

Predominant Role of Hypoxia-Inducible Transcription Factor (Hif)-1 α versus Hif-2 α in Regulation of the Transcriptional Response to Hypoxia¹

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Abstract

Tumor hypoxia induces the up-regulation of a gene program associated with angiogenesis, glycolysis, adaptation to pH, and apoptosis via the hypoxia-inducible transcription factors (Hifs) 1 and 2. Disruption of this pathway has been proposed as a cancer therapy. Here, we use short interfering RNAs to compare specific inactivation of Hif-1 α or Hif-2 α and show markedly different cell type-specific effects on gene expression and cell migration. Remarkably, among a panel of hypoxia-inducible genes, responses were critically dependent on Hif-1 α but not Hif-2 α in both endothelial and breast cancer cells but critically dependent on Hif-2 α in renal carcinoma cells.

Introduction

Hypoxia is an important process in the progression and treatment resistance of many human cancers (1). The majority of human cells share a common mechanism of oxygen sensing mediated by Hifs³ 1 and 2. These proteins are heterodimers consisting of α subunits, Hif-1 α and Hif-2 α (also known as endothelial PER-ARNT-SIM domain protein 1) that dimerize with the constitutively expressed aryl hydrocarbon receptor nuclear translocator (also known as Hif-1 β ; reviewed in Ref. 2). Both Hif- α molecules are subject to similar regulatory processes involving enzymatic hydroxylation of conserved prolyl and asparaginyl residues that target them for degradation via the VHL ubiquitin E3 ligase complex (reviewed in Ref. 3). Moreover, in transfection assays, both transcription factors activate a range of hypoxia response elements with similar efficacy (4, 5).

Despite these striking similarities, genetic studies have provided firm evidence for nonredundant functions. Targeted inactivation of Hif-1 α and Hif-2 α in embryonic stem cells is associated with different patterns of response to hypoxia and low glucose stress (6), and different developmental defects are observed in Hif-1 α ^{-/-} and Hif-2 α ^{-/-} mouse embryos (for review see Ref. 3). In part, differences may relate to distinct patterns of cellular expression. For instance, in the kidney, whereas both transcription factors are abundantly expressed, Hif-1 α is the predominant form in epithelial cells, whereas Hif-2 α is predominant in interstitial fibroblast and endothelial cells (7). However, many cancers and cell lines express both isoforms. The expression of the two Hif- α isoforms at similar levels in this setting might be predicted to lead to a level of redundancy. Nevertheless, overexpression of Hif-2 α , but not Hif-1 α , promoted growth of renal

cell carcinoma cells (8, 9) yet inhibited growth of breast cells (10), suggesting distinct effects on biology. These findings raise important questions as to what extent Hif-1 α and Hif-2 α have overlapping or redundant transcriptional functions in the cancer setting, whether expression of particular Hif transcriptional targets are always linked to expression of a particular Hif- α isoforms, or whether transcriptional selectivity varies according to cell background.

We have used siRNAs to specifically inhibit Hif-1 α and Hif-2 α production in human breast and renal carcinoma cell lines and in a human endothelial cell line, which express differing levels of Hif-1 α and Hif-2 α , ranging from isolated expression of Hif-1 α to isolated expression of Hif-2 α . The role of each molecule on induction of specific transcriptional targets with a variety of functions in the hypoxic response was then investigated.

