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Abstract

Tumor hypoxia often directly correlates with aggressive phenotype, metastasis progression, and resistance to chemotherapy. Two transcription factors [hypoxia-inducible factor-1α (HIF-1α) and HIF-2α] are dramatically induced in hypoxic areas and regulate the expression of genes necessary for tumor adaptation to the conditions of low oxygen; however, the relative contribution of these factors is controversial. We used RNA interference–mediated inactivation of HIF-1α or HIF-2α followed by microarray analysis to identify genes specifically regulated by either HIF-1 or HIF-2 in hypoxia. We found that, in the MCF7 cell line, the vast majority of hypoxia-responsive genes (>80%) were dependent on the presence of HIF-1α. However, a small group of genes were preferentially regulated by HIF-2α. Promoter analysis for this group of genes revealed that all of them have putative binding sites for ETS family transcription factors, and 10 of 11 HIF-2α-dependent genes had at least one potential hypoxia-responsive element (HRE) in proximity to an ETS transcription factor binding site. Knockdown of ELK-1, the most often represented member of ETS family, significantly reduced hypoxic induction of the HIF-2α-dependent genes. Physical and functional interaction between ELK-1 and HIF-2α were supported by coimmunoprecipitation of these two proteins, luciferase reporter assay using CITED2 promoter, and binding of ELK-1 protein to the promoters of *CITED2* and *WISP2* genes in proximity to a HRE. These data suggest that the choice of the target genes by HIF-1 or HIF-2 depends on availability and cooperation of HIFs with other factors recognizing their cognate elements in the promoters. (Cancer Res 2006; 66(11): 5641-7)

Introduction

To survive, tumor cells must adapt to the conditions of low oxygen originating from delayed blood vessel development during tumor growth. The adaptive process involves induction of angiogenesis, increased glucose consumption, and a switch to the glycolytic pathway of energy production. This response is regulated by two transcription factors [hypoxia-inducible factor-1 (HIF-1) and HIF-2; refs. 1, 2], which belong to the basic helix-loop-helix PAS family transcription factors. Under normal oxygen tension, the α subunits of these factors undergo rapid proteasomal degradation mediated by the von Hippel-Lindau tumor suppressor protein (VHL; refs. 3–8). The interaction between HIF-α and VHL is dependent on hydroxylation of specific proline residues in HIF-α subunits by a family of HIF prolyl-4-hydroxylase enzymes that require oxygen, iron, and 2-oxoglutarate as cofactors (9–13). Therefore, a decrease in oxygen concentration or iron chelation results in HIF-α stabilization, translocation into the cell nucleus, and heterodimerization with the constitutively expressed HIF-β/aryl hydrocarbon receptor nuclear translocator subunit to form an active transcription complex.

HIF-1 and HIF-2 recognize the same DNA consensus sequence within the hypoxia-responsive elements (HRE) in the promoters of their target genes and are able to activate the same set of genes. Despite these similarities, the functions of these factors are not redundant. Targeted deletion of either HIF-1α or HIF-2α in mouse embryos shows that these genes do not compensate for each other in development (14–19).

Because hypoxia is a common feature for solid tumors of different origin (20) and degree of hypoxia correlates directly with poor prognosis (21–23), HIFs are considered a potential target for therapeutic intervention. Therefore, it is important to understand which of the HIFs are primarily responsible for the tumor-associated phenotype.

Several studies addressed the target specificity of HIF-1 or HIF-2 transcription factors by microarray or serial analysis of gene expression analysis. These studies used VHL-deficient renal carcinoma cell lines with ectopic expression of VHL (24–27) or forced expression of HIF-1α and HIF-2α (26, 28) or by comparing gene expression from HIF-1α knockout cells to control cells (29, 30).

More recent studies approached this problem by using RNA interference (RNAi) for inactivation of either HIF-1α or HIF-2α (31, 32), but only a few hypoxia-regulated genes were chosen for analysis. In one study, all selected genes were responsive to the HIF-1α small interfering RNA (siRNA) in cell lines, where both HIF-1α and HIF-2α were endogenously expressed. In the renal clear cell carcinoma cells with no HIF-1α protein expression, HIF-2α was able to substitute for HIF-1α in hypoxic activation of the same genes (31). Another study showed similar results with the exception of erythropoietin (EPO), which was identified as a HIF-2-dependent (but not HIF-1-dependent) gene in two different cell lines (32). It is noteworthy that HIF-1α was originally cloned as a factor inducing EPO (33). Importantly, the same study shows that forced expression of the HIF-α subunits can change target gene specificity. These data support the idea that the relative effect of HIF-1α and HIF-2α may be cell type dependent, although the mechanism of target selectivity remains unclear.

