# Hypoxia-Inducible Factor 1α Mediates Anoikis Resistance via Suppression of α5 Integrin

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## Abstract

**The transcription factor hypoxia-inducible factor 1 (HIF-1) α is abundantly expressed in the majority of human carcinomas and their metastases. HIF-1α controls central metastasis-associated pathways such as glycolysis, angiogenesis, and invasion. Functional inhibition of HIF-1α leads to impaired metastasis formation in murine tumor models. However, the precise molecular mechanisms underlying the metastasis-promoting role of HIF-1α have not been fully characterized. The ability of transformed epithelial cells to initiate the metastatic cascade relies on their ability to escape anoikis, a default program of apoptosis induction following loss of integrin anchoring to the extracellular matrix. Therefore, we addressed the function of HIF-1α in anoikis resistance and anchorage-independent growth. Inhibition of HIF-1α via RNA interference resulted in up-regulation of α5 integrin on the cell surface of human gastric cancer cells, whereas other integrins remained unaffected. Integrin α5 induction occurred at the level of transcription and was dependent on elevated intracellular superoxide in HIF-1α-knockdown cells. HIF-1α–deficient cells displayed significantly increased anoikis susceptibility due to up-regulated α5 integrin. Finally, colony formation in soft agar was shown to be dependent on HIF-1α as HIF-1α–deficient cells displayed a 70% reduction in anchorage-independent proliferation. Results obtained by RNA interference could be entirely confirmed by application of the pharmacologic HIF-1α-inhibitor 2-methoxyestradiol. Hence, our data argue for a pivotal role for HIF-1α in anoikis control via suppression of α5 integrin. HIF-1α–inhibiting drugs might therefore offer an innovative strategy for anti-metastatic cancer therapy.** [Cancer Res 2008;68(24):10113–20]

## Introduction

Lack of oxygen (hypoxia) is a hallmark of solid tumor growth and represents an independent prognostic factor in a diverse range of human cancers (1). Hypoxia is associated with local invasion, metastatic spread, and resistance to chemotherapy as well as radiotherapy, ultimately limiting patients prognosis (1). The transcription factor hypoxia-inducible factor 1 (HIF-1) constitutes the principal mediator of cellular adaptation to hypoxia. HIF-1 is a heterodimeric protein consisting of a ubiquitously expressed β-subunit (also known as aryl hydrocarbon receptor nuclear translocator) and a hypoxia-inducible α subunit (1). HIF-1 is centrally involved in a multitude of physiologic and pathophysiologic processes such as embryonal development, glycolysis, erythropoiesis, myeloid-cell mediated inflammation, bactericidal activity of phagocytes, and matrix synthesis (2). In human cancer, HIF-1α overexpression is associated with increased patient mortality, e.g., in malignant breast, colon, and lung tumors (2). This association is mainly based on the HIF-1–mediated regulation of genes that possess pivotal roles in central features of cancer pathogenesis including: immortalization, cellular dedifferentiation, genetic instability, vascularization, glycolytic metabolism, as well as invasion and metastasis (2). Inhibition of HIF-1α by means of RNA interference or chemical compounds has proven antitumoral activity in different murine models of cancer (2). Furthermore, inhibitors of HIF-1 are being tested in clinical studies as anticancer agents (nine actively recruiting phase I and II studies as of April 2008).[4] Elevated apoptotic tumor cell fractions have frequently been observed in experimental tumors after blockade of HIF-1α. However, the precise function of HIF-1α in apoptosis regulation remains controversial and the molecular mechanisms governing the putative antiapoptotic role of HIF-1α *in vivo* remain to be elucidated (2). By applying a lentiviral-mediated RNA interference system, we observed that HIF-1α–deficient gastric cancer cells are defective in migration, invasion, and adhesion to endothelial cells.[5] To identify candidate mechanisms linking HIF-1α to these biological effects, we examined whether integrin expression patterns had changed after functional inactivation of HIF-1α. Surprisingly, we were able to show a specific up-regulation of α5 integrin on human gastric cancer cells after loss of HIF-1α. Our functional analysis revealed a markedly reduced ability of HIF-1α–deficient cells to evade anoikis, and we provide experimental evidence for a causal role of α5 integrin in restoring anoikis susceptibility.

## Materials and Methods

**Cell culture and chemicals.** The human gastric cancer cell lines AGS (CRL-1739; American Type Culture Collection) and MKN28 (JCRB Cell Bank) were grown in monolayer cultures in recommended medium. 2-Methoxyestradiol (2ME2) and diphenyleneiodonium (DPI) chloride were purchased from Sigma-Aldrich and dissolved in DMSO at a concentration of 30 and 10 mmol/L, respectively.

**Plasmid construction and production of lentiviral vectors.** Short hairpin RNA (shRNA) sequences against human HIF-1α and scrambled

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

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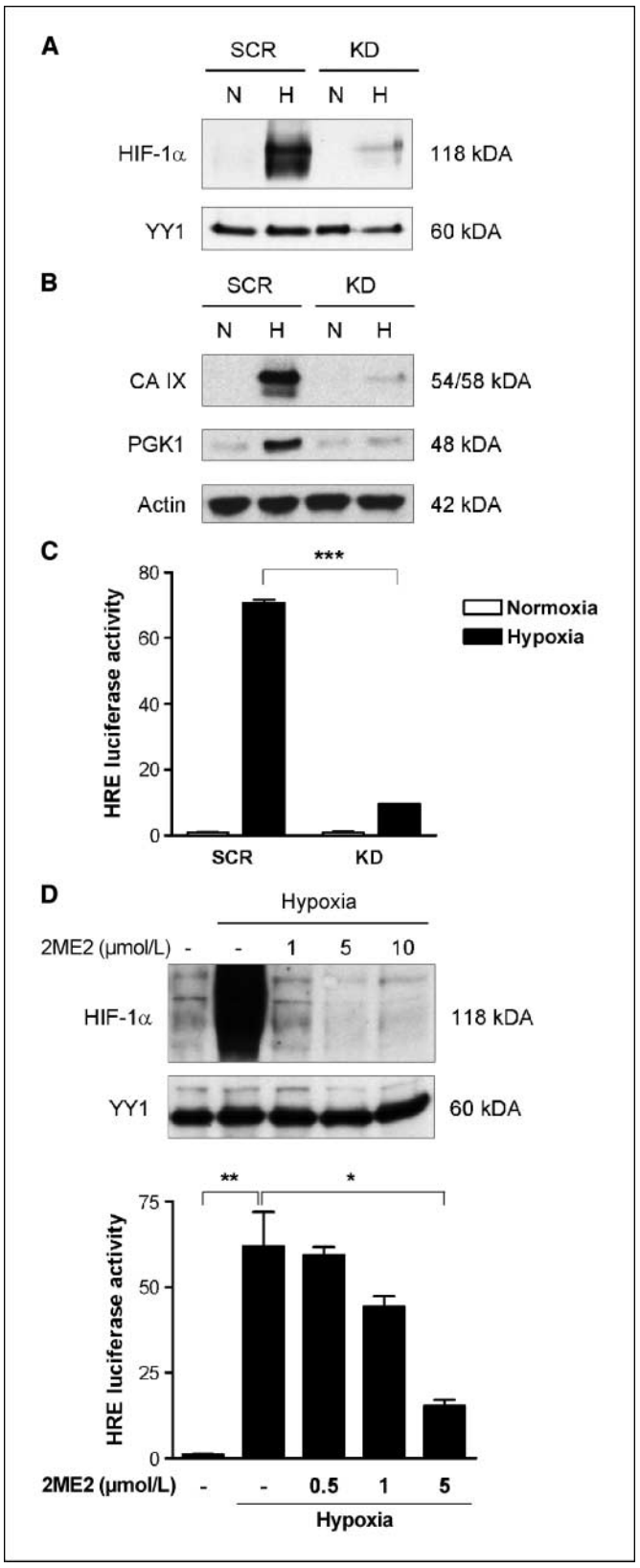
doi:10.1158/0008-5472.CAN-08-1839