Materials and Methods

siRNA Duplexes. The siRNA oligonucleotides were designed after the recommendations of Elbashir *et al.* (11) and were synthesized and high-performance liquid chromatography purified at Transgenomic Laboratories (Glasgow, United Kingdom). The Hif-1 α siRNA duplex targeted nucleotides 1521–1541 of the Hif-1 α mRNA sequence (NM001530) and comprised of: sense 5'-CUGAUGACCAGCAACUUGAdTdT-3' and antisense 5'-UCAAG-UUGCUGUCAUCAGdTdT-3'. The Hif-2 α siRNA duplex targeted nucleotides 1260–1280 of the Hif-2 α mRNA sequence (NM001430) and comprised of sense 5'-CAGCAUCUUUGAUAGCAGUdTdT-3' and antisense 5'-ACUGCUAUCAAAGAUGCUGdTdT-3'. The inverted Hif-1 α control duplex did not target any gene and comprised of sense 5'-AGUUAACGAC-CAGUAGUCdTdT-3' and antisense 5'-GACUACUGGUCGUUGAdTdT-3'. Duplexes were prepared by mixing 50- μ M concentrations of antisense and sense oligonucleotides with annealing buffer [30 mM HEPES (pH 7.0), 100 mM potassium acetate, and 2 mM magnesium acetate], heat denaturing for 1 min at 85°C, and annealing at 37°C for 1 h. Duplex formation was confirmed by electrophoresis through 5% low melting temperature agarose (NuSieve GTG; FMC Bioproducts, Rockland, ME). Additional siRNA duplexes used for confirmation of the specificity of particular effects were prepared as above and targeted to nucleotides 1510–1530 (AACGACACAGAACTGATGAC) of the Hif-1 α mRNA sequence and nucleotides 328–348 (AAATCAGCTTCCT-GCGAACAC) of the Hif-2 α mRNA sequence.

Cell Culture. MDA 435 cells, MDA 468 cells (breast cancer), 786-0 cells (renal cancer), and HUVECs (endothelial) were obtained from the Cancer Research United Kingdom cell service. Breast and renal cancer cells were grown in DMEM supplemented with 10% FCS (Gibco), L-glutamine (2 μ M), penicillin (50 IU/ml), and streptomycin sulfate (50 μ g/ml). HUVECs were grown in the media supplemented as above but with 20% FCS plus endothelial cell growth supplement and heparin (Sigma) and grown on plates coated with 2% gelatin/PBS. Experiments were performed on dishes of cells in normoxia (humidified air with 5% CO₂) or hypoxia [hypoxic conditions were generated in a Napco 7001 incubator (Precision Scientific) with 0.1% O₂, 5% CO₂, and balance N₂].

siRNA Treatment of Cells. Cells were plated onto 10-cm² cell culture dishes and grown to 30–50% confluence before transfection. The duplexes were diluted to give a final concentration of 20 nM in Opti-Mem I (Invitrogen Life Technologies, San Diego, CA). Twenty-five μ l of Oligofectamine transfection reagent (Invitrogen Life Technologies) were added, and the mixture

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³ The abbreviations used are: Hif, hypoxia-inducible factor; CA9, carbonic anhydrase 9; GLUT-1, glucose transporter-1; HUVEC, human umbilical vein endothelial cell; siRNA, short interfering RNA; uPAR, urokinase-type plasminogen activator receptor; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

incubated at room temperature for 25 min. The cells were rinsed with Opti-Mem I to remove any residual serum and incubated with the oligonucleotide duplexes in serum-free conditions for 4 h at 37°C. Serum was then added back to the culture, and cells were incubated for an additional 24 h before beginning an experiment.

RNA Preparation and RNase Protection Assay. Cells were rinsed with PBS and drained thoroughly. RNA was extracted from the cells using the solution D method described by Chomczynski and Sacchi (12) and assessed by absorbance at 260/280 nm. The RNase protection assay protocol and generation of ³²P-labeled RNA probes to Hif-1 α , Hif-2 α , and U6 small nuclear RNA has been described previously (4). Protected fragments were resolved on an 8% polyacrylamide gel and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Blotting. Cells were washed thoroughly with PBS before being homogenized in a lysis buffer containing 8 M urea, 10% SDS, 1 M DTT, and protease inhibitors. Samples were electrophoresed on a 10% SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedfordshire, United Kingdom). Proteins were detected using monoclonal antibodies to Hif-1 α (Signal Transduction Laboratories), Hif-2 α (4), CA9 (13), GLUT-1 (Alpha Diagnostic, San Antonio, TX), glyceraldehyde-3-phosphate dehydrogenase (Abcam, Cambridge, United Kingdom), and BNip3 (14) at 1:1,000, 1:1,000, 1:500, 1:250, 1:2,000, and 1:20,000, respectively. As a loading control, a mouse monoclonal antibody to β -tubulin (Sigma) was used at 1:20,000. Overnight primary antibody incubation was followed by incubation with goat antimouse or rabbit horseradish peroxidase (Dako) and enhanced chemiluminescence developing reagents (Amersham). Blots were exposed to film for between 30 s and 2 min.

