Evaluating Hypoxia-Inducible Factor-1α as a Cancer Therapeutic Target via Inducible RNA Interference In vivo

Leiming Li, Xiaoyu Lin, Michael Staver, Alexander Shoemaker, Dimitri Semizarov, Stephen W. Fesik, and Yu Shen


Abstract

Validating potential targets is an important step in the drug discovery process. In this study, we tested the feasibility of using inducible RNA interference (RNAi) in vivo to obtain an unbiased evaluation on the efficacy of inhibiting hypoxia-inducible factor-1α (HIF-1α) in established tumors. We showed that HIF-1α inhibition resulted in transient tumor stasis or tumor regression, and inhibiting HIF-1α in early-stage tumors was found to be more efficacious than inhibiting HIF-1α in more established tumors. A differential requirement of HIF-1α for tumor growth was also observed among different tumor types. Examination of tumors resistant to HIF-1α inhibition suggested that the resistance might result from a less hypoxic tumor environment and the level of HIF-1α expression in tumors may be a useful marker for predicting tumor response to HIF-1 inhibition. This study shows that inducible RNAi is a versatile tool for evaluating cancer targets in vivo. In addition to broad implications on in vivo validation of cancer targets, results from this study will also be instructive for practical applications of HIF-1–based cancer therapeutics. (Cancer Res 2005; 65(16): 7249-58)

Introduction

Validating potential targets in animal models is one of the most critical steps in drug discovery. However, current approaches for evaluating cancer targets in vivo have various limitations. RNA interference (RNAi) is a process for silencing gene expression using double-stranded RNA (1–5). Many attempts have been made to use small interfering RNAs (siRNA) or short hairpin RNAs (shRNA) to inhibit targets in tumors for validating cancer targets in vivo (6–8). However, using these methods, the siRNA is only delivered to a small percentage of the tumor cells, which makes it difficult to predict the potential efficacy of small-molecule inhibitors against the target. To better evaluate the in vivo efficacy of a target, knockdown of the target should occur in >90% of the tumor cells, a level that is often achievable with small-molecule inhibitors. In addition, it would be advantageous to have the flexibility of initiating target inhibition at various stages of tumor development to allow the evaluation of target inhibition on tumor maintenance and metastasis. The recent development of regulated shRNA expression systems provides a potential solution for validating cancer targets in vivo (9–13). Using such systems, cancer cell lines could be established to express shRNAs against the target under the tight control of an inducing reagent. These cell lines would allow us to create xenograft tumors to evaluate the effects of target knockdown at different stages of tumor development.

Hypoxia-inducible factor-1 (HIF-1) is a transcriptional factor that regulates cellular response to hypoxia (14–18). HIF-1α is the regulatory subunit of HIF-1. The requirement of HIF-1α for tumor growth has been examined by comparing the growth of tumors derived from HIF-1α-deficient and HIF-1α wild-type cells (19–23). Although most of the studies suggested that the loss of HIF-1α function results in slower tumor growth, one study showed that the loss of HIF-1α function leads to more aggressive tumors (22). The significance of inhibiting HIF-1 in established tumors have also been shown using small-molecule inhibitors of the HIF-1 pathway (24, 25). However, these small-molecule inhibitors often have other activities in addition to inhibiting the HIF-1 pathway, making it difficult to distinguish whether tumor responses observed using these inhibitors are solely due to the inhibition of HIF-1 pathway or a combined inhibitory effect on multiple biological processes. Therefore, it will be beneficial to develop a highly specific method to abrogate the HIF-1 pathway in established tumors for the evaluation of HIF-1 as a cancer therapeutic target.

In this study, we tested the feasibility of using inducible RNAi in vivo to obtain an unbiased evaluation of the potential therapeutic effect of inhibiting HIF-1α in established tumors. We used the previously reported 202-inducible system to establish various cancer cell lines that express an shRNA-targeting HIF-1α under the control of doxycycline (13). Using these cell lines, we generated xenograft tumors and carefully evaluated the effect of inhibiting HIF-1α in two tumor types.