[4] http://www.clinicaltrials.gov

[5] N. Rohwer, S. Lobitz, Jöns, M. Vieth, W. Kemmner, B. Wiedenmann, T. Cramer, M. Höcker, submitted.

control oligonucleotides (TIB MOLBIOL) were published elsewhere (3, 4). Oligonucleotides were inserted into *BsrG*I and *Xba*I restriction sites of the lentiviral bicistronic vector pPR1, which allows for coexpression of GFP (5). Lentiviruses were produced by transient transfection of pPR-HIF-1α or pPR-scr with packaging vectors in 293T cells using the calcium-phosphate method. Vector titers were determined by transducing 293T cells with serial dilutions of concentrated lentivirus, and green fluorescent protein (GFP) was used to quantitate the transduced cell fraction by flow cytometry 60 h after transduction. Gastric cancer cell lines stably expressing siRNAs were generated by transduction with lentiviruses at a multiplicity of infection of 10 for 24 h. Transduction efficiency of target cells was determined by flow cytometry analysis of GFP using a FACSCalibur (Becton Dickinson).




**Western blot analysis.** Immunoblot was performed as described in detail before (6). Antibodies were against PGK1, YY1 (Santa Cruz Biotechnology), CA IX (clone M75; a kind gift of Jaromir Pastorek, Institute of Virology, Slovak Academy of Science, Bratislava, Slovakia; ref. 7), α5 integrin (BD Transduction Laboratories), αv integrin (Chemicon), HIF-1α (R&D Systems), and β-actin (Sigma-Aldrich).

**Transient transfection and reporter luciferase assay.** Transfections were performed as described in detail before (6).

**Quantitative real-time PCR analysis.** Total cellular RNA was extracted with Trizol reagent (Invitrogen), and first strand cDNA was synthesized with an oligo (dT) primer and a SuperScript First Strand Synthesis System (Invitrogen). Quantitative real-time PCR analysis was performed by using TaqMan PCR Universal Mastermix (Applied Biosystems) with α5 integrin TaqMan probe und primers (Hs00233743_m1; Applied Biosystems). To normalize the amount of input RNA, PCR reactions were done with probe and primers for β-actin (probe, 5′-(6FAM)CCA TCCTAAAAGCCACCC-CACTTCTCTCTA(BHQ~6FAM)-3′; forward primer, 5′-TGCATTGTTACAG-GAAGTCCCTT-3′; reverse primer, 5′-GGG AGA GGA CTG GGC CAT-3′).

**Analysis of integrin expression by flow cytometry.** Cell surface expression of integrins was determined as described in detail before (8).

**Measurement of intracellular superoxide levels.** Intracellular superoxide anion levels were estimated using the fluorescent dye dihydroethidium (DHE), obtained from Sigma-Aldrich. After 24 h cultivation, cells were trypsinized, harvested by centrifugation, resuspended in PBS containing 10 μmol/L DHE for 25 min at 37°C, and thereafter washed with ice-cold PBS. Dye oxidation was determined by flow cytometry with excitation and emission settings of 488 and 585 nm, respectively. The mean fluorescence intensity of at least $1 \times 10^5$ cells was analyzed and corrected for autofluorescence from unlabeled cells.

**Determination of cell cycle distribution and apoptosis by flow cytometry.** Cell cycle distribution and apoptosis fraction were determined from DNA histograms as described before (8). Apoptosis was also quantitated based on the mitochondrial membrane potential using JC-1 staining (Molecular Probes). Briefly, cells were harvested, centrifuged at 14,000 *g* for 40 s, resuspended in medium containing JC-1 (5 μg/mL) at 37°C

**Figure 1.** Inhibition of HIF-1α in AGS gastric cancer cells by RNA interference or via treatment with the chemical compound 2ME2. *A,* AGS cells stably expressing siRNA against HIF-1α (*KD*) or scrambled control siRNA (*SCR*) were cultivated for 16 h under normoxia (*N*) or hypoxia (*H*), and HIF-1α protein levels were analyzed in nuclear extracts by Western blot analysis, with YY1 serving as nuclear loading control. AGS KD cells were unable to express HIF-1α protein under hypoxic culture. *B,* expression of HIF-1 target genes *PGK1* and *CA IX* was determined by Western blot of total cell lysates, with β-actin serving as cytoplasmic loading control. AGS KD and SCR cells were subjected for 24 h to either normoxia or hypoxia. Inhibition of HIF-1α protein by RNAi resulted in decreased expression of HIF-1 target genes PGK1 and CA IX in AGS KD cells under hypoxic conditions. *C,* confirmation of loss of HIF-1α function by HRE-luciferase reporter assay. AGS KD and SCR cells were cotransfected with a HRE-luc reporter and phRL-null *Renilla* as an internal control and incubated under either normoxia or hypoxia for 24 h. Inhibition of HIF-1α resulted in a significant decrease of HRE-luc reporter activity under hypoxic conditions (***, $P < 0.0001$; unpaired Student's *t* test). Luciferase activity, normalized to *Renilla* luciferase activity, was expressed relative to that of transfected control cells (*SCR*) under normoxia, set at 1.0. Results shown are representative of three independent experiments; *columns,* mean of triplicate determinations; *bars,* SE. *D,* AGS WT cells were treated for 24 h with increasing concentrations of 2ME2 and inhibition of HIF-1α was analyzed by Western blot analysis (*top*) and HRE-luc reporter assay (*bottom*). Treatment with 2ME2 led to a dose-dependent reduction of HIF-1α protein and in agreement to a significant decrease of HRE-luc reporter activity under hypoxia (**, $P = 0.0035$; *, $P = 0.036$; unpaired Student's *t* test).