Measurement of VEGF and uPAR. Supernatant was harvested from treated cells and centrifuged to remove cell debris. Secreted VEGF and uPAR were measured in the supernatant using the respective Quantikine ELISA kit (R&D Systems, Abingdon, United Kingdom) as per the manufacturer's instructions. The amount of VEGF and uPAR in the supernatant was normalized to the final number of cells in the dish from which it was harvested.

Cell Migration Assay. Cells treated with siRNA as described above were incubated in 0.1% oxygen for 16 h, removed from the culture dish using 2 mM EDTA, and resuspended in 1% FCS media. A total of 200 μ l of serum-free media containing 1.5×10^4 cells was placed into the top of migration chambers with 8- μ m filters (24-well plate format; Falcon), which were standing in wells containing 700 μ l of media containing 10% FCS. The cells were incubated at 37°C for 4 h, after which the chambers were removed from the wells and coded for analysis by a blinded observer. Cells that had migrated to the bottom of the filter were fixed with 2.5% glutaraldehyde for 15 min, rinsed thoroughly with PBS, and stained with 0.1% crystal violet for 2 min. The total number of cells on the bottom of each filter was counted under a microscope, and each experiment was performed in triplicate on at least three occasions.

Results

Specificity of siRNAs Targeted to Hif-1 α and Hif-2 α . We synthesized siRNA oligonucleotides that specifically target Hif-1 α or Hif-2 α mRNAs for degradation and transfected these into cells 24 h before hypoxic stimulation. RNA extracted from the treated cells was subjected to RNase Protection Assay analysis for Hif-1 α and Hif-2 α . MDA 468 and HUVECs expressed transcripts encoding both Hif-1 α and Hif-2 α (Fig. 1), whereas the MDA 435 cells did not express Hif-2 α mRNA (Fig. 1), and the 786-0 cells did not express Hif-1 α mRNA (Fig. 1). Treatment of the cells with the siRNAs ablated the expression of Hif-1 α and Hif-2 α mRNA specifically in that the Hif-1 α siRNA did not affect the Hif-2 α gene expression and *vice versa* (Fig. 1). Inverted siRNA controls of the Hif-1 α and Hif-2 α siRNAs had no effect on the expression of either gene; the inverted Hif-1 α siRNA was used as the control in all experiments described (Figs. 1–4). When cells were transfected with both siRNAs, expression of Hif-1 α and Hif-2 α was ablated. No cell toxicity was noted after transfection with either of the siRNAs or with Oligofectamine alone (described as the negative control). To confirm the specificity of the technique, siRNAs targeted to another region of the Hif-1 α and