In our study, we combined RNAi-mediated inactivation of HIF-1α and HIF-2α with microarray analysis. We found that the

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

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majority of hypoxia-regulated genes responded to inactivation of HIF-1α, but not HIF-2α. A small number of genes required the presence of HIF-2α for expression in hypoxic conditions. Promoter analysis of this group of genes revealed that all of them had at least one ETS transcription factor binding site in their promoters, and 10 of 11 genes had a potential HRE, RCGTG, in proximity to the ETS consensus sequence. We then inhibited the expression of ELK-1, the most represented ETS family member and found that several HIF-2-dependent genes bearing ELK-1 binding sites were either no longer induced by hypoxia (*CITED2* and *WISP2*) or had reduced induction by hypoxia (*IGFBP3*). Together with previously published cooperation of HIF-2α with ETS-1 during transcriptional activation of FLK-1 receptor (34), these data imply that interaction between HIF-2 and ETS factors maybe a more general phenomenon necessary for the proper HIF-2 target gene selection.

Materials and Methods

Cell culture and hypoxic treatment. MCF7 cells were cultured in DMEM supplemented with 10% FCS (Biosource, Camarillo, CA), L-glutamine, and antibiotics. Hypoxia treatment was carried out in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) with the mixture containing 0.5% oxygen, 5% CO_2, and 94.5% nitrogen for 8 hours.

siRNA duplexes and transfection. siRNA to HIF-1α and HIF-2α has been described previously (24). ELK-1 siRNA was purchased from Ambion (Austin, TX). MCF7 cells were plated at a density 4×10^5 cells/60 mm plate. Transfection was routinely done at 60% to 70% cell confluency using 8 μL Mirus TransIT-TKO transfection reagent (Mirus, Inc., Madison, WI) and 60 nmol/L siRNA duplexes according to the manufacturer's instructions. The complexes of siRNA and transfection reagent were prepared in 200 μL antibiotic- and serum-free Opti-MEM medium (Life Technologies, Carlsbad, CA) and added to the cells in the complete medium. Twenty-four hours later, the transfection procedure was repeated, and cells were subjected to hypoxia treatment the day after the second transfection. No siRNA was added to the transfection mixture for mock-transfected cells.

Microarray analysis. Total RNA was purified using Trizol reagent (Life Technologies). Total RNA (5 μg) was labeled, hybridized, and processed according to the Affymetrix (Santa Clara, CA) technical manual. The labeled probes were hybridized to human genome U133A GeneChip arrays, and data were collected on Affymetrix Scanner 3000 using GeneChip Operating Software (GCOS; Affymetrix). Information about the chip, gene annotations of probes, statistical algorithms of signal intensity calculation, and the software are available at http://www.Affymetrix.com. Signal intensities were determined by one-step Tukey's biweight algorithm and normalized to an average value of 500 for each chip excluding lowest 2% and highest 2% of the signals. Each experimental group had four independent transfections with the scramble duplex as a control or with two different HIF-1α or HIF-2α siRNAs repeated twice. Statistical comparison between groups was done by class comparison algorithm of "BRB Array Tools" software developed by Biometric Research Branch, National Cancer Institute (NCI), Bethesda, MD (http://linus.nci.nih.gov/~brb/tool.htm), consisting of a modified two-sample F test at α level of 0.01. Additional filters were applied to these statistically significant genes based on GCOS signal values and detection calls. Genes associated with the "absent" call ($P > 0.065$) in all arrays or average signal values <200 in all of the classes were eliminated. We first selected the genes differentially regulated by hypoxia in scramble-transfected MCF7 cells. The probes significantly different between scramble and mock-transfected cells were excluded from this set. All other genes with $P < 0.01$ and >1.5-fold difference between normoxic and hypoxic cells were subjected to further analysis.

To identify genes responding to HIF-1α or HIF-2α siRNA, we applied a predicted pattern classification. Four different expected gene expression patterns considered are presented in Supplementary Table S1. Because siRNA does not completely inactivate its target genes, we did not expect complete ablation of hypoxic response but allowed at least half of the response value compared with control scramble duplex. The correlation coefficient between the expected patterns and the replicate averages of signal values for each gene were calculated. In our study, four different vascular endothelial growth factor (VEGF) probes were induced by hypoxia and exhibited correlation coefficients >0.9 with the pattern predicted for HIF-1α-responsive gene. Thus, the cutoff for the correlation coefficient was set up at 0.9 using VEGF probe as a known hypoxia-inducible gene. Therefore, if the expression pattern of any gene had a correlation coefficient >0.9 with any of the expected expression patterns, it was classified accordingly.

Quantitative reverse transcription-PCR. Total RNA was purified using Trizol reagent, and 1 μg total RNA was reverse transcribed in 50 μL reaction using Taqman reverse transcription reagents (Applied Biosystems, Inc., Foster City, CA). Reverse-transcribed cDNA (5 μL) was subjected to PCR according to Applied Biosystems technical recommendations. The Taqman probes were purchased from Assay-on-demand predesigned set (Applied Biosystems). Three replicate reactions were run for each RNA sample.