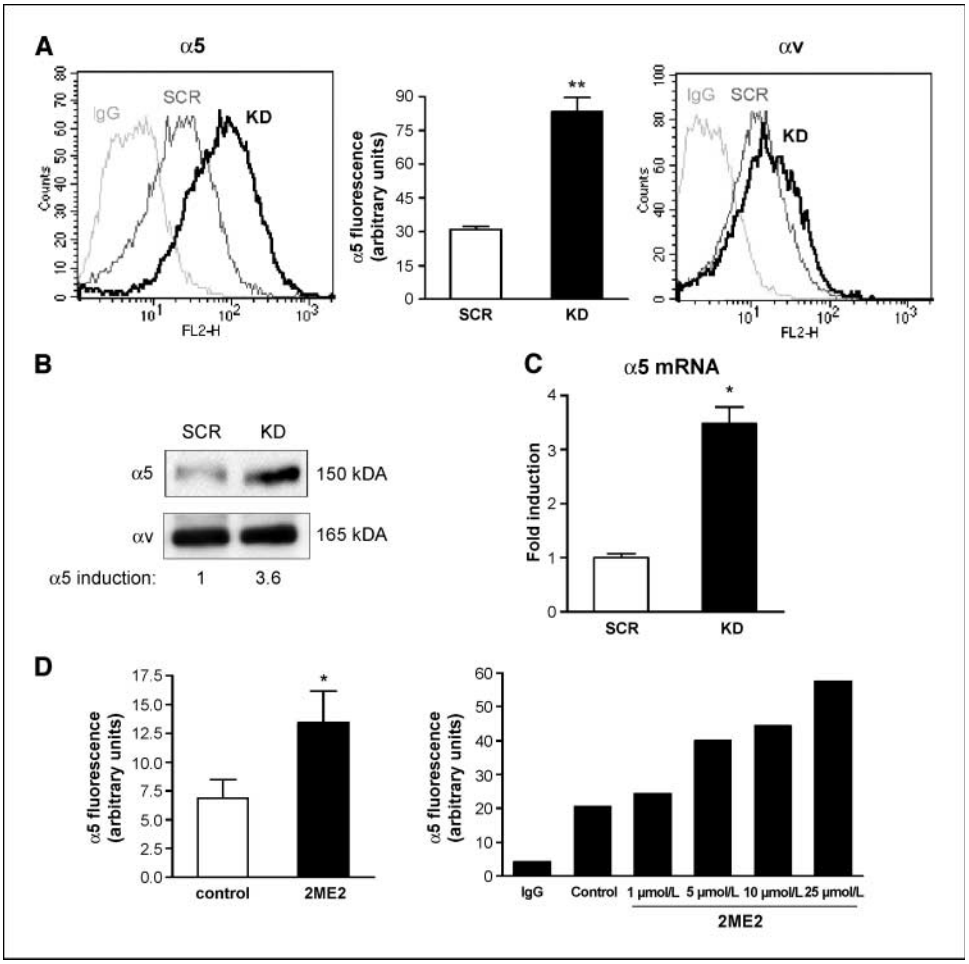



**Figure 2.** Effects of HIF-1α inhibition on α5 integrin expression. *A,* AGS KD (*black*) and SCR (*gray*) were incubated with antibody against α5 integrin (*left*) or αv integrin (*right*) or immunoglobulin (*IgG*; *light gray*) as isotype control and subsequently analyzed by flow cytometry. Shown are representative histograms of four experiments. *Columns,* mean of four experiments; *bars,* SE (**, $P = 0.0055$; paired Student's *t* test; *middle*). *B,* analysis of α5 and αv integrin expression in AGS KD and SCR cells by Western blot using whole cell lysates. Inactivation of HIF-1α by RNAi resulted in a significant increase of α5 integrin. *C,* expression of α5 integrin mRNA was analyzed relative to β-actin by quantitative real-time PCR. Inhibition of HIF-1α increased α5 integrin mRNA abundance (*, $P = 0.0156$; unpaired Student's *t* test). Data were expressed relative to α5 mRNA levels in control cells (*SCR*), set at 1.0. *Columns,* mean; *bars,* SE. *D,* AGS WT cells were incubated with 10 μmol/L (*left*) or increasing concentrations (*right*) of 2ME2 and α5 integrin expression was determined by fluorescence-activated cell sorting (FACS) analysis. Pharmalogical inhibition of HIF-1α by 2ME2 provoked a significant and dose-dependent induction of α5 integrin. *Columns*, mean of three experiments; *bars,* SE (*, $P = 0.0262$; paired Student's *t* test; *left*). *Columns,* mean of one representative experiment (*right*).

for 15 min, and washed twice with PBS. A minimum of $1 \times 10^5$ cells were measured on a FACSCalibur to quantitate J-aggregate formation.

**Quantitation of active caspase-3.** Active caspase-3 was quantified by flow cytometry using an Alexa Fluor 488–conjugated antibody against the cleaved form of caspase-3 (9669; Cell Signaling Technology) according to the manufacturer's protocol.

**Anoikis protection assay.** Cells ($2.5 \times 10^5$) were seeded in 6-well plates, allowed to adhere, and pretreated with 10 μmol/L 2ME2 for 24 h. Subsequently, $2.5 \times 10^5$ cells were replated onto either polyHEMA-coated dishes or regular tissue culture ware in the presence or absence of a function blocking α5 integrin antibody (clone P1D6; Chemicon) or mouse immunoglobulin control antibody (Dako). After 16 h, anoikis was evaluated based on the fraction of cells harboring active caspase-3.

**Anchorage-independent growth assay.** Anchorage-independent growth was assessed by human tumor clonogenic assay as described before (8). For determination of anchorage-independent growth in cells treated with 2ME2, every third day, 2ME2 was added to the cells in the indicated concentrations.