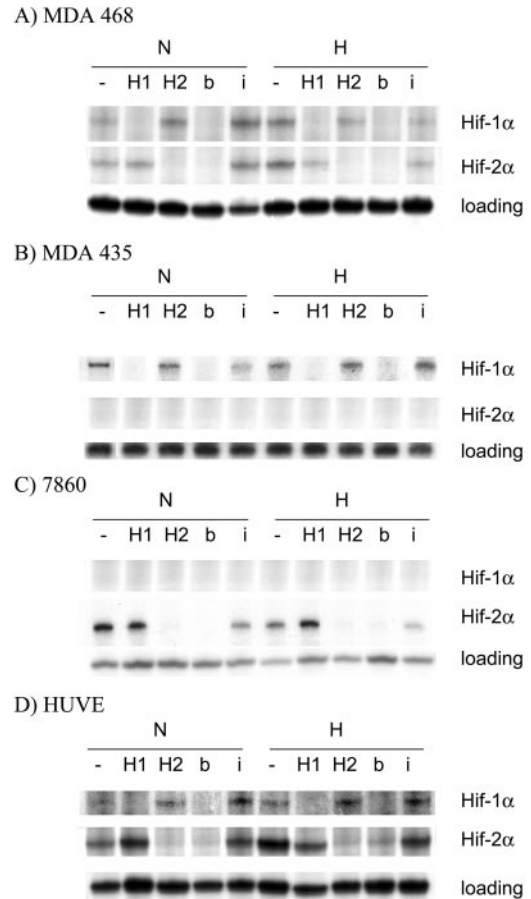


Fig. 1. RNase protection analysis of RNA extracted from MDA 468 cells (A), MDA 435 cells (B), 786-0 cells (C), and HUVECs (D). Cells were mock transfected (–) or subjected to siRNA directed to Hif-1 α (1), Hif-2 α (2), both Hif-1 α and Hif-2 α (b), or inverted control (i) before subsequent incubation for 16 h in 20% oxygen (N) or 0.1% oxygen (H). Specific down-regulation of Hif-1 α or Hif-2 α mRNA occurred after siRNA for each respective transcript or both transcripts. The inverted siRNA control had no effect on mRNA levels. Quantification of U6 small nuclear RNA was used as a loading control.

Hif-2 α mRNAs were synthesized and transfected into MDA 468 cells, which were then subjected to hypoxic stimulation. The results obtained with these siRNAs were the same as described above in respect to specificity of Hif-1 α and Hif-2 α targeting (data not shown).

Expression of Hypoxically Induced Genes by Human Cell Lines After Treatment with siRNA for Hif-1 α and Hif-2 α . The HIF system up-regulates the production of proteins with a wide range of functions in the homeostatic and apoptotic response (2, 3, 13, 15) to hypoxia and cell death in many different human cell types. To investigate the importance of Hif-1 α and Hif-2 α in conferring such responses in different cell backgrounds, we analyzed the expression of CA9 (acid metabolism), BNip3 (cell death), GLUT-1 (glucose/energy metabolism), VEGF (angiogenesis), and uPAR (proteolytic pathway of invasion) in MDA 435 cells, MDA 468 cells (breast carcinoma), 786-0 cells (renal carcinoma), and HUVECs (endothelial) after treatment with Hif-1 α and/or Hif-2 α siRNA. Protein levels were measured using Western blot analysis (CA9, BNip3, and GLUT-1) or ELISA (VEGF and uPAR).

Analysis of the breast carcinoma cell lines revealed that MDA 468 cells expressed both Hif-1 α and Hif-2 α protein (Fig. 2), whereas MDA 435 cells expressed only Hif-1 α protein (data not shown). In both cell lines, hypoxic induction of CA9, BNip3, GLUT-1, VEGF, and uPAR protein was inhibited by treatment with Hif-1 α siRNA but not affected by Hif-2 α siRNA. Silencing both Hif-1 α and Hif-2 α had the same effect as silencing with Hif-1 α , and the inverted control

