–9.
13. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1α dependent mechansm. J Biol Chem 2006;281:9030–7.
14. Pouyssegur J, Franchi A, Pages G. pHi, aerobic glycolysis and vascular endothelial growth factor in tumour growth. In: Novartis Found Symp; 2001:John Wiley & Sons, Ltd; 2001. p. 186–96.
15. Alper SL. Molecular physiology of SLC4 anion exchangers. Exp Physiol 2006;91:153–61.
16. Romero MF, Fulton CM, Boron WF. The SLC4 family of HCO_3^- transporters. Pflugers Arch 2004;447: 495–509.
17. Izumi H, Torigoe T, Ishiguchi H, et al. Cellular pH regulators: potentially promising molecular targets for cancer chemotherapy. Cancer Treat Rev 2003;29: 541–459.
18. Ivanov S, Liao SY, Ivanova A, et al. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 2001;158:905–19.
19. Swietach P, Vaughan-Jones RD, Harris AL. Regulation of tumor pH and the role of carbonic anhydrase 9. Cancer Metastasis Rev 2007;26:299–310.
20. Thiry A, Dogne JM, Masereel B, Supuran CT. Targeting tumor-associated carbonic anhydrase IX in cancer therapy. Trends Pharmacol Sci 2006;27:566–73.
21. Ivanov SV, Kuzmin I, Wei MH, et al. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. Proc Natl Acad Sci U S A 1998;95:12596–601.
22. Wykoff CC, Beasley NJ, Watson PH, et al. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 2000;60:7075–83.
23. Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. Nat Rev Drug Discov 2008;7:168–81.
24. Pastorekova S, Zatovicova M, Pastorek J. Cancer-associated carbonic anhydrases and their inhibition. Curr Pharm Des 2008;14:685–98.
25. Whittington DA, Waheed A, Ulmasov B, et al. Crystal structure of the dimeric extracellular domain of human carbonic anhydrase XII, a bitopic membrane protein overexpressed in certain cancer tumor cells. Proc Natl Acad Sci U S A 2001;98:9545–50.
26. Dayan F, Roux D, Brahimi-Horn MC, Pouyssegur J, Mazure NM. The oxygen sensor factor-inhibiting hypoxia-inducible factor-1 controls expression of distinct genes through the bifunctional transcriptional character of hypoxia-inducible factor-1α. Cancer Res 2006;66:3688–98.
27. Hussain SA, Ganesan R, Reynolds G, et al. Hypoxia-regulated carbonic anhydrase IX expression is associated with poor survival in patients with invasive breast cancer. Br J Cancer 2007;96:104–9.
28. Zavada J, Zavadova Z, Pastorek J, Biesova Z, Jezek J, Velek J. Human tumour-associated cell adhesion protein MN/CA IX: identification of M75 epitope and of the region mediating cell adhesion. Br J Cancer 2000;82: 1808–13.
29. Svastova E, Zilka N, Zat'ovicova M, et al. Carbonic anhydrase IX reduces E-cadherin-mediated adhesion of MDCK cells via interaction with β-catenin. Exp Cell Res 2003;290:332–45.
30. Dorai T, Sawczuk IS, Pastorek J, Wiernik PH, Dutcher JP. The role of carbonic anhydrase IX overexpression in kidney cancer. Eur J Cancer 2005;41:2935–47.
31. Svastova E, Hulikova A, Rafajova M, et al. Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. FEBS Lett 2004; 577:439–45.
32. Swietach P, Wigfield S, Cobden P, Supuran CT, Harris AL, Vaughan-Jones RD. Tumor-associated carbonic anhydrase 9 spatially co-ordinates intracellular pH in three-dimensional multicellular growths. J Biol Chem 2008;283:20473–83.
33. van de Wetering M, Oving I, Muncan V, et al. Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep 2003; 4:609–15.
34. Trastour C, Benizri E, Ettore F, et al. HIF-1α and CA IX staining in invasive breast carcinomas: prognosis and treatment outcome. Int J Cancer 2007;120:1451–8.
35. L'Allemain G, Paris S, Pouyssegur J. Growth factor action and intracellular pH regulation in fibroblasts. Evidence for a major role of the Na+/H+ antiport. J Biol Chem 1984;259:5809–15.
36. Cardone RA, Bellizzi A, Busco G, et al. The NHERF1 PDZ2 Domain Regulates PKA-RhoA-p38-mediated NHE1 Activation and Invasion in Breast Tumor Cells. Mol Biol Cell 2007;18:1768–80.
37. Counillon L, Pouyssegur J. The expanding family of eucaryotic Na(+)/H(+) exchangers. J Biol Chem 2000;275: 1–4.
38. Karumanchi SA, Jiang L, Knebelmann B, Stuart-Tilley AK, Alper SL, Sukhatme VP. VHL tumor suppressor regulates Cl-/HCO_3^- exchange and Na+/H+ exchange activities in renal carcinoma cells. Physiol Genomics 2001;5:119–28.
39. Pouyssegur J, Franchi A, L'Allemain G, Paris S. Cytoplasmic pH, a key determinant of growth factor-induced DNA synthesis in quiescent fibroblasts. FEBS Lett 1985;190:115–9.
40. Sterling D, Reithmeier RAF, Casey JR. A transport metabolon. Functional interaction of carbonic anhydrase II and chloride/bicarbonate exchangers. J Biol Chem 2001;276:47886–94.
41. Becker HM, Deitmer JW. Carbonic anhydrase II increases the activity of the human electronic Na+/HCO_3^- cotransporter. J Biol Chem 2007;282:13508–21.
42. Becker HM, Deitmer JW. Non-enzymatic proton handling by carbonic anhydrase II during H+-lactate cotransport via monocarboxylate transporter 1. J Biol Chem 2008;283:21655–67.
43. Lu J, Daly CM, Parker MD, et al. Effect of human carbonic anhydrase II on the activity of the human electogenic Na/HCO3 cotransporter NBCe1-A in *Xenopus* Oocytes. J Biol Chem 2006;281:19241–50.
44. Morgan PE, Pastorekova S, Stuart-Tilley AK, Alper SL, Casey JR. Interactions of transmembrane carbonic anhydrase, CAIX, with bicarbonate transporters. Am J Physiol Cell Physiol 2007;293:C738–48.
45. Walenta S, Mueller-Klieser WF. Lactate: mirror and motor of tumor malignancy. Semin Radiat Oncol 2004; 14:267–74.
46. Rofstad EK, Mathiesen B, Kindem K, Galappathi K. Acidic extracellular pH promotes experimental metastasis of human melanoma cells in athymic nude mice. Cancer Res 2006;66:6699–707.
47. Parkkila S, Rajaniemi H, Parkkila A-K, et al. Carboninc anhydrase inhibitor suppresses invasion of renal cancer cells *in vitro*. Proc Natl Acad Sci U S A 2000;97:2220–4.
48. Robertson N, Potter C, Harris AL. Role of carbonic anhydrase IX in human tumor cell growth, survival and invasion. Cancer Res 2004;64:6160–5.
49. Melillo G. Targeting hypoxia cell signaling for cancer therapy. Cancer Metastasis Rev 2007;26:341–52.
50. Semenza GL. Evaluation of HIF-1 inhibitors as anticancer agents. Drug Discov Today 2007;12:854–7.