**Immunocytochemical detection of α5 integrin.** Cells grown onto sterile coverslips were washed with PBS before fixation in acetone/methanol (1:1) for 2 min at room temperature. Cells were washed twice with PBS, blocked for 20 min in PBS with 2% nonfat dried milk, washed twice again, and were then incubated with a mouse monoclonal antibody to human α5 integrin (Abcam) in 0.1% bovine serum albumin/PBS overnight at 4°C. Cells were washed four times in PBS before incubation with a goat anti mouse Cy3 antibody (1 h at room temperature) followed by 2 additional washes with PBS and final fixation in ethanol. Cells were mounted in Elvanol and immunofluorescence was evaluated on a Zeiss Axioskop.

**Statistical analysis.** Statistical analysis was done using Prism 4.0 software (GraphPad Software). Statistical significance was determined by two-tailed Student's *t* test for unpaired or paired observations ($P < 0.05$).

## Results and Discussion

Robust metastatic proficiency is a hallmark of human gastric carcinomas and the clinical outcome of gastric cancer patients is critically determined by the extent of local invasion and systemic dissemination. Invasiveness in turn was found associated with HIF-1α expression via analysis of gastric cancer specimens by us and other investigators (9).[5] In view of this background, we chose AGS and MKN28 human gastric cancer cell lines as suitable cell models to study the role of HIF-1α in cellular processes that promote metastasis. To achieve sound functional inactivation of HIF-1α *in vitro*, we constructed a lentiviral RNA interference system. AGS and MKN28 cells stably expressing either siRNA specifically targeting HIF-1α (knockdown, "KD") or unspecific control siRNA (scrambled, "SCR") were generated by lentiviral transduction. This approach yielded a highly efficient knockdown, as Western blot analysis showed a near complete failure of AGS KD cells to induce HIF-1α protein under hypoxic conditions (Supplementary Fig. S1*A*; Fig. 1*A*). Furthermore, functional inactivation of HIF-1α was achieved in AGS KD cells as shown by greatly reduced protein expression of HIF-1α target genes carbonic anhydrase IX (CA IX) and phosphoglycerate kinase 1 (PGK1; Fig. 1*B*) and a significant reduction of a luciferase reporter plasmid driven by

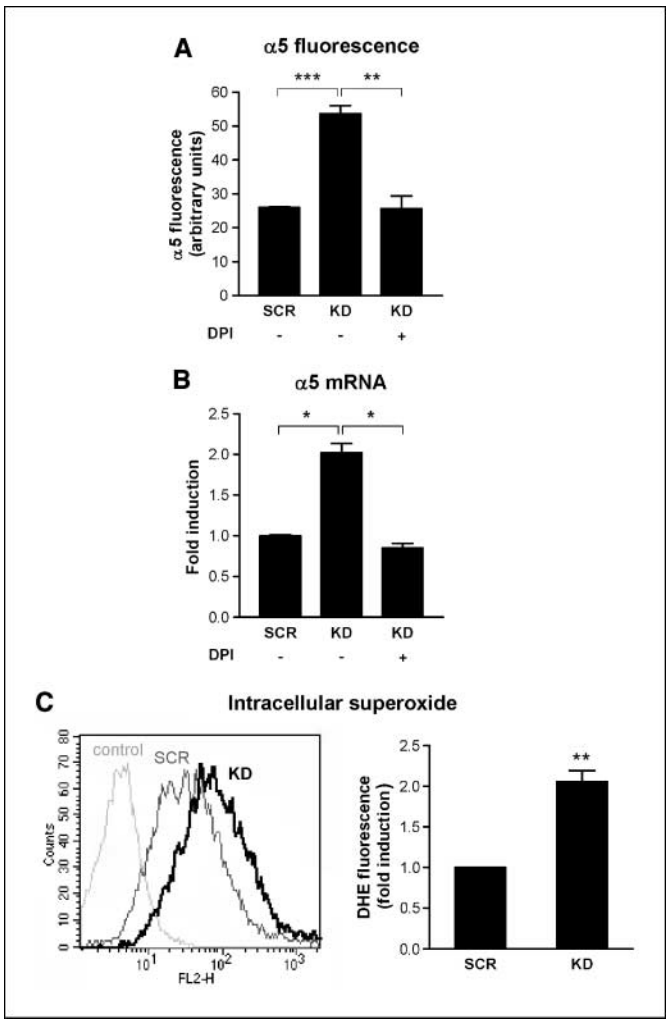



**Figure 3.** Contribution of ROS to α5 integrin induction in AGS KD cells. *A* and *B,* effect of the NADPH oxidase inhibitor DPI on α5 integrin expression on the surface (*A*) and α5 mRNA levels (*B*). Treatment of AGS KD cells with 5 μmol/L DPI for 24 h resulted in a significant decrease of α5 integrin expression to baseline levels. *A,* α5 integrin expression was determined by FACS analysis. *Columns,* mean of three experiments (***, $P = 0.0003$; **, $P = 0.0031$; unpaired Student's *t* test); *bars,* SE. *B,* α5 integrin transcription levels were expressed relative to that of untreated control cells (*SCR*), set at 1.0. *Columns,* mean (*, $P \leq 0.0123$; unpaired Student's *t* test); *bars,* SE. *C,* analysis of superoxide anion levels. AGS KD and SCR cells were cultured for 24 h under normoxia and stained with 10 μmol/L DHE, and dye oxidation was determined by flow cytometry. *Left*, a representative histogram of three experiments. *Columns,* mean of three independent experiments (*right*; **, $P = 0.0013$; unpaired Student's *t* test); *bars,* SE.

hypoxia-responsive elements (HRE) under hypoxic conditions (Fig. 1*C*). In a complementary pharmacologic approach, HIF-1α protein induction and transcriptional activity were inhibited by the compound 2ME2. Exposure of AGS cells to 2ME2 reduced the levels of nuclear HIF-1α protein under hypoxia in a dose-dependent manner (Fig. 1*D, top*). As a consequence, hypoxia-induced transcriptional activity of HIF-1α was suppressed by 2ME2 with comparable dose dependency (Fig. 1*D, bottom*). Taken together, these results show that HIF-1α could be functionally inactivated either by means of a lentivirus-based siRNA system or via treatment with the chemical compound 2ME2.