Materials and Methods

Cell lines, short hairpin RNAs, and small interference RNAs. D54MG-tetR and MDA-MB435LM-tetR, the D54MG and MDA-MB435LM-derived cell lines that constitutively express tetR, were established using the pCDNA6/TR plasmid (Invitrogen, Carlsbad, CA) according to standard protocols. To establish inducible knockdown cells, D54MG-tetR and MDA-MB435LM-tetR cells were cotransfected with a linear hygromycin marker (Clontech, Palo Alto, CA) and a plasmid that use the 2O2 promoter to express an shRNA against the target. The following shRNA sequences were used in the study: “GACAGUACAGGAUGUCUGCUUCUUAGAGCAAGCAUCUCUGACUCUCUGCU” for HIF-1α, “GGAGACACUUAGCGUGACUGCUCCUGAAGCUGACUCUCUGACUCUCUGACUCUCU” for luciferase, and “GGAGACACUGCGACAGAGUCUCUCUUAGAGCAACUCUGCUGCGCUGACUC” for HIF-2α. To induce shRNA expression, cells were incubated in medium containing 1 μg/mL doxycycline for 36 hours. For hypoxia treatment, cells were incubated in an environment of 1.5% O2 and 5% CO2 at 37°C for 16 hours.

Reporter assay. Cells in a 96-well plate were transfected with pGL3-control or pHRE, a reporter construct containing the hypoxia responsive element (HRE) from the enolase promoter, together with pRL-TK (Promega, Madison, WI) using LipofectAMINE 2000 (Invitrogen). Three hours after transfection, the medium was replaced by fresh medium with or without 1 μg/mL doxycycline. Thirty-six to 48 hours after transfection, cells were
subjected to hypoxia treatment for 16 hours and the luciferase activity was determined using the Dual-Luciferase Assay System (Promega).

Xenograft models. All cell lines used to generate xenograft tumors were subjected to the IMpact profile 1 Test (18 agents) at The University of Missouri Research Animal Diagnostic and Investigative Laboratory, and each cell line was found negative for all 18 infectious agents tested. Scid mice of 6 to 8 weeks of age were obtained from the Charles River Laboratory (Wilmington, MA). Cells were injected s.c. into the hind quarters of mice as a 1:1 mixture with matrigel (BD Biosciences, San Jose, CA). D54MG-derived cells were injected into scid male at 5 × 10^6 cells per site, and MDA-MB-435LM-derived cells were injected into scid female at 1 × 10^6 cells per site. Tumors were measured twice weekly using a microcaliper, and the volume dimension was calculated according to the formula: (long dimension) × (short dimension)^2 / 2. Doxycycline (Sigma, St. Louis, MO) was given through a 1:1 mixture with matrigel (BD Biosciences, San Jose, CA) and 3,3′-diaminobenzidine (Sigma) was used for staining of all markers. For immunohistochemical staining of HIF-1α, methyl green (Vector Laboratories, Burlingame, CA) was used for counter staining. For all other markers, Hematoxylin 7211 (Richard-Allan Scientific, Inc., Kalama-zoo, MI) was used for counter staining. To quantify tumor necrosis, the standard H&E procedure was followed to detect necrotic regions (Eoin-X and Hematoxylin 7211) were both purchased from the Richard-Allan Scientific. To minimize systemic errors and avoid the cross-comparison of immunohistochemistry on samples from different experiments or samples processed at different times, immunohistochemical staining for the same antigen was done on all samples from one experiment as one batch at the same time.

Image acquisition. Immunohistochemical images were acquired using the ACISII automated imaging system (Chroma Vision, San Juan Capistrano, CA). Briefly, slides were loaded onto the imaging system, and the machine automatically adjusted the focus and exposure time and captured images of a section using a 10 × objective lens by scanning the entire section. Depending on the size of the section, 80 to 400 separate images were acquired to cover the entire section, and these images were automatically composited into one image and exported for analysis. In this study, >200 nonoverlapping images were typically acquired to cover an average section with a size of 50 mm^2.

Image analysis. The images exported from the ACISII automated imaging system were analyzed using the “AxioVision 4” software. To ensure an unbiased analysis, the pictures were coded without revealing the identities of the samples to the analyst during the image analysis process. To determine the necrotic index, the necrotic regions were identified based on H&E staining using the criteria of shrinkage or loss of the cell nucleus. The necrotic regions in a section were manually circled, and the software calculated the size of the encircled regions. Tumor necrotic index is calculated using the formula: the size of necrotic regions/the size of the whole section. To determine the percentage of positive staining areas for HIF-1α, Ki67, active caspase 3, and Glut1, a control slide was used to set the threshold for positive staining of a particular antigen and saved as the standard. The same standard was applied for all sections stained against the same antigen in one batch. To exclude necrotic regions in analysis, the necrotic areas were first manually identified based on the counter staining. The entire section excluding the necrotic regions was selected as the area of interest, and the software automatically calculated the size of positive staining areas in the area of interest and the overall size of the area of interest. The percentage of positive staining area of a marker was calculated using the formula: size of the positive staining area / size of the area of interest (the entire section minus necrotic regions). To determine the microvesSEL density, the CD31-positive staining areas were scored using the same method as other markers. The software automatically calculated the number of vessels and the size of each vessel in the section. Three different analyses were done regarding the tumor vasculature based on the CD31 staining. These include the number of vessels per square unit (vessel density), the percentage of CD31-positive areas in the section (both the number and the size of the vessels were taken into consideration using this analysis), and the size distribution of vessels in each section. The same conclusion was reached using each analytic method, and only the percentage of positive CD31 staining areas were presented.