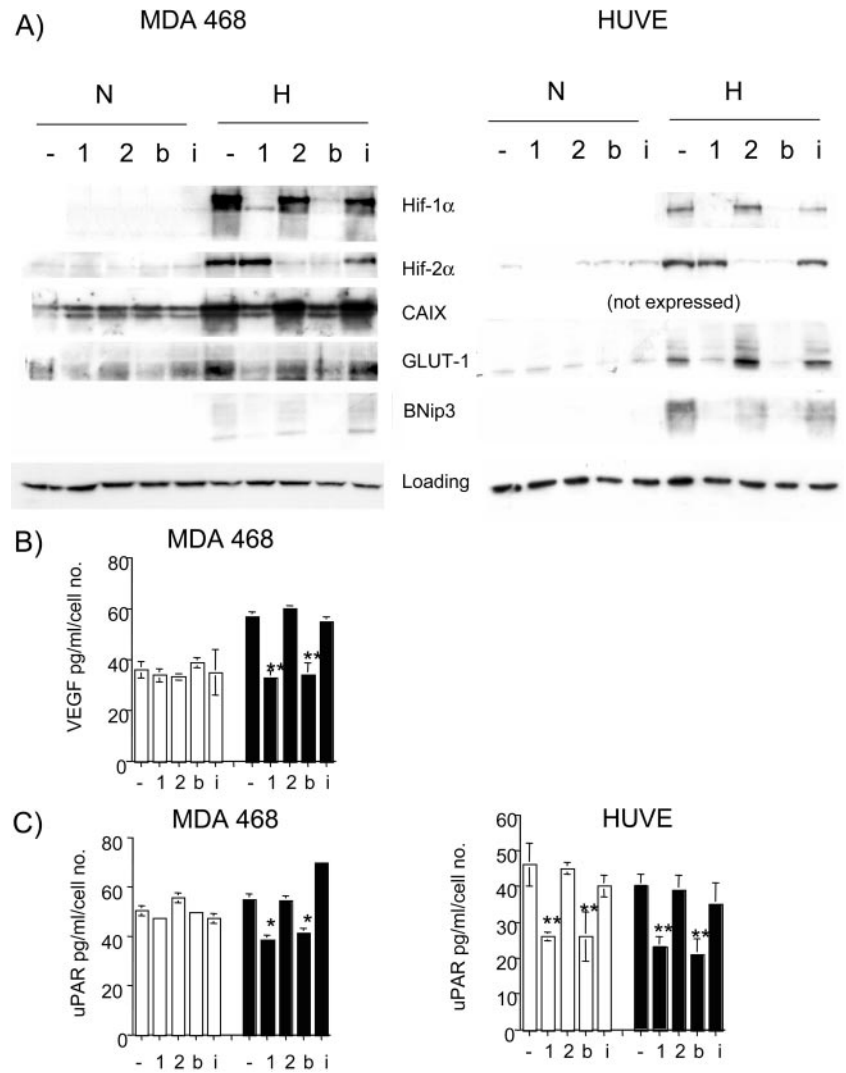


Fig. 2. A, Western blot analysis of protein extracted from MDA 468 cells and HUVECs after treatment as described in Fig. 1. Specific down-regulation of Hif-1 α or Hif-2 α protein occurred after siRNA for each respective transcript or both transcripts. The inverted siRNA control had no effect on protein levels. Hypoxic induction of CA9, GLUT-1, and BNip3 protein was blocked after siRNA for Hif-1 α but not after siRNA for Hif-2 α . siRNA against both genes also resulted in the down-regulation of the target genes. B, VEGF levels and uPAR levels (C) in media conditioned by MDA 468 (B and C) and HUVECs (C), normalized to final cell number. Normoxic or hypoxic treatment of cells is indicated by \square and \blacksquare , respectively. Experiments were performed in triplicate at least three times, and results from one representative experiment are shown. One-tailed, student *t* tests comparing each treatment with the hypoxic mock control were performed, and significance is indicated by * for $P < 0.05$ and ** for $P < 0.01$.

siRNA had no effect on the expression of any of the genes (Fig. 2; data not shown). The same results were obtained when MDA 468 cells were transfected with the confirmatory siRNAs (data not shown).

Similar to the MDA 468 cell lines, HUVECs expressed both Hif-1 α and Hif-2 α protein after hypoxic stimulus (Fig. 2). Hypoxia did not induce HUVECs to express CA9 or secrete VEGF but did increase the levels of expression of BNip3, GLUT-1, and uPAR. Pretreatment of HUVECs with siRNA to Hif-1 α ablated the hypoxic induction of BNip3, GLUT-1, and uPAR, but Hif-2 α siRNA treatment had no effect on protein production (Fig. 2).

The renal carcinoma cell line 786-0 expressed Hif-2 α but not Hif-1 α , and because this cell line lacks functional VHL, expression of Hif-2 α was seen constitutively under normoxic conditions (Fig. 3). VEGF and GLUT-1 proteins were also constitutively expressed, but BNip3 and CA9 proteins were not expressed at detectable levels. uPAR was constitutively expressed by 786-0 cells but at 2-fold lower levels than by breast or endothelial cells. Treatment of cells with siRNA to Hif-2 α reduced the expression of GLUT-1 and VEGF, whereas siRNA to Hif-1 α had no effect (Fig. 3). Expression of uPAR was not affected by siRNA to Hif-1 α or Hif-2 α .