In our initial biological characterization of HIF-1α–deficient AGS and MKN28 cells, proliferation and viability were not different from SCR control cells.[4] However, a distinct reduction of migration and invasion capacity was noted.[5] Both migration and invasion rely on the dynamic and controlled interaction with the extracellular matrix (ECM) environment and are crucially regulated by the integrin family of large glycoproteins. Integrins function as transmembrane receptors that mediate cell adhesion to ECM and participate in signal transduction (10). Besides migration and adhesion, integrins are centrally involved in the regulation of other protumorigenic processes such as angiogenesis and metastasis (10). Changes in integrin expression frequently occur in the context of malignant transformation and have furthermore been linked to the metastatic phenotype, although individual integrins may function as promotors or suppressors of tumor progression. Interestingly, hypoxia has been shown to induce integrins on a variety of human cancer cell lines *in vitro* (11–13). In line with these findings, inhibition of HIF-1α resulted in down-regulation of β1 integrin on pancreatic cancer cells (14). Accordingly, we compared the surface presentation of a panel of integrins in HIF-1α–competent and HIF-1α–deficient AGS cells by flow cytometry (Supplementary Fig. S2). Overall integrin expression was comparable in AGS wild-type (WT) and AGS SCR cells, confirming that the lentiviral infection per se had not changed integrin expression. In contrast, a significant and selective induction of α5 integrin was apparent on the surface of AGS KD cells (Fig. 2*A*). The α5 subunit represents the limiting constituent of the fibronectin receptor, α5β1 integrin, and a highly attractive candidate target of HIF-1α regulation. Thus, we went on and corroborated the increased content of α5 integrin in AGS KD cells by immunoblotting experiments (Fig. 2*B*). Immunfluorescence microscopy revealed punctuate α5 fluorescence throughout the area of substrate attachment in SCR control cells. This pattern was similarly observed in AGS KD cells, which furthermore displayed intense membrane staining, consistent with a more abundant presentation of α5 integrin at the surface of HIF-1α–deficient AGS cells (Supplementary Fig. S3). A subsequent quantitative analysis of α5 integrin mRNA levels by real-time PCR documented a pronounced increase in AGS KD cells, indicating that α5 integrin induction occurred at the level of transcription (Fig. 2*C*).

To obtain independent evidence for the proposed HIF-1α–dependent regulation of α5β1 integrin, 2ME2 was added to AGS WT cells for pharmacologic inhibition of HIF-1α (Fig. 2*D*). Indeed, 2ME2 treatment resulted in a dose-dependent induction of α5 integrin presentation at the concentrations that were required to inhibit HRE reporter gene activity (please compare to Fig. 1*D*), consistent with a reduction of HIF-1α activity underlying integrin regulation. Thus, pharmacologic inhibition of HIF-1α confirmed its capacity to control the cellular α5 integrin complement.

Next, we aimed at identifying the molecular mechanism controlling the up-regulation of α5 integrin upon loss of HIF-1α. Integrin expression is regulated by numerous signaling pathways, consistent with the need to flexibly adjust the communication interface with the cellular surrounding. In this context, the level of reactive oxygen species (ROS) constitutes an intriguing intersection of integrin regulatory and HIF-1α effector pathways. ROS (including superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radicals) are natural byproducts of aerobic cellular metabolism. ROS are considered to be involved in carcinogenesis and act as important regulators of cell proliferation, apoptosis, and migration (15). They were shown to modulate mRNA expression of different integrin subunits in mammary epithelial cells (16). More specifically, increased α5 integrin expression of AGS cells upon colonization with *Helicobacter pylori* was reported to occur via elevated

intracellular ROS (17). To determine whether intracellular ROS levels contributed to α5 integrin induction in HIF-1α–deficient cells, AGS KD cells were treated with the flavoprotein inhibitor DPI. DPI has long been characterized as an inhibitor of mitochondrial complex I, both *in vitro* and *in vivo* (18). The vast majority of intracellular ROS are being produced by the mitochondrial electron transport chain (19). Here, complex I and complex III represent the major sites of ROS generation (19). Remarkably, treatment with DPI for 24 hours completely blocked the induction of α5 integrin on the surface and α5 integrin mRNA levels in HIF-1α–deficient AGS cells (Fig. 3*A* and *B*). This definite result strongly suggested elevated intracellular ROS levels as the molecular mediators of α5 integrin up-regulation upon loss of HIF-1α. To establish the causal role of HIF-1α for the redox potential of AGS cells, intracellular ROS levels were determined in AGS KD and SCR cells by flow cytometry. Cells were incubated with the nonfluorescent compound DHE, which in the presence of superoxide anions is oxidized to the fluorescent hydroethidium. We found that the intracellular superoxide levels in AGS KD cells were 2-fold higher than those in AGS SCR cells (Fig. 3*C*), indicating that functional inactivation of HIF-1α in AGS cells resulted in significant and functional elevation of intracellular oxidative stress. These results are well in line with recent reports demonstrating either increased ROS levels in normoxic HIF-1α–deficient mouse embryonic fibroblasts or reduced ROS in renal cancer cells harboring constitutive HIF–1α expression due to mutation of the von Hippel-Lindau tumor suppressor (20, 21). As mitochondria represent the prime source of intracellular ROS, HIF-1α activity may counteract ROS production via regulatory functions at the mitochondrial level. These include (*a*) incremental reduction of mitochondrial activity due to enhanced use of glycolysis even under conditions of sufficient oxygen availability (the Warbug effect; refs. 22, 23), (*b*) inhibition of pyruvate shuttling into the mitochondria (24) by the HIF-1 target gene pyruvate dehydrogenase kinase 1, which blocks mitochondrial pyruvate entry via inactivation of pyruvate dehydrogenase (25), and (*c*) direct inhibition of mitochondrial biogenesis, as was shown in renal cancer cells (21). Our analysis of human gastric cancer cells clearly showed elevated levels of ROS upon inactivation of HIF-1α and is therefore well in line with the above outlined pathway. Furthermore, our functional link between ROS and α5 integrin expression suggests that HIF-1–mediated suppression of ROS may constitute a critical pathway with respect to the acquisition of invasive and metastatic properties.