Immunoprecipitation and Western blotting. For immunoprecipitation, 10^7 MCF7 or 5×10^6 786-O cells were incubated in 0.5% oxygen overnight. All following procedures were done in the presence of 100 μmol/L desferrioxamine to maintain high levels of HIFs. Nuclear fraction was prepared by high-salt extraction with subsequent dilution to the final NaCl concentration of 150 mmol/L and immunoprecipitated with anti-ELK-1 antibody I-20 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Immune complexes were collected with protein A-Sepharose (Amersham, Piscataway, NJ), washed, and resolved in SDS-PAGE. For Western blotting, cells were lysed in radioimmunoprecipitation assay buffer supplemented with 1 mmol/L sodium orthovanadate, protease inhibitor mix (Boehringer Mannheim, Indianapolis, IN), and 1 mmol/L phenylmethylsulfonyl fluoride. Total protein (60 μg) was fractionated in 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was probed with anti-HIF-1α antibody (Transduction Laboratories, San Jose, CA), anti-HIF-2α antibody (Santa Cruz Biotechnology), or anti-ELK-1 antibody overnight followed by washing and incubation with horseradish peroxidase–conjugated secondary antibody (Pierce, Rockford, IL) and developed using SuperSignal West Pico chemiluminescent substrate (Pierce).

Reporter assay. Hep3B or MCF7 cells were seeded onto 12-well plates at 10^5 or 1.5×10^5 cells per well, respectively. Next day, cells were transfected using Fugene 6 reagent (Roche, Indianapolis, IN) with 0.25 μg luciferase reporter gene, 0.4 μg pCMV6-XL4-ELK-1 (OriGene, Rockville, MD), and 0.1 μg pcDNA3-HIF-1α (P564A) or HIF-2α (P531A) expression vectors. In 48 hours, cells were lysed in passive lysis buffer (Promega, Madison, WI) and tested for luciferase activity. Samples were normalized by cotransfection either with 0.01 μg pCMV-Renilla or to protein concentration when ELK-1 effect was tested.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation was done using chromatin immunoprecipitation assay kit (Upstate, Charlottesville, VA) following the manufacturer's instructions. Briefly, MCF7 cells were treated with hypoxia overnight, and 2×10^6 cells were resuspended in SDS lysis buffer for sonication and further immunoprecipitation with 4 μg anti-ELK-1 (I-20)× antibody (Santa Cruz Biotechnology). Normal rabbit IgG or no antibody was used as a negative control. After immune complexes were collected, washed, and eluted according to the manufacturer's protocol, 5 μL DNA was subjected to 32 cycles of PCR. The primers for *CITED2* genes were 5′-CAAGTCAATGAACCAAACGG-3′ (forward) and 5′-ATAGATAACGTGGTAATCGC-3′ (reverse); the primers for *WISP2* genes were 5′-AGGATGGGAAGCGAAGCAAG-3′ (forward) and 5′-TCTTCGGTGTGCCTCTCATG-3′ (reverse).

Results

We chose to use the MCF7 cell line because of the robust induction of both HIF-1α and HIF-2α in hypoxia and the well-documented role of HIFs in breast cancer (22). We first showed that we can effectively and specifically inactivate HIF-1α or HIF-2α subunits in MCF7 cells using two different siRNA oligonucleotides for each factor. Quantitative reverse transcription-PCR (RT-PCR)

analysis revealed that remaining amounts of HIF-1α or HIF-2α mRNA were close to 10% of their respective levels in control cells transfected with scramble oligonucleotide (Fig. 1*A*). Western blotting of the samples exposed to hypoxia confirmed the diminished levels of HIF-α proteins after transfection with siRNA (Fig. 1*B*). A time course study of the induction of four known hypoxia-inducible genes showed that peak mRNA levels were reached between 4 and 8 hours of hypoxia (Supplementary Fig. S1A); thus, we selected an 8-hour time point for analysis to minimize secondary target induction. To eliminate genes that may unspecifically respond to scramble, we did a comparison between scramble and mock-transfected cells (where no siRNA was added in the transfection). Statistical analysis showed that several genes indeed changed their level in scramble-transfected cells compared with mock-transfected control, but, for most of them, the magnitude of change was <1.5-fold. Genes that responded to the scramble transfection at higher ratios were eliminated from the analysis. Supplementary Fig. S1B delineates the comparisons used for microarray analysis. To identify genes regulated by HIF-1 or HIF-2, cells were transfected with two different HIF-1α or HIF-2α siRNA oligonucleotides in two independent experiments (for a total of four samples in each group) and compared with hypoxia-treated samples from four independent transfections with scramble. The global gene expression patterns were examined by principal component analysis of 11,878 transcripts detected in at least half of the arrays. A projection on the three principal components covering highest variance (39% of the total) shows grouping of similar samples (Supplementary Fig. S2). The 39% variance of 11,878 transcripts indicates that there are considerable differences in gene expression between the groups.

All genes altered by hypoxia were placed in a HIF-independent, HIF-1-dependent, HIF-2-dependent, or responding equally to both transcription factor groups using the pattern recognition algorithm as described in Materials and Methods. The average normalized gene expression levels for each category followed the expected expression patterns (Fig. 2). For example, genes classified as HIF-1 dependent had reduced expression levels in the HIF-1α, but not the HIF-2α, siRNA-treated cells. The results show that most of the hypoxia-responsive genes are regulated by HIF-1 only. Of 325 probes responding to hypoxia, 268 probes (240 up-regulated and 28 down-regulated) were in the HIF-1-dependent group, 19 probes responded equally to the inactivation of either HIF-1α or HIF-2α, and 24 probes (18 up-regulated and 6 down-regulated) were changed in hypoxia, independent of HIF-1α or HIF-2α. The complete list of genes in each group can be found in Supplementary Table S2. There were 14 probes specifically up-regulated by HIF-2α, and none down-regulated in this category. These 14 probes included two probes each for CITED2 and IGFBP3; so, overall, 12 distinct genes were represented. Quantitative RT-PCR validation of the genes from different groups confirmed the microarray data (Fig. 3).