Vascular endothelial growth factor ELISA. The Quantikine human VEGF ELISA kit (R&D Systems) was used to measure the VEGF levels in culture supernatants. To measure VEGF in xenograft tumors, the tumors were homogenized in Celllytic-M (Sigma) with protease inhibitors, and the amount of VEGF was measured using the QuantiGlo human VEGF kit (R&D Systems).

Quantitative reverse transcription-PCR. Quantitative reverse transcription-PCR (QPCR) analysis for human HIF-1α was done using the I-Cycler machine (Bio-Rad, Richmond, CA). Gene-specific primers and Taqman probe sequences were as follows: forward primer, 5′-ATGAAACAATAGGTCGCAAGTGG-3′; reverse primer, 5′-CTGAGGT-TTGGTTACTGGTGTTATCATATA-3′; probe, 5′-356FAM/TTGCACTGCA-CAGGCCACATTCAATCG-3′. This primer/probe set was designed to hybridize to a unique sequence in the human HIF-1α mRNA to avoid cross-hybridization to the microtissues. The specificity of the primer/probe set was confirmed by two independent QPCR analyses using total RNAs from the HT1080 (human) cells and the NIH-3T3 (mouse) cells. Whereas the primers detected HIF-1α mRNA in the human cells, no HIF-1α transcripts was detected in the mouse cells. QPCR analyses for lactate dehydrogenase (LDH) and phosphoglycerate kinase 1 (PGK1) were done using the ABI-7700 instrument with ABI’S “assay in demand” validated primer/probe mixtures (LDHA, PGK1). Each reaction was run in triplicate. Data analysis was done using a standard curve method.

Statistical analyses. The single-factor ANOVA test was used to compare the transient tumor regression in D54_Hif tumors with the knockdown initiated at day 2 after inoculation, 85, 190, or 275 mm^2. The “Exponential Growth Test” was used to compare tumor growth rates. In this analysis, tumors were assumed to grow in an exponential manner, and the logarithmic value of tumor volumes versus time was fit in a linear regression slope test. The ANOVA test and the exponential growth test were preformed using the Prism 4 software (GraphPad Software, San Diego, CA). Nonpaired Student’s t test was used for the statistical analysis of data from VEGF ELISA and immunohistochemical quantifications of Glut1. In all analyses, P < 0.05 was considered statistically significant.

Results

Establishment of cancer cell lines that knock down hypoxia-inducible factor-1α under the control of doxycycline. To assess the potential therapeutic effect of inhibiting HIF-1, we established D54_Hif, a D54MG-derived cell line that expresses a shRNA against HIF-1α under the control of doxycycline. Compared with D54_Luc, a control cell line that expresses an shRNA against...
luciferase upon doxycycline induction, the D54_Hif cells produced a similar level of HIF-1α protein in the absence of doxycycline, suggesting that the 202 inducible system is very tightly regulated (Fig. 1A, D54_Hif, Dox− versus D54_Luc, Dox−). Upon doxycycline induction, the D54_Hif cells exhibited complete knockdown of the HIF-1α protein (Fig. 1A, D54_Hif, H), and an 80% reduction of the HIF-1 reporter activity (Fig. 1B, pHRE, D54_Hif). Transfection of these cells with a potent shRNA against HIF-2α did not result in further inhibition of the reporter activity (data not shown), indicating that HIF-1α is the predominant form in these cells. QPCR analysis indicated that doxycycline treatment in D54_Hif cells resulted in decreased transcription of HIF-1 downstream factors such as PGK1 and LDH (Fig. 1C, D54_Hif, H, PGK, and LDH). As a control, doxycycline treatment in D54_Luc cells caused a reduction of the luciferase reporter activity (Fig. 1B, D54_Luc, pGL3) but not the knockdown of HIF-1α protein (Fig. 1A, D54_Luc, H) or inhibition of HIF-1 target genes (Fig. 1C, D54_Luc, H, PGK, and LHD). These results show that expression of an shRNA against HIF-1α results in the impairment of HIF-1 dependent transcription, and this inhibitory effect results from the specific inhibition of HIF-1α rather than from a nonspecific effect of shRNA expression or doxycycline treatment.