Cell Migration Induced by Hypoxia Is Affected by Pretreatment with siRNA to Hif-1 α or Hif-2 α Depending on the Cell Type. Intratumoral hypoxia is correlated with increased risk of invasion in human cancer (1), and hypoxia increases the invasion of colon carcinoma cells (16).

To elucidate which hypoxia-induced transcription factor is involved in this process, we analyzed MDA 468 and HUVE cells treated with siRNA for Hif-1 α or Hif-2 α and normoxia or hypoxia in a cell migration assay. Cells subjected to hypoxia showed increased migration compared with the cells that had remained in normoxia, and treatment with inverted siRNA or mock transfection had no effect on the migration response. In MDA 435 cells, the hypoxic response was inhibited by treatment with siRNA directed to Hif-1 α but not to Hif-2 α . However, in MDA 468 and HUVECs, hypoxically induced migration was inhibited by pretreatment of the cells with either Hif-1 α or Hif-2 α siRNA. Treatment of cells with both siRNAs inhibited the hypoxically induced migration response in both cell lines but not more than with either alone (Fig. 4).

Discussion

In this study, we used siRNAs that specifically target degradation of mRNAs encoding Hif-1 α or Hif-2 α . After treatment with siRNA, the expression of Hif-1 α or Hif-2 α mRNA and protein was greatly reduced under hypoxic conditions. The effects of these siRNAs were analyzed in two human breast carcinoma cell lines, a human endothelial cell line, and a human renal carcinoma cell line containing an inactivating mutation in VHL.

Our results indicate that in the breast carcinoma and endothelial cell

Fig. 3. A, Western blot analysis of protein extracted from 786-0 cells after treatment as described in Fig. 1. 786-0 cells do not express Hif-1 α , but specific down-regulation of Hif-2 α protein occurred after siRNA for Hif-2 α . The inverted siRNA and Hif-1 α siRNA had no effect on Hif-2 α protein levels. GLUT-1 protein is reduced after siRNA for Hif-2 α but not after siRNA for Hif-1 α . Somewhat unusually, there was a modest induction of GLUT-1 after hypoxic stimulus, which was also inhibited by siRNA for Hif-2 α . siRNA for both genes also resulted in the down-regulation of the target genes. B, VEGF levels and uPAR levels (C) in media conditioned by the above cells normalized to final cell number. Normoxic or hypoxic treatment of cells is indicated by \square and \blacksquare , respectively. Experiments were performed in triplicate at least three times, and results from one representative experiment are shown. One-tailed, student *t* tests comparing each treatment with the hypoxic mock control were performed, and significance is indicated by * for $P < 0.05$ and ** for $P < 0.01$.