The fact that the majority of hypoxia-regulated genes are responding predominantly to HIF-1α could be explained by an abundance of HIF-1α when compared with HIF-2α. Although it is difficult to compare the protein levels due to different antibody efficiencies, quantitative RT-PCR showed ~10-fold higher levels of HIF-1α mRNA than HIF-2α mRNA in MCF7 cells (data not shown). Therefore, the question arises why some genes are still preferentially responding to HIF-2α, although some of them, such as *IGFBP3* and *CITED2*, were previously reported to be activated by HIF-1α in several cell types and *in vitro* assays. To gain insight into the transcriptional regulation of these genes, we analyzed the promoter regions of the 12 HIF-2-dependent genes using Genomatix (Germany) software. One gene was rejected by the software (*TRAM2*, Hs.310230) due to limited information. When common transcription factors present in the promoters of these genes were analyzed, 90% of the promoters contained ETS family transcription factor binding sites. Although some genes have more than one promoter, each gene in this group has a putative ETS factor binding site(s) in at least one promoter. Previously, one case of ETS-1 cooperation with HIF-2α has been reported for the *FLK-1* gene (34). The promoter of this gene has a HRE next to the ETS-1 binding site, and full activation of the FLK-1 was achieved by ETS-1 interaction with HIF-2α, but not HIF-1α. Therefore, we analyzed the presence of potential HRE in the vicinity to the ETS factor binding sites for the HIF-2-dependent genes. Ten of 11 genes (Table 1) contained a HRE within 60 bp of the ETS recognition site. *CITED2* was the only gene in this group that has a well-characterized HRE in its promoter, localized between −1,202 and −1,159 bp from the transcription start site (TSS; ref. 35). This fragment also contains a putative binding site for the ELK-1 transcription factor, a member of ETS family. Only one gene on our list, *SOX9*, was found to have an ETS family binding site in the absence of a predicted HRE.

The ETS family of transcription factors is a large group with at least 25 different family members. To test the hypothesis that HIF-2 cooperates with ETS factors to activate certain genes in hypoxia, we inhibited one of the most ubiquitous ETS transcription factors (ELK-1) by siRNA and tested the effect on hypoxia-induced HIF-2-dependent genes. ELK-1 was chosen because it was present in ETS-HRE tandems of three genes (*IGFBP3, CITED2*, and *WISP2*; Fig. 4*A* and *B*). Analysis of hypoxia-mediated induction of the three genes bearing ELK-1 close to HRE showed that *CITED2* and *WISP2* no longer responded to hypoxia in the absence of ELK-1, whereas

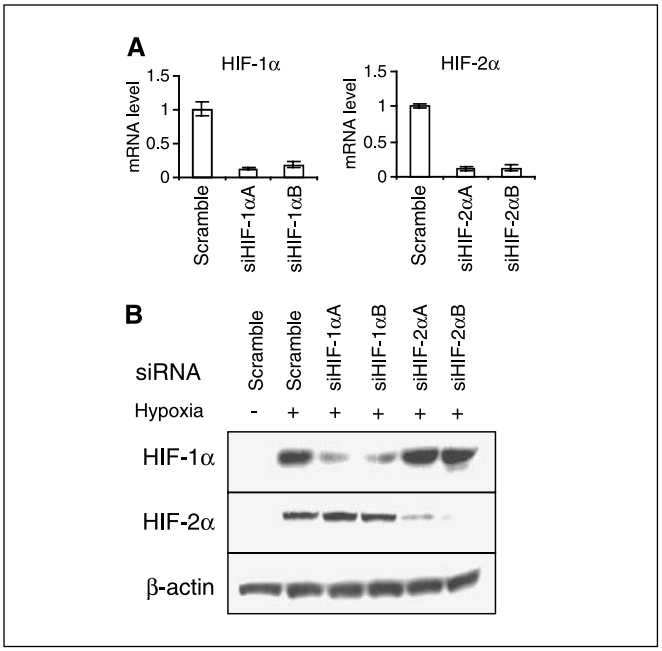

Figure 1. HIF-1α and HIF-2α inactivation by siRNA. *A,* quantitative RT-PCR of RNA from MCF7 cells 24 hours after transfection with two different HIF-1α siRNA (*left*) or HIF-2α siRNA (*right*). Scramble oligonucleotide was used as a control. *B,* Western blot analysis of HIF-1α and HIF-2α in MCF7 cells transfected with HIF-1α or HIF-2α siRNA and subjected to 8 hours to hypoxia (0.5% oxygen).

induction of *IGFBP3* by hypoxia was diminished (Fig. 4*C*). To validate that interaction with ELK-1 is specific for HIF-2-dependent genes, we selected several genes from HIF-1 regulated group that also have putative ELK-1 binding sites in their promoters. Three genes analyzed, *VEGF, HK1*, and *NDRG1*, showed no change in their ability to respond to hypoxia in the absence of ELK-1 (Fig. 4*D*).