In the context of tumor biology, reduced α5β1 expression was reported in many human tumors including breast and colonic cancer cells (26, 27). Furthermore, experimental overexpression of α5β1 suppressed tumorigenicity of Chinese hamster ovary or human colonic carcinoma cells, suggesting α5β1 functions as a tumor suppressor (26, 28). Consistent with this notion, ras-transformed cells present a reduction of α5β1 integrin expression and conversely, reconstitution of the tumor suppressor

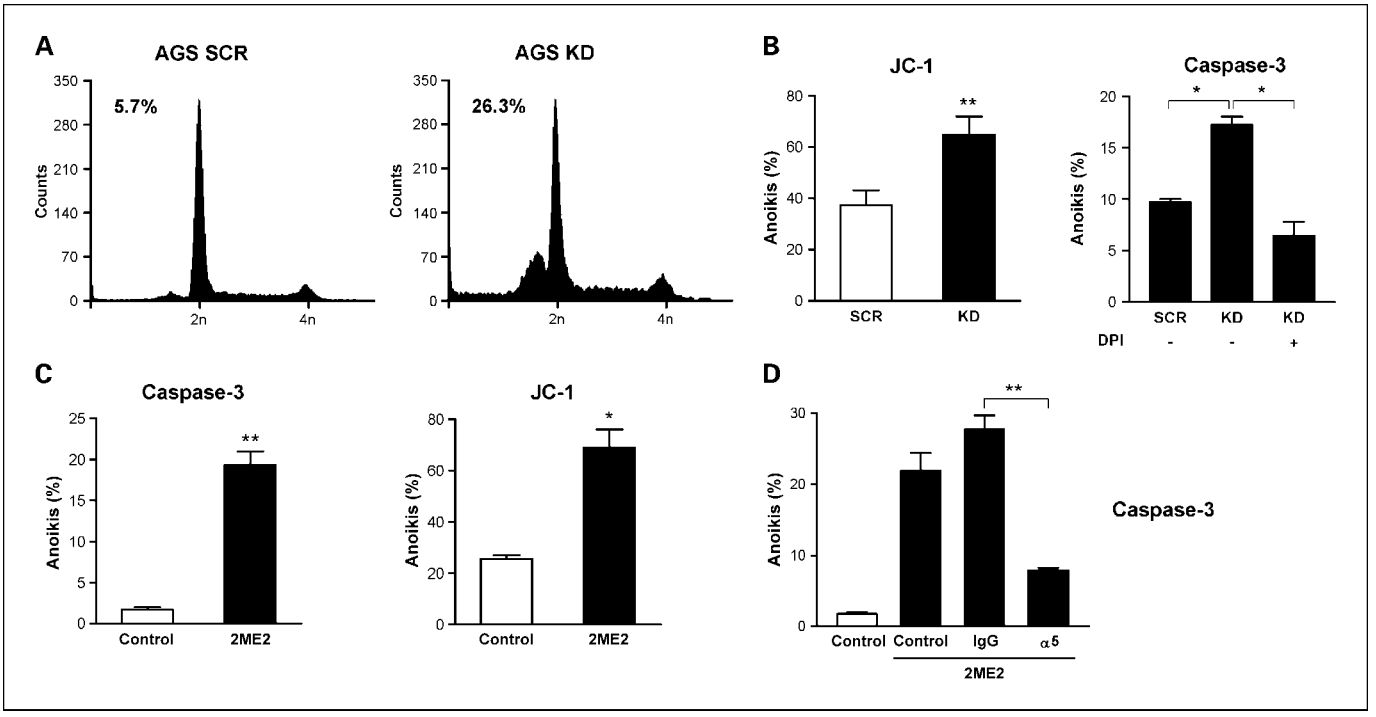



**Figure 4.** Effects of HIF-1α inhibition on anoikis. For induction of anoikis, AGS cells were cultivated on polyHEMA-coated dishes and subjected to apoptosis detection assays. *A,* AGS KD and SCR were cultivated for 48 h on polyHEMA and cell cycle distribution was determined by propidium iodide staining with subsequent FACS analysis. Shown are representative histograms. The fractions of cells in pre-$G_1$ are given as percentage of total cell number. A 4- to 5-fold increase of anoikis was detected in AGS KD cells compared with control cells. *B,* anoikis of AGS KD and SCR cells was assayed by JC-1 staining between 24 and 72 h after cultivation on polyHEMA (*left*) or by cleavage of caspase-3 (*right*). *Columns,* mean; *bars,* SE (*left*). AGS KD cells showed a significant increase of anoikis (**, $P = 0.0087$; unpaired Student's *t* test). *Right,* effect of DPI on anoikis. AGS KD cells were treated for 24 h with 5 μmol/L DPI, and anoikis was quantitated by activation of caspase-3. *Columns,* mean; *bars,* SE (*, $P \leq 0.0196$; unpaired Student's *t* test). Treatment with DPI rescued AGS KD cells from anoikis. *C,* AGS WT cells were pretreated with 10 μmol/L 2ME2 for 24 h and then replated onto either polyHEMA-coated or uncoated dishes. After 16 h, anoikis was quantitated by activation of caspase-3 (*left*) or JC-1 staining *(right*) followed by FACS analysis. *Columns,* mean of three experiments (*, $P = 0.0264$; **, $P = 0.0095$; unpaired Student's *t* test); *bars,* SE. Pharmalogical inhibition of HIF-1α by 2ME2 caused a significant induction of anoikis. *D,* AGS WT cells were pretreated with 10 μmol/L 2ME2 for 24 h and exposed to anoikis conditions either in the absence or in the presence of 15 μg/mL anti-α5 antibody (clone P1D6) or mouse control immunoglobulin for 16 h. Anoikis induction was determined by activation of caspase-3. Results are from two independent experiments (**, $P = 0.0099$; unpaired Student's *t* test). Incubation with a blocking α5 integrin antibody led to escape from anoikis in 2ME2-treated AGS cells.