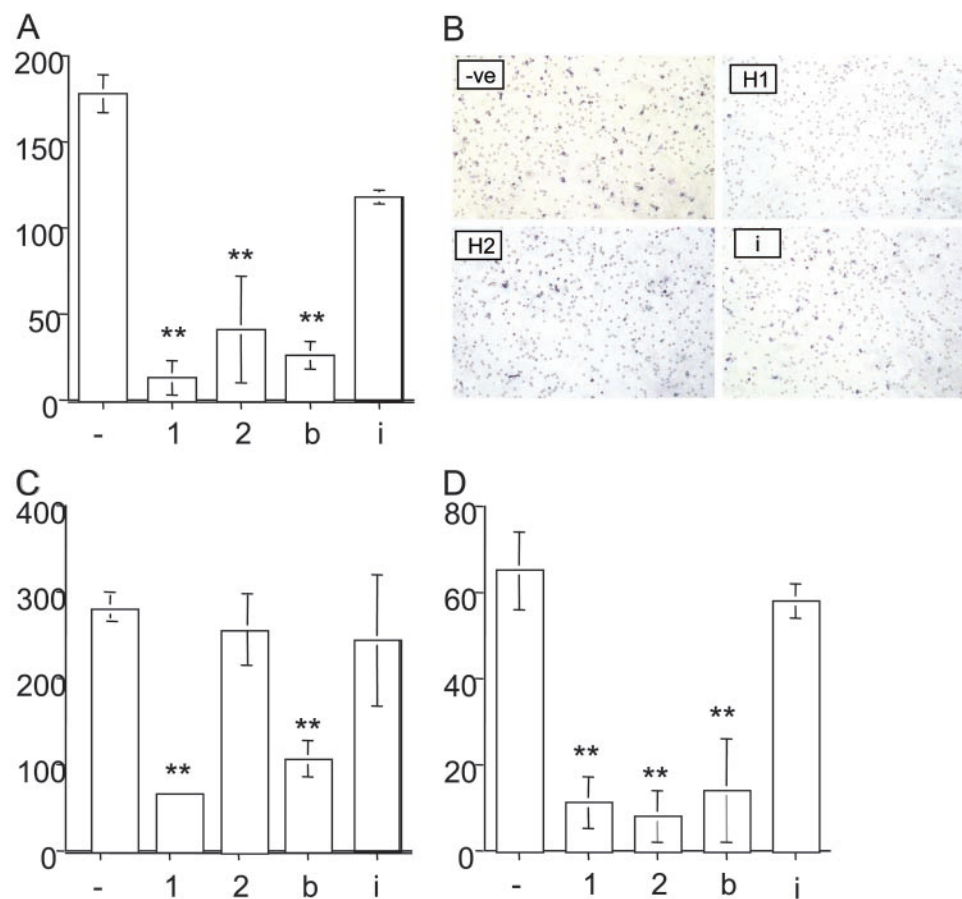
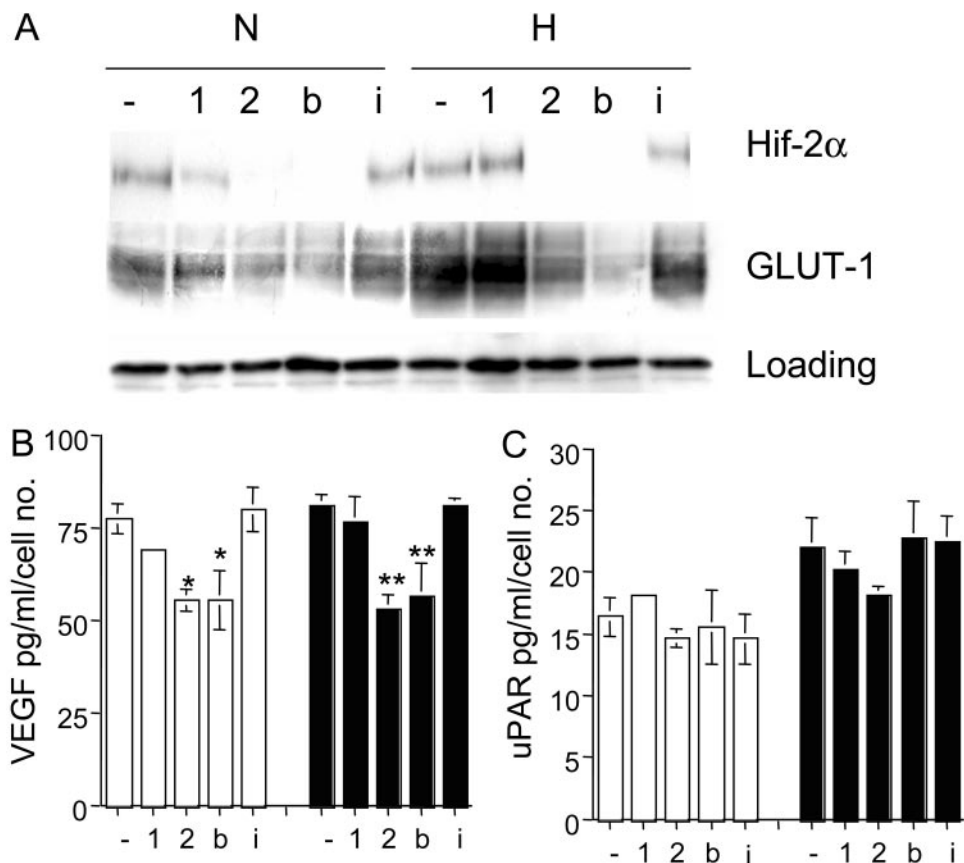