The promoter of the *CITED2* gene has been previously cloned, and HREs have been identified by Bhattacharya et al. (35). We used two constructs with the CITED2 promoter to test transcriptional activation by HIF-1α and HIF-2α. The 2-kb promoter-luciferase construct (−2,186/+65) showed a 3-fold activation when cotransfected with HIF-1α P564A and ~25-fold activation with HIF-2α P531A (Fig. 5*A, left*). We used these proline-mutated HIF constructs to ensure higher stability of HIF proteins under normoxic conditions. Three consensus HREs (5′-RCGTG) were identified within a 32-nucleotide sequence at −1,196/−1,165 and shown to be responsible for hypoxia and desferrioxamine-dependent activation of the *CITED2* gene expression (35). A short fragment of 44 bp containing these HREs cloned upstream of a thymidine kinase (TK)-luciferase construct showed >100-fold activation by HIF-2α, whereas HIF-1α induced luciferase activity by 4.5-fold (Fig. 5*A, right*).

Because an ELK-1 consensus site (catacgGGACgtgcagc) within the CITED2 promoter is also located in the proximity to the HRE, we tested whether ELK-1 cooperatively increases HIF-2α transcriptional activity. We used a native CITED2 promoter-luciferase construct (−2,186/+65) in two different cell lines. We found that, in MCF7 cells, coexpression of ELK-1 with HIF-1α modestly increased luciferase activity from 2.2-fold (HIF-1α alone) to 2.6-fold (HIF-1α plus ELK-1), whereas activation with HIF-2α increased from 9.7-fold (HIF-2α alone) to 14.9-fold (HIF-2α plus ELK-1; Fig. 5*B*). Similar data were obtained with Hep3B cells (Fig. 5*C*). Because ELK-1 alone slightly activates the reporter construct, the modest increase of HIF-1α transcriptional activity by coexpression with ELK-1 can be explained by additive effect of these two transcription factors. On the other hand, increase in HIF-2α activity by ELK-1 is much greater than would be expected from an additive effect. These data imply that ELK-1 cooperatively increases HIF-2α activity in the transcriptional activation of the CITED2 promoter.

We next used chromatin immunoprecipitation to determine whether ELK-1 protein binds in proximity to the HREs in the promoter regions of HIF-2-dependent genes. Using anti-ELK-1 antibody and PCR primers designed around ELK-1/HRE sites, we found ELK-1 transcription factor bound to the promoters of two genes tested, *CITED2* and *WISP2* (Fig. 6*A*). We also did a classic coimmunoprecipitation assay to find out whether ELK-1 and HIF-2α physically interact with each other. We used the nuclear fraction of hypoxia-treated MCF7 and 786-O cells that do not express HIF-1α protein but express high levels of HIF-2α protein due to the mutational inactivation of pVHL. Anti-ELK-1 antibody (but not normal IgG) successfully precipitated the complex of ELK-1 with HIF-2α (Fig. 6*B*). No corresponding HIF-1α band was identified in MCF7 cells when the same blot was stripped and reprobed with anti-HIF-1α antibody.

Collectively, our data confirm that the ELK-1 transcription factor plays a direct role in hypoxic induction of HIF-2-dependent genes.

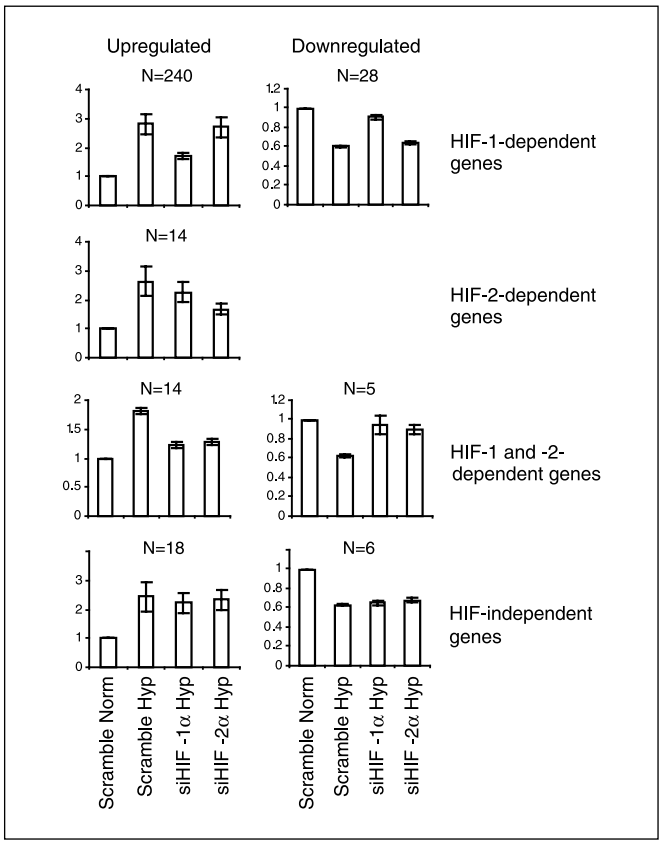

Figure 2. Results of microarray analysis. Average normalized signal value for the probes in each category are calculated relative to the control cells under normoxic conditions. The actual values of gene expression in each group follow the predicted pattern of gene behavior. *N,* number of probes. *Columns,* mean; *bars,* SD. No probes were down-regulated by HIF-2α.