Doxycycline treatment induces hypoxia-inducible factor-1α knockdown in xenograft tumors. To determine whether target knockdown could be induced in xenograft tumors, we created s.c. tumors using the D54_Hif cells and examined the mRNA levels of HIF-1α in tumors after doxycycline treatment. An 80% reduction of the HIF-1α mRNA was observed in tumors from mice that received doxycycline for 3 days, and the knockdown was sustained over the 12-day treatment period (Fig. 2A, D54_Hif). Examination of the tumor samples by immunohistochemistry indicated a clear reduction of the HIF-1α protein from day 3 onward (data not shown). To determine whether doxycycline can penetrate larger tumors to induce target knockdown, we examined HIF-1α knockdown in tumors that were treated with doxycycline for <7 days with sizes ranging from 150 to 1,059 mm³. If the ability of doxycycline to induce target knockdown is impaired in larger tumors, a differential degree of HIF-1α knockdown should be expected for different sized tumors. However, equivalent degrees of HIF-1α knockdown were observed in all of the tumors at the mRNA level regardless of the tumor size (Fig. 2B). A robust knockdown of HIF-1α in all of these tumors was also confirmed by immunohistochemical analyses (data not shown). These results indicate that doxycycline can penetrate tumors of different sizes and reach a concentration that is sufficient for the induction of shRNA expression. To determine whether the ability of doxycycline to induce target knockdown would be impaired with long-term doxycycline treatment or when the tumors reach a very large size, we further analyzed the knockdown of HIF-1α in tumors from mice treated with doxycycline for 45 days. The average tumor size in these mice was 2,000 mm³. Compared with similar sized control tumors from water-treated mice, doxycycline treatment resulted in a robust knockdown of HIF-1α (Fig. 2C, QPCR analysis). The degree of target knockdown in these 2,000 mm³ tumors is comparable with that of 150 to 1,059 mm³ tumors (Fig. 2C versus B). Furthermore, immunohistochemistry analyses showed that the HIF-1α protein was reduced to a barely detectable level in these 2,000 mm³ tumors compared with similar sized tumors without

---

**Figure 1.** Establishment of D54MG derived HIF-1α inducible knockdown cell lines. A, D54_Luc and D54_Hif cells were incubated in the presence or absence of 1 μg/mL doxycycline. After 36 hours, cells were either untreated (N) or subjected to hypoxia treatment (H) for additional 16 hours. The cells were lysed and analyzed by Western blotting using antibodies against HIF-1α (top) or HIF-1β (bottom). B, D54_Luc and D54_Hif cells were transfected with either pGL3-control/pRL-TK (left) or pHRE/pRL-TK plasmids (right) in the presence or absence of 1 μg/mL doxycycline. Thirty-six hours following transfection, cells were subjected to hypoxia treatment. Luciferase activities were determined 16 hours after hypoxia treatment. C, D54_Luc and D54_Hif cells were incubated in the presence or absence of 1 μg/mL doxycycline. After 36 hours, cells were either untreated (N) or subjected to hypoxia treatment (H) for an additional 16 hours. Total RNA were isolated and used for QPCR analysis of the levels of the indicated genes.
doxycycline treatment (Fig. 2C, immunohistochemical images and quantification, D54_Hif, Dox versus Control). These results indicate that strong suppression of target expression can be sustained for a long period of time, even when tumors reach a very large size. As a control, doxycycline treatment of tumors generated using the D54_Luc cells did not affect HIF-1α expression at both mRNA and protein levels (Fig. 2A and C, D54_Luc), indicating that the reduction of HIF-1α in D54_Hif tumors upon doxycycline treatment results from the expression of a shRNA against HIF-1α.