Fig. 4. Migration analysis of cells treated with a mock transfection (-) or siRNA for Hif-1 α (1), Hif-2 α (2), both Hif-1 α and Hif-2 α (b), or inverted control (i) before subsequent incubation for 16 h in 0.1% oxygen. The number of cells that had migrated through an 8- μ m filter was counted, and the mean and SD of three replicates in a representative experiment is shown graphically. A and B, hypoxically induced migration of MDA 468 cells is inhibited by treatment with siRNA for both Hif-1 α and Hif-2 α . B shows photographs of the bottom of a representative selection of migration chambers, with blue cells visible around the smaller round pores of the filter. C, hypoxically induced migration of MDA 435 cells was inhibited by treatment with siRNA for Hif-1 α , not Hif-2 α , whereas migration of HUVECs was inhibited by siRNA for both Hif-1 α and Hif-2 α (D).

lines, the major Hif- α isoform required for induction of a set of well-characterized hypoxic genes is Hif-1 α . Surprisingly, even in cells expressing both Hif- α isoforms, Hif-2 α did not substitute in regulating any of these genes when Hif-1 α was inactivated. Nevertheless, functional analysis of the endothelial and breast carcinoma cell lines revealed that both Hif-1 α and Hif-2 α are required for hypoxia-induced cell migration in cell lines that express both proteins, suggesting that there are other actions of Hif-2 α that have not been revealed in our studies of gene expression. Overall, however, the importance of Hif-1 α in these cells is in concordance with other studies that have reported Hif-1 α as a positive factor in tumor growth (17) and carcinoma cell invasion (16) in different cells. The hypothesis that Hif-1 α is the major hypoxia-induced transcription factor involved in breast carcinogenesis is supported by evidence that one of the breast carcinoma cell lines used in this study has lost Hif-2 α expression, and stable transfection of this cell line with Hif-2 α resulted in its impaired growth as xenograft tumors compared with the parental line (10).

In contrast with the above results, we found that in the VHL-defective 786-0 renal carcinoma line, in which the native Hif-1 α gene is not expressed, some of the hypoxia-inducible transcripts were now critically dependent of Hif-2 α . VHL is required for proteolytic regulation of both Hif-1 α and Hif-2 α , and in VHL defective cells both isoforms are stabilized. However, there is an unusual bias toward enhanced Hif-2 α mRNA expression in clear cell renal carcinoma that is not observed in the renal tubular epithelium from which these tumors are derived (7) but arises during tumor development (18). This may be because of an additional action of VHL on the Hif system (19, 20) and/or additional non-VHL mediated actions on Hif α isoforms that arise during the oncogenic process. The current results suggest the existence of another distinct interface between the HIF system and renal carcinogenesis that makes connections between Hif-2 α expression and certain hypoxia-inducible mRNAs. The finding that the Hif-2 α pathway appears to be specifically activated in clear cell renal carcinogenesis by several steps strongly suggests a causal role for Hif-2 α in development of the cancer. Interestingly, this is supported by comparison of results from two groups that have examined the expression of mutant forms of Hif-1 α or Hif-2 α that escape VHL-mediated destruction on the tumor suppressor effect of expressing wild-type VHL in renal cell carcinoma cells. These studies have shown that stabilized Hif-2 α but not Hif-1 α reverses VHL tumor suppressor function (8, 9).

In conclusion, these studies have, for the first time, directly compared functional inactivation of Hif-1 α and Hif-2 α in different cancer cell lines. The findings indicate that the actions are distinct and differ according to cell background and suggest that these differences are important in tumor development.

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