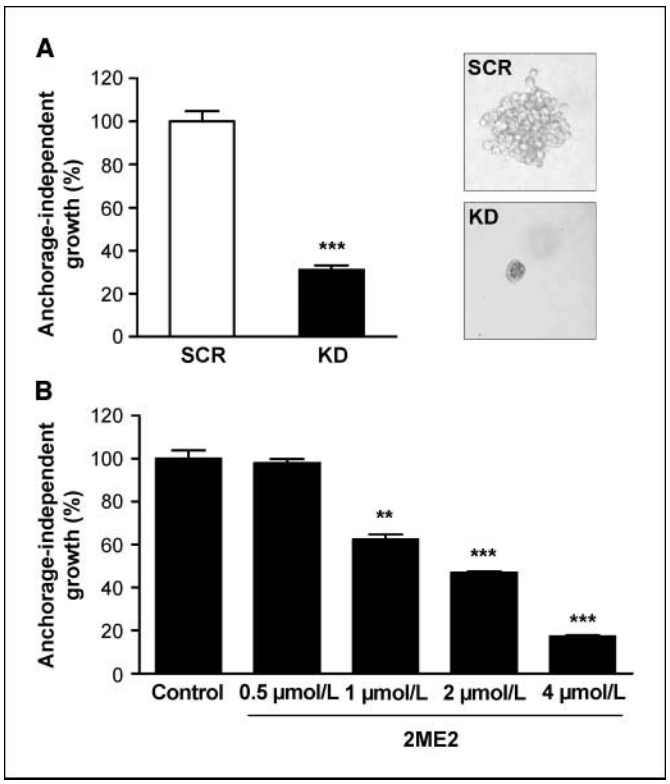



**Figure 5.** Effects of HIF-1α inhibition on anchorage-independent growth. Anchorage-independent growth was studied by soft agar colony formation assays. *A,* AGS KD and SCR cells were incubated for 12 d under normoxic conditions and vital colonies were counted. *Columns,* mean colony numbers derived from triplicate determinations of one representative from three independent experiments (*left*; ***, $P = 0.0002$; unpaired Student's *t* test); *bars,* SE. *Right,* colonies of AGS KD and AGS SCR cells. Loss of HIF-1α protein by RNAi inhibits anchorage-independent growth in normoxia. *B,* to evaluate anchorage-independent growth under treatment with 2ME2, the compound was added every third day in the indicated concentrations and vital colonies were counted after 12 d. *Columns,* mean of triplicate determinations from a representative of three experiments (**, $P = 0.0012$; ***, $P \leq 0.0005$; unpaired Student's *t* test); *bars,* SE. Treatment with 2ME2 resulted in a significant and dose-dependent reduction of anchorage-independent growth.

p16 increased the cellular α5β1 complement (8). However, α5β1 may to some extent support tumor growth. For instance, impaired vascularisation was noted in xenograft tumors derived from α5-deficient cells, suggesting that tumor cell derived α5β1 may promote tumor growth by orchestrating tumor-associated angiogenesis (29). When bound to ECM components, α5β1 integrin cooperates with growth factors to activate mitogenic and antiapoptotic signaling pathways. In contrast, loss of matrix contact generates proapoptotic signals reminiscent of death receptor activation. In consequence, epithelial cells that lack appropriate ECM contacts undergo a specific form of apoptosis called "anoikis" (30). Anoikis thus represents a central safeguard mechanism to preclude survival of epithelial cells in inappropriate locations. Importantly, the tumor suppressor function of the fibronectin receptor α5β1 integrin in epithelial cells has been linked to its ability to induce anoikis (31).

Therefore, we next compared the apoptosis fraction of HIF-1α–competent and HIF-1α–deficient AGS cells in either adherent cultures or after suspension culture on polyHEMA-coated dishes (anoikis conditions). The functional inactivation of HIF-1α did not result in different apoptosis fractions of adherent AGS cells as determined by the pre-$G_1$ fraction of cell cycle analyses and caspase-3 activity assays (Supplementary Fig. S4). In contrast, a marked difference in anoikis susceptibility was evident from the pre-$G_1$ fraction of cell cycle analysis, with a significantly increased anoikis fraction present in HIF-1α–deficient AGS KD cells (Fig. 4*A*). This difference in anoikis susceptibility was additionally confirmed and quantitated using JC-1, an early and sensitive marker of apoptosis, which reflects the integrity of the mitochondrial membrane potential (Fig. 4*B, left*).

To provide further support for the functional link between HIF-1α and anoikis, we used complementary pharmacologic approaches to test whether (*a*) inhibition of α5 integrin induction by DPI could revert the anoikis susceptible phenotype of AGS KD cells, or (*b*) conversely, suppression of HIF-1α activity by 2ME2 could sensitize AGS WT cells to anoikis. First, AGS KD cells were treated for 24 hours with DPI and subjected to apoptosis detection. As shown in Fig. 4*B* (*right*), the inhibition of ROS production by DPI rescued AGS KD cells from anoikis, demonstrating a pivotal role of ROS for the anoikis susceptible phenotype of HIF-1α–deficient gastric cancer cells. Second, we examined anoikis in AGS WT cells that were pretreated with 2ME2 (Fig. 4*C*). To avoid confounding toxic effects of prolonged 2ME2 treatment, we focused our analysis on early apoptosis induction using the mitochondrial membrane integrity and the presence of active caspase-3 as two independent approaches for quantitative determination of early apoptotic cells. Both variables revealed a significant induction of anoikis in 2ME2-treated cells, confirming enhanced susceptibility to anoikis after functional inactivation of HIF-1α (Fig. 4*C*).

This experimental approach was then exploited to address the functional relevance of α5 integrin for anoikis induction after pharmacologic inhibition of HIF-1α (Fig. 4*D*). AGS cells were pretreated with 2ME2 and exposed to anoikis conditions either in the absence or in the presence of an antibody to α5 integrin, and anoikis induction was determined based on the abundance of active caspase-3. Antibody treatment profoundly reduced 2ME2-induced anoikis almost to control levels, confirming a requirement for α5 integrin. This is in excellent agreement with the ability of DPI to reverse both, α5 integrin induction and anoikis in AGS KD cells, and firmly supports a functional role of α5 integrin in HIF-1α–dependent control of anchorage dependence.

Anchorage-independent survival has a major effect on tumorigenicity *in vivo*, a feature that is modeled *in vitro* by colony formation in agar suspension. Accordingly, we subjected HIF-1α–competent and HIF-1α–deficient AGS cells to culture in soft agar and monitored colony formation (Fig. 5). Compared with AGS SCR control cells, colony formation of AGS KD cells was profoundly suppressed and remaining colonies were notably smaller in size (Fig. 5*A*). Similarly, treatment of AGS WT cells with 2ME2 led to a dose-dependent suppression of colony formation over the concentration range required for HIF-1α functional inhibition (Fig. 5*B*). These results are in line with an earlier report that overexpression of HIF-1α in transformed melanocytes lead to an increase in the number of colonies in soft agar (32). Furthermore, anchorage-independent growth of human colon cancer cell lines has been shown to be reduced after functional inactivation of HIF-1α (33). However, the latter study also reported a growth defect of HIF-1α–deficient cells under anchorage-dependent growth conditions, raising the possibility that reduced colony formation resulted from cell cycle arrest rather than apoptosis induction. In sharp contrast, the anchorage-dependent proliferation of the gastric carcinoma cells used in the current study was not at all affected by knockdown of HIF-1α. Moreover, AGS cells displayed little spontaneous apoptosis under