Small interfering RNA–mediated inhibition of hypoxia-inducible factor-1α delays D54MG tumor growth. To assess the effect of inhibiting HIF-1α in established tumors, we created s.c. tumors using the D54_Hif cells and initiated the knockdown of HIF-1α when tumors reached an average size of 190 mm³. HIF-1α knockdown resulted in two phases of tumor growth. In the initial phase (Fig. 3A, days 1-11), tumors continued to grow but at a slightly slower growth rate compared with tumors with functional HIF-1α (linear regression slope test, k = 0.067 versus 0.096; P = 0.226). In the second phase, tumors remained static before regaining growth (Fig. 3A, day 11 and afterwards). Although the overall tumor growth seemed slower in the doxycycline-treated group compared with the control group (linear regression slope test, k = 0.059 versus 0.104; P = 0.0029), all tumors in the treated group eventually grew to very large sizes (Fig. 3A, Hif). As a control, xenograft tumors generated from D54_Luc cells grew at the same rate regardless of the presence or absence of doxycycline treatment, indicating that the slower growth phenotype of tumors expressing the HIF-1α shRNA was a consequence of HIF-1α knockdown (Fig. 3A, Luc). A similar inhibitory effect of HIF-1α knockdown on tumor growth was observed using two independent clones that express HIF-1α shRNA upon doxycycline treatment, showing that the observed effect is consistent and not due to an aberrant clone (data not shown). These results suggest that the loss of HIF-1α in established tumors cause a transient crisis, which resulted in tumor stasis. However, tumors quickly adapt to the loss of HIF-1α and continue to grow to a large size. To examine whether tumors adapted to the loss of HIF-1α by increasing the expression of HIF-2α, we examined HIF-2α expression in tumors treated with doxycycline or water for 6, 12, and 23 days. All tumors have only barely detectable sporadic HIF-2α staining, and no increase of HIF-2α staining was observed in doxycycline-treated tumors (data not shown). In contrast, HIF-2α staining in the 786-O tumors revealed a very high level of HIF-2α expression, indicating that the lack of HIF-2α staining in D54 tumors is not due to a poor sensitivity of the HIF-2α antibody (data not shown). These results suggest that tumors adapt to the loss of HIF-1α through a mechanism that does not involve compensatory HIF-2α expression.

Inhibition of hypoxia-inducible factor-1α during the early stages of tumor growth is more efficacious than inhibition of hypoxia-inducible factor-1α in more established tumors. Tumors at different stages of growth could have differential growth rates and response to HIF inhibition. To test this, we induced HIF-1α knockdown in established tumors (Fig. 3B) and quantified tumor sizes (Fig. 3C). Tumors at different stages of growth could have differential growth rates and response to HIF inhibition. To test this, we induced HIF-1α knockdown in established tumors (Fig. 3B) and quantified tumor sizes (Fig. 3C). The difference in growth rates of tumors at different stages of growth is more evident when tumors are treated with doxycycline for 6 days (linear regression slope test, k = 0.096; P = 0.039 versus 0.104; P = 0.0029). These results suggest that tumors adapt to the loss of HIF-1α through a mechanism that does not involve compensatory HIF-2α expression.
requirements for HIF-1. To address whether the effect of HIF-1α inhibition on tumor growth will be affected by the stage of tumor development, HIF-1α knockdown was initiated either at the second day after inoculation or when tumors reached an average size of 85 or 275 mm³. Two phases of tumor growth were also observed when the knockdown was initiated at the second day after inoculation or at 85 mm³. However, compared with the tumor stasis observed when the knockdown was initiated at 190 mm³, tumor regression was observed when the knockdown was initiated at day 2 after inoculation or at 85 mm³ (Fig. 3B, Hif, left and middle versus Fig. 3A, Hif). When the knockdown was initiated after the tumors reached an average size of 275 mm³, the negative effect of HIF-1α inhibition on tumor growth was greatly diminished and no tumor regression was observed (Fig. 3B, right). The duration of tumor regression in each mouse from the four treatment groups (knockdown at day 2 after inoculation, or when tumors reached an average size of 85, 190, and 275 mm³) were examined and the differences between groups were found to be statistically significant (P < 0.00001, single-factor ANOVA test). Dashed lines, average of each group.

Figure 3. The effect of HIF-1α knockdown on tumor growth. A, after tumors reached the average size of 190 mm³, mice were supplied with (Dox) or without (Control) doxycycline, and tumor growth was monitored (15 mice/group). B, tumor growth in water-treated mice or in mice that were supplied with doxycycline the second day after inoculation (left, 15 mice per group) or when the average tumor size reached 85 mm³ (middle, 10 mice per group) or 275 mm³ (right, 10 mice per group). Points, means of tumor size (n = 10-15); bars, SE (A and B). C, duration of tumor regression from individual doxycycline treated mice in (A) and (B) was plotted. "Duration of regression" is defined as the period of time that starts when a tumor begin to reduce in size and ends when the tumor regain growth and reach the preregression size. Because of the two phases of tumor growth after doxycycline treatment, tumors typically start to regress around day 11 after doxycycline treatment. The difference between groups is statistically significant (P < 0.00001, single-factor ANOVA test).

Taken together, these results suggest that early-stage tumors are more sensitive to HIF-1α inhibition compared with more established tumors.