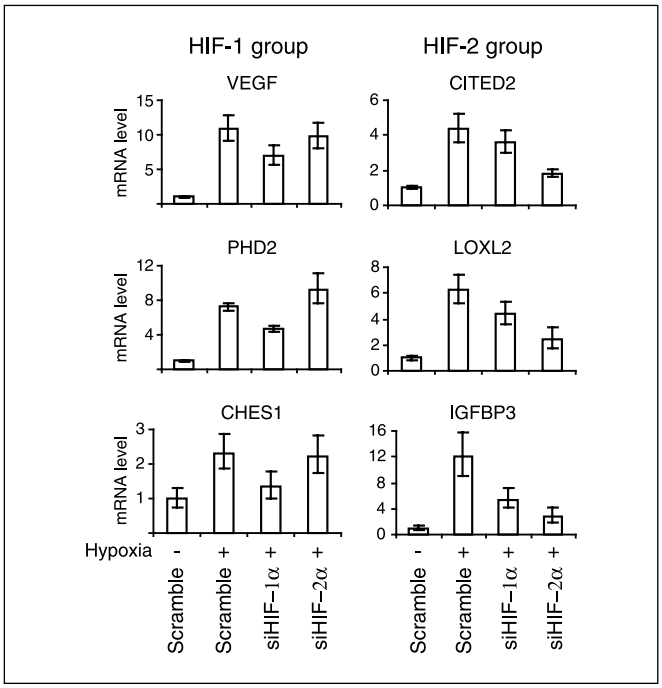

Figure 3. Quantitative RT-PCR validates microarray data. The selected HIF-1-dependent or HIF-2-dependent genes show less induction in hypoxia when the corresponding transcription factor was inactivated by RNAi. Data are the amount of each transcript relative to control cells transfected with scramble oligonucleotide under normoxic conditions. *Columns,* mean of three replicate experiments; *bars,* SE.

Table 1. Localization of ETS factors and HREs in the promoters of HIF-2-dependent genes

Gene name	Unigene ID	Promoter	TSS position	ETS factor	ETS position	HRE position
HIST1H4H	Hs.421737	P877397	1200	NRF2	148-164	149-153
EFNA1	Hs.399713	P912177	501, 914	GABP	441-457	409-413
IGFBP3	Hs.450230	P935978	501, 560	ELK1	432-448	446-450
IGFBP 3	Hs.450230	P935978	501, 560	ELF2	533-549	611-615
LOXL2	Hs.83354	P848219	501	ETS2	28-44	106-110
TBC1D3	Hs.562407	P311853	501	PU1	226-242	288-292
ILVBL	Hs.78880	P936164	501	NRF2	280-289	331-335
FAM13A1	Hs.442818	P951803	501	ETS1	530-546	605-609
HEY1	Hs.234434	P944652	566, 569	CETS1	112-128	104-109
CITED2	Hs.82071	P932762	1251	ELK1	1109-1125	1054-1058
CITED2	Hs.82071	P932762	1251	ELK1	46-62	54-58
WISP2	Hs.194679	P901924	501, 823	ELK1	907-923	872-876
WISP2	Hs.194679	P901925	501	ETS1	487-503	509-513

NOTE: Promoter numbers are given according to the Genomatix Suite nomenclature. Both ETS and HRE position numbers are from the beginning of the promoter. The TSS position shows the length of the upstream sequence analyzed.

Discussion

In this study, we used RNAi to specifically inactivate HIF-1α or HIF-2α to identify their contribution in hypoxia-regulated gene expression. The inactivation of HIF-α genes by siRNA eliminates several drawbacks of forced expression of HIF-α subunits, such as unequal expression levels and possible overwhelming of the balance in protein complexes acting to achieve regulation of genes in hypoxia. We observed that >80% of genes regulated by hypoxia were responsive to HIF-1α inactivation, suggesting that, in MCF7 cells, HIF-1 is the major transcription factor responsible for cellular adaptation to hypoxic conditions.