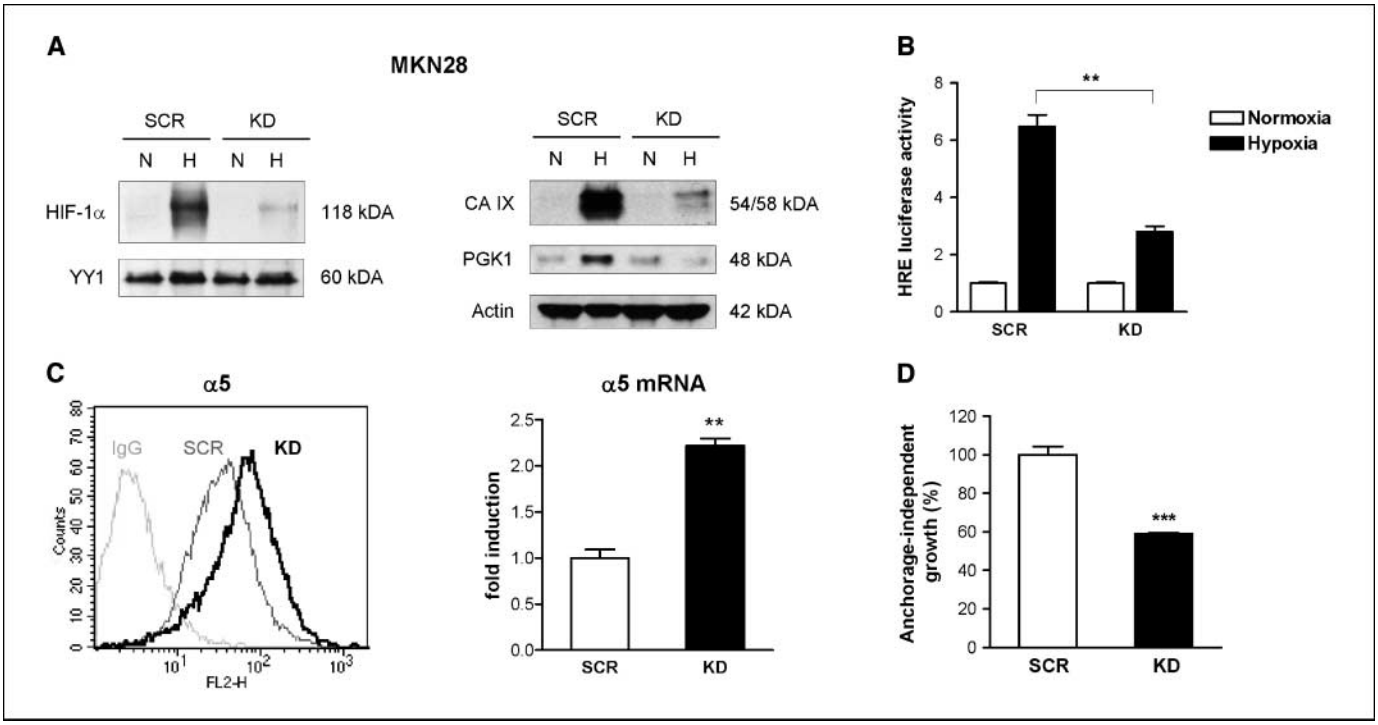



**Figure 6.** Inhibition of HIF-1α in MKN28 gastric cancer cells. *A,* inactivation of HIF-1α by RNA interference was analyzed by Western blot analysis of HIF-1α (*left)* and HIF-1 target genes PGK1 and CA IX (*right*). HIF-1α protein and HIF-1 target genes PGK and CA IX were markedly decreased in MKN28 KD cells under hypoxic conditions. *B,* loss of HIF-1α function was confirmed by HRE-luc reporter assay. *Columns,* mean of three independent experiments, each performed in triplicate; *bars,* SE (**, $P = 0.0012$; unpaired Student's *t* test). RNA interference against HIF-1α resulted in a significant decrease of HRE-luc reporter activity under hypoxic conditions. *C,* α5 integrin expression in MKN28 KD and SCR cells was determined by FACS analysis (*left*) and by quantitative real-time PCR (*right*). Inactivation of HIF-1α by RNAi resulted in an increase of α5 integrin on the surface (*left*) and α5 mRNA (*right*). *Left*, a representative histogram. *Right*, transcription levels of α5 integrin were analyzed relative to β-actin. *Columns,* mean (**, $P = 0.0096$; unpaired Student's *t* test); *bars,* SE. *D,* anchorage-independent growth of MKN28 KD and SCR cells was examined using a soft agar colony formation assay under normoxic conditions. *Columns,* mean of a representative of three experiments, each performed in triplicate (***, $P = 0.0007$, unpaired Student's *t* test); *bars,* SE. Inhibition of HIF-1α suppressed anchorage-independent growth.

anchorage-dependent growth conditions, and these low apoptosis rates were not influenced by HIF-1α inhibition (Supplementary Fig. S4). Hence, our results constitute the first report about a permissive role of HIF-1α for tumor cell survival under anchorage-independent conditions in normoxic microenvironments.

To extend these findings to other gastric cancer cells, key observations were confirmed in MKN28 cells. Characterization of the RNA interference against HIF-1α in this cell line is provided in Fig. 6. MKN28 KD cells showed a complete loss of HIF-1α protein expression under hypoxic conditions and a significant decrease in transcriptional activity of HIF-1α as determined by target protein expression and HRE-luciferase activity (Supplementary Fig. S1*B*; Fig. 6*A* and *B*). In good agreement with our observations on AGS cells, MKN28 KD cells induced surface α5 integrin expression and α5 integrin mRNA levels (Fig. 6*C*) and displayed a significant inhibition of colony formation in soft agar (Fig. 6*D*), indicating that the HIF-1α–dependent control of anchorage-independent growth is not restricted to the AGS cell system.

In summary, the herewith presented experiments argue for a central role of HIF-1α in the control of anoikis via suppression of α5 integrin. The exploration of HIF-1α–inhibiting compounds in clinical studies of gastric cancer might inaugurate efficient and novel treatment regimes for this fatal disease.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

Received 5/15/2008; revised 9/9/2008; accepted 10/6/2008.

**Grant support:** Deutsche Forschungsgemeinschaft (CR 133/2-2), Berliner Krebsgesellschaft (CRFF200804; T. Cramer), and Deutsche Forschungsgemeinschaft (Graduiertenkolleg 276/4, "Signalerkennung und –umsetzung"; N. Rohwer).

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We thank Daniel C. Chung (Harvard Medical School, Boston, MA) for providing plasmids containing shRNA against human HIF-1α and Jaromir Pastorek (Slovak Academy of Sciences, Bratislava, Slovakia) for the CA IX antibody.

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*Cancer Res* 2008;68:10113-10120.