The transient tumor stasis/regression upon hypoxia-inducible factor-1α knockdown is correlated with a transient drop and recovery of the glucose transporter 1 level in tumors. To gain insight into how HIF-1α inhibition leads to tumor stasis or regression, we first examined whether the loss of HIF-1α could affect cell growth. Under both normoxic and hypoxic conditions, the D54_Hif cells have almost completely overlapping growth curves in the presence or absence of HIF-1α (Fig. 4A, solid lines versus dotted lines), suggesting that the inhibition of HIF-1α does not affect cell growth in vitro. Based on the observed transient tumor stasis/regression, we speculated that any causative changes at the molecular level should precede tumor stasis/regression. Therefore, tumor samples from mice that were doxycycline treated or untreated for 6 and 12 days were collected and analyzed for various markers. No significant differences in Ki67 and active caspase 3 were detected in tumors from doxycycline-treated versus untreated mice (Fig. 4B, Ki67 and Active caspase 3), indicating the lack of apparent difference in tumor cell proliferation and apoptosis in the presence or absence of HIF-1α signal. In contrast, a clear increase in tumor
necrosis was observed in the treated versus the control tumors (Fig. 4B, Necrosis and C). Because activation of angiogenesis and glycolysis are two major consequences of HIF-1 signaling, we further examined microvessel density, VEGF production, and the Glut1 protein levels in tumors with or without HIF-1α knockdown. In tumors from mice treated with doxycycline, the VEGF level dropped from days 6 to 12 (Fig. 5A, VEGF). Similar levels of inhibition (30-50%) as at day 12 were also observed in tumors from mice treated with doxycycline for longer periods (data not shown), indicating that sustained inhibition of VEGF was achieved in tumors with HIF-1α knockdown. Although a >50% reduction of VEGF was observed in tumors with HIF-1α knockdown, we failed to detect a significant reduction of microvessel density in these tumors (data not shown). These results suggest that HIF-1α knockdown in D54MG-derived tumors do not lead to a significant change in tumor vasculature. It has been reported that although there is no gross alteration of tumor vasculature in hif-1α−/− tumors, VEGF expression in hif-1α−/− tumors exhibited a distinct punctuated pattern (20). To determine whether HIF-1α knockdown might lead to a similar spatial change in VEGF expression, we examined VEGF expression in tumors by immunohistochemical staining. In addition to the predominant VEGF staining surrounding necrotic areas, sporadic VEGF staining was also present throughout the tumor (Fig. 5B, Control, Necrotic area, and Sporadic staining). Interestingly, although the VEGF staining surrounding the necrotic areas was greatly reduced in HIF-1α knockdown tumors (Fig. 5B, Necrotic areas), the sporadic VEGF staining in these tumors was not affected (Fig. 5B, Sporadic staining), suggesting that the sporadic VEGF expression is independent of HIF-1α. The Glut1 level in tumors exhibited an interesting trend after HIF-1α knockdown. In tumors from water-treated mice, Glut1 levels were increased from days 0, 6, and 12, presumably due to the increase in tumor hypoxia caused by the increase in tumor size (Fig. 5A, Glut1, control). In contrast, in tumors from doxycycline-treated mice, a small but reproducible reduction of Glut1 at day 6 was observed (Fig. 5A and C, Glut1, day 6). However, the Glut1 level in doxycycline-treated tumors quickly rebounded to a level that was higher than the pretreatment level at day 12 (Fig. 5B, Glut1, Dox). The transient tumor stasis/regression observed after doxycycline treatment seemed to track with the transient drop and recovery of Glut1, suggesting a potential link between the effect of HIF-1α inhibition on glycolysis and tumor growth.

Figure 4. HIF-1α knockdown in tumors is associated with an increase in necrosis. A, growth of D54_Hif and D54_Luc cells in vitro in the presence (Dox+) or absence of doxycycline (Dox−) was determined under both hypoxia (H) or normoxia (N) conditions using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay. B, mice bearing the D54_Hif tumors with an average size of 200 mm³ were treated with or without (Control) doxycycline for 0, 6, or 12 days, and analyzed by IHC for Necrosis, Ki67 and active caspase 3. The image acquisition and analysis were done as described in Materials and Methods. Points, means of three tumors from each treatment group; bars, SD. C, representative images of H&E-stained tumor samples from mice treated with doxycycline (day 12) or water (Control, day 12) for 12 days. *, necrotic region.
Hypoxia-inducible factor-1α is not required for MDA-MB435LM tumor growth. To determine whether tumors of different types also require HIF-1α for rapid growth, we established MDA_Hif, the MDA-MB435LM-derived stable clone that express an shRNA against HIF-1α under the control of doxycycline. These cells exhibited complete knockdown of HIF-1α and a >80% inhibition of the HIF-1 reporter upon doxycycline treatment (Fig. 6, MDA_Hif, H and B, pHRE, MDA_Hif). Surprisingly, knockdown of HIF-1α in MDA_Hif-derived tumors did not have any significant effect on tumor growth (Fig. 6C, MDA_Hif, Control versus Dox). The lack of growth inhibition was not due to the loss of shRNA expression in these tumors, because QPCR analysis clearly showed that HIF-1α was knocked down in the doxycycline-treated group at both early and late time points (Fig. 6D). Furthermore, immunohistochemical staining of HIF-2α revealed barely detectable HIF-2α expression in the MDA tumors before doxycycline treatment and after doxycycline treatment for 6 and 12 days (data not shown), indicating that the lack of response to HIF-1α knockdown is not due to a compensatory increase of HIF-2α expression in these tumors. Similar results were observed in tumors derived from four different types of tumors.
additional MDA-derived inducible knockdown clones, indicating that the lack of tumor growth defect upon HIF-1α knockdown was not due to an aberrant clone (data not shown).