Other studies applied siRNA to HIF-1α and HIF-2α to observe the changes in the transcription of selected genes (31, 32). Similar to our observation, Sowter et al. (31) found that, in cell lines expressing both HIFs, the *VEGF, Glut1, CA9, Bnip3*, and *uPAR* genes were responsive to HIF-1α inactivation only. In addition, the authors also tested an effect of HIF-1α or HIF-2α inactivation on hypoxia-induced cell migration. Inhibition of HIF-1α as well as HIF-2α by siRNA compromised the ability of cells to migrate in an *in vitro* test, implying that there is a subset of HIF-2 target genes that play role in the hypoxia-mediated increase in cell migration. We found several HIF-2α-dependent genes that are potentially involved in cell motility and oncogenesis. *LOXL2* was recently identified as a gene with elevated expression in metastatic breast cancer cell lines (36), and MCF7 cells expressing LOXL2 were more invasive and metastatic in mouse xenograft models (37). Another

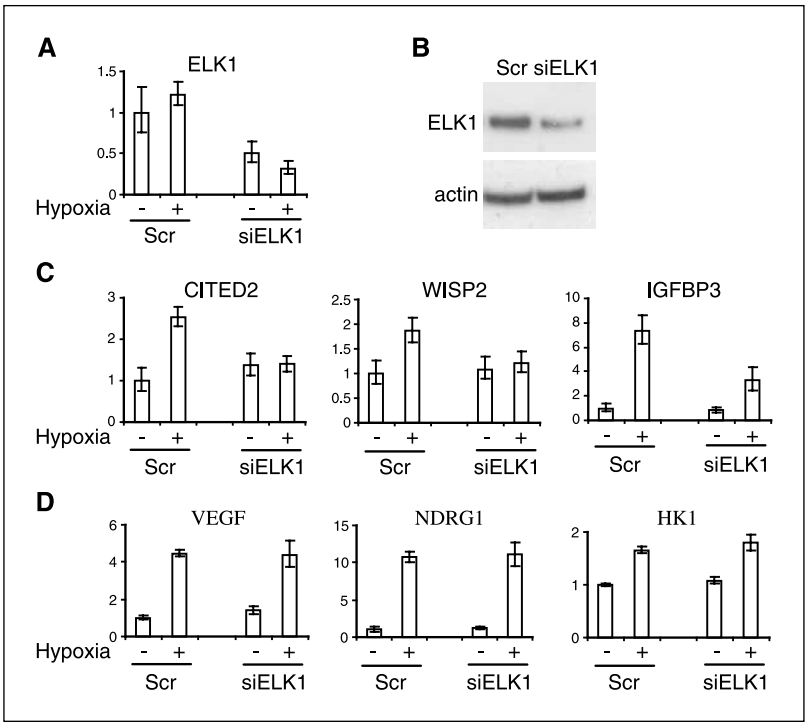

Figure 4. Knockdown of ELK-1 diminishes hypoxic induction of HIF-2-dependent genes but not HIF-1-dependent genes. *A,* quantitative RT-PCR analysis of ELK-1 after transfection of MCF7 cells with scramble (*Scr*) control or siELK-1. *B,* Western blot analysis of ELK-1 protein. *C* and *D,* quantitative RT-PCR analysis of the hypoxia-inducible genes after transfection with siELK-1. Genes selected from HIF-2-dependent group (*C*) and HIF-1-dependent group (*D*) relative to their expression in the scramble-transfected MCF7 cells under normoxic conditions. *Columns,* mean of the two independent transfection experiments with three repeats in each; *bars,* SE.

HIF-2α regulated gene, *WISP2*, was highly expressed in breast cancer cell lines compared with normal mammary epithelia (38) and is required for the estrogen-stimulated cell proliferation in the MCF7 cell line (39). In agreement with the recent study of Wang et al. (28), we found that CITED2 is preferentially regulated by HIF-2α. CITED2 is involved in cell proliferation and oncogenesis; however, under hypoxic condition, it limits HIF activity by competing for transcriptional activator CBP/p300 (35). It is not clear if CITED2 can promote oncogenic transformation of hypoxic cells.

Recently, the *EPO* gene was identified as an exclusive target of HIF-2α in two different cell lines, hepatocellular carcinoma Hep3B and human neuroblastoma cells Kelly (32). Analysis showed that DNA sequences surrounding HRE are important for the HIF-2α responsiveness. It is therefore likely that HIF-2α binds to the EPO-HRE in conjunction with other nuclear factors that recognize DNA sequences adjacent to the HRE. The authors suggested that the candidate nuclear factor was HNF4, identified previously as a factor required for the full hypoxic response of the endogenous *EPO* gene in Hep3B (40). In our study, EPO was not expressed in the MCF7 cell line under any conditions (hypoxia or normoxia). It is therefore possible that, in different cell lines, the choice of the target genes by HIF-1 or HIF-2 may depend on availability and cooperative effect of tandem factors recognizing their cognate elements in the promoters.