To understand the molecular mechanism that leads to the resistance to HIF-1α inhibition in MDA-derived tumors, we first examined the MDA_Hif cells in vitro for their response to hypoxia treatment. The accumulation of HIF-1α and the activation of various HIF-1α target genes were observed in this cell line under hypoxic conditions (Fig. 6A, MDA_Hif, N versus H, and Fig. 7A, PGK and LDH, MDA_Hif, N versus H), and the addition of doxycycline caused reduction of HIF-1 downstream targets such as PGK1 and LDH at the transcription level under hypoxia (Fig. 7A, PGK and LDH, MDA_Hif, H). These results suggest that the MDA_Hif cells retain the normal response to hypoxia, and the responses is regulated by HIF-1α. Although both the MDA_Hif and D54_Hif cells are responsive to hypoxia, striking differences were observed in the levels of secreted VEGF protein between these two cell lines. Compared with the D54_Hif cells, the MDA_Hif cells produce a very high level of VEGF under normoxic conditions, and this basal level of VEGF production was not dependent on HIF-1α (Fig. 7B, MDA_Hif, N versus D54_Hif, N). To determine whether the very high level of HIF-1α-independent VEGF expression might lead to a different tumor environment in the MDA tumors, we examined the vessel density and HIF-1α expression in MDA_Hif tumors by CD31 and HIF-1α staining. Compared with D54MG tumors, the MDA tumors have a slightly higher vessel density and dramatically reduced HIF-1α staining in tumors (Fig. 7C, HIF-1α and CD31), indicating that these tumors are properly vascularized but the HIF-1 signal is less activated in the MDA tumors compared with the D54 tumors.

Furthermore, unlike tumors derived from D54_Hif cells, similar levels of VEGF were detected in the MDA_Hif tumors from doxycycline-treated or untreated mice (Fig. 7C, VEGF), indicating that the production of VEGF in MDA tumors is not dependent on HIF-1α activity.

**Discussion**

The RNAi-based methods offer a potentially reliable and cost effective approach for target validation in vivo. In this study, we explored the use of inducible RNAi in xenograft tumors for evaluating a potential cancer target, HIF-1.

Consistent with the results from studies using HIF-1α-deficient cells (19, 21), we observed impaired tumor growth in D54MG-derived tumors when HIF-1α was inhibited at an early stage of tumor development. Reduction of various HIF-1 targets such as PGK1, LDH, and VEGF at the mRNA or protein level was observed in the inducible knockdown cells, and the degree of inhibition is comparable with what has been reported using hif-1α−/− cells (19, 21, 22). This indicates that the efficiency of in vitro and in vivo target inhibition by shRNA is comparable with that achieved by targeted gene ablation. However, the inducible RNAi approach offers several advantages such as the flexibility of choosing different human cancer cell lines and the ability of testing different treatment schedules. Using this system, we were able to capture information that is difficult to obtain using other methods.

One unexpected finding from this study was the dramatically different response of early-stage and later-stage tumors to HIF-1α inhibition. In the initial stage of growth, the tumor masses are transitioning from normoxia to hypoxia, which results in the
activation of HIF-1α and angiogenesis. Therefore, tumors at this stage might be more vulnerable to HIF-1 inhibition. It is not clear whether the diminished response to HIF-1α inhibition in later-stage tumors is a unique feature of the D54MG-derived tumors or is broadly applicable to other tumor types. If the reverse association of tumor size and efficacy is applicable to other tumor type, later-stage patients with established large tumors may not respond well to HIF-1 inhibitors.