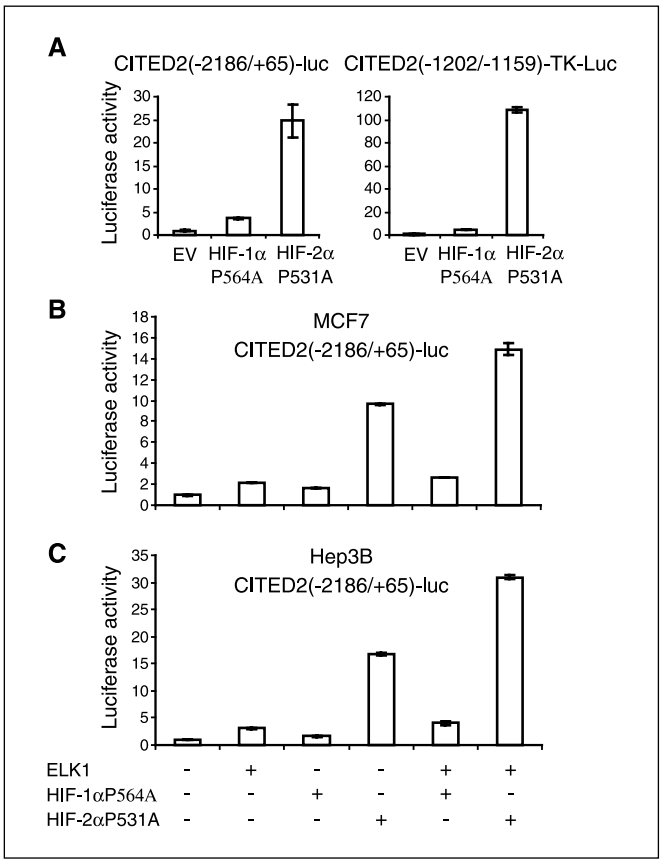

Figure 5. CITED2 promoter is preferentially activated by HIF-2α and further enhanced by ELK-1. *A,* MCF7 cells were transfected with either the native CITED2 promoter-luciferase (*Luc*) construct (−2,186/+65; *left*) or the hypoxia-responsive fragment of this promoter-TK-luciferase construct (−1,202/−1,159; *right*) together with pCMV-Renilla and empty vector (*EV*) or expression vectors encoding HIF-1α P564A or HIF-2α P531A. MCF7 (*B*) and Hep3B (*C*) cells were transfected with the native CITED2 promoter-luciferase reporter and either HIF-1α P564A or HIF-2α P531A alone or in combination with the ELK-1 expression vector. Data relative to the empty vector control. *Columns,* mean; *bars,* SE.

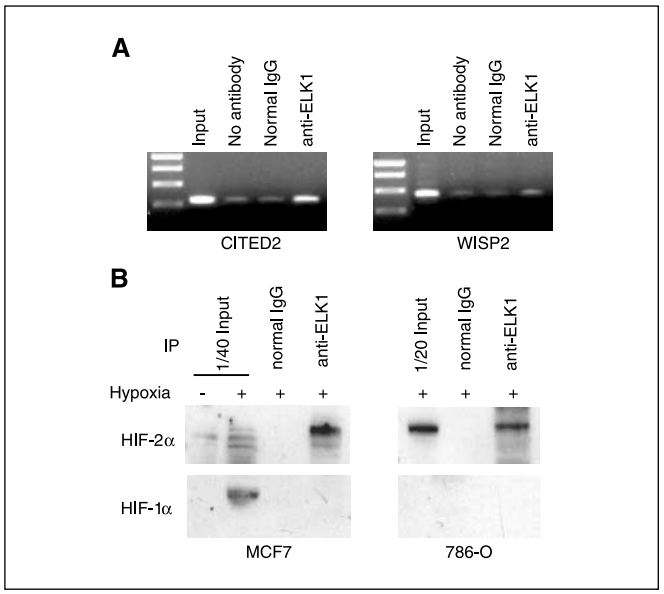

Figure 6. ELK-1 protein binds to the promoters of CITED2 and WISP2 at sites close to HREs and coprecipitates with HIF-2α protein. *A,* chromatin immunoprecipitation done with MCF7 cells treated with hypoxia. The primers for PCR were designed around ELK-1/HRE sites. *B,* immunoprecipitation (*IP*) with anti-ELK-1 antibody was followed by Western blot with anti-HIF-2α or anti-HIF-1α antibody using nuclear extracts from MCF7 or 786-O cells treated with hypoxia.

Our data suggest that HIF-2 cooperates with ETS family transcription factors in MCF7 cells, a finding supported by the data for another member of the ETS family. Elvert et al. (34) showed that VEGF receptor-2 (FLK-1) is activated by the cooperative interaction between HIF-2α and ETS-1, and HIF-1α cannot substitute HIF-2α in this interaction. The ETS family of transcription factors consists of a large number of evolutionarily conserved proteins controlling cell proliferation, angiogenesis, and metastasis during tumor progression. They can also mediate interaction between tumor and stromal cells and play critical role during epithelial-mesenchymal transition (41). Importantly, ELK-1 is activated by hypoxia via phosphorylation by mitogen-activated protein kinase (42, 43).

This is one of the first attempts to understand how similar HIFs are selecting their target genes. We identified a necessary but not sufficient factor in HIF-2-dependent gene expression. It seems unlikely that mapping of all ETS binding sites next to HRE in the promoters will be enough to say which HIF will be responsible for the activation. Other transcription factors may selectively cooperate with HIFs and determine the preference of HIF-1 over HIF-2 and vice versa. Identification of these factors will help to explain the cell type-dependent differences in the genetic response to hypoxia as well as differences in the magnitude and timing of hypoxia-induced gene expression.

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References

1. Semenza GL. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551–78.
2. Wenger RH. Cellular adaptation to hypoxia: O_2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O_2-regulated gene expression. FASEB J 