The two phases of tumor growth observed after HIF-1α inhibition are intriguing. Although maximum knockdown of HIF-1α was observed at day 3, reduction of tumor size only started to manifest around day 12. This prolonged delay suggests that unlike cytotoxic drugs, HIF-1 inhibitors may not produce an acute tumor response and require sustained dosing to obtain efficacy. It is surprising that tumors are able to quickly adapt to the loss of HIF-1α and continue to grow. The adaptation process does not seem to involve compensatory HIF-2α expression because we do not observe a significant increase of HIF-2α expression in tumors after up to 23 days of doxycycline treatment. Among all the markers examined, the Glut1 level seems to track the transient tumor stasis/regression observed after HIF-1α knockdown. However, further studies will be needed to examine whether the Glut1 level

**Figure 7.** Weak HIF-1α expression and HIF-1α-independent VEGF expression in MDA tumors in vivo. A, the MDA_Hif and MDA_Luc cells were incubated in the presence or absence of 1 μg/mL doxycycline (Dox). After 36 hours, cells were either untreated (N) or subjected to hypoxia treatment (H) for additional 16 hours. Total RNAs were prepared for the analysis of indicated genes by QPCR. B, D54_Luc, D54_Hif, MDA_Luc, and MDA_Hif cells were treated as in (A). Culture medium was collected from each treatment and assayed for VEGF using a human VEGF-specific ELISA kit. Final results were presented as pg of VEGF in every 1,000 cells. C, HIF-1α expression in doxycycline-treated or water-treated (control) similar-sized tumors derived from D54_Hif and MDA_Hif were determined by immunohistochemistry (left) according to the protocol described in Materials and Methods. To compare the MVD in D54_Hif- and MDA_Hif-derived tumors, similar-sized tumors from untreated mice were examined using an anti-CD31 antibody (middle). The VEGF levels in MDA-derived tumors that were treated with doxycycline or water (Control) for 12 days were determined using ELISA analysis of tumor homogenates (right). The amount of VEGF was normalized to the amount of proteins in the homogenate. Points, averages of three tumors in each group; bars, SD.
The flexibility of choosing different cancer cells using the inducible RNAi approach allowed us to examine the efficacy of HIF-1a inhibition in different tumor types and to identify the MDA tumor as a resistant tumor type. Compared with the D54 cells, the VEGF expression pattern for the MDA cells/tumors are more complex. In cell culture, VEGF production is partially HIF-1a dependent, whereas in tumors, the VEGF production is HIF-1a independent. In addition to the different patterns of VEGF expression in the D54 and MDA cells/tumors, the MDA tumors have very low levels of HIF-1a expression compared with the D54 tumors. Because the MDA cells exhibited a similar accumulation of HIF-1a in response to hypoxia treatment as the D54 cells, the low level of HIF-1a staining in the MDA tumors suggests that the MDA tumors might be much less hypoxic compared with the D54 tumors. If this hypothesis is true, the discrepancy of VEGF tumors might be much less hypoxic compared with the D54 tumors. If this hypothesis is true, the discrepancy of VEGF expression is easily explained. As a result of the less hypoxic environment, the less hypoxic environment can also explain the lack of response to HIF-1a inhibition in the MDA tumors because less hypoxic tumors are likely less dependent on the HIF-1 pathway for rapid growth.

In summary, our studies show that inducible RNAi is a versatile tool for evaluating cancer targets in vivo. As a proof of principle, we used this approach to evaluate HIF-1a as a cancer target and identified various factors that could influence the efficacy of abrogating the HIF-1a pathway in established tumors. It is important to note that even under sustained HIF-1a knockdown, we failed to completely arrest tumors in the D54MG glioma model. This suggests although tumors driven by HIF, such as the VHL-negative RCCs, are likely respond to HIF inhibition, HIF-1a inhibitors as monotherapy might not achieve a robust therapeutic effect in other tumor types, especially in late-stage patients with large tumors. Future studies are under way to evaluate the effect of HIF-1a inhibition in combination with other cancer therapeutics, which could be instructive for defining a broader application of HIF-1a specific inhibitors in cancer patients.

Acknowledgments
Received 12/13/2004; revised 6/7/2005; accepted 6/9/2005.
Grant support: Abbott Laboratories’ Discovery Innovation.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Jacqueline O’Connor for technical support.

References
Evaluating Hypoxia-Inducible Factor-1α as a Cancer Therapeutic Target via Inducible RNA Interference In vivo

Leiming Li, Xiaoyu Lin, Michael Staver, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/16/7249

Cited articles
This article cites 25 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/16/7249.full#ref-list-1

Citing articles
This article has been cited by 18 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/16/7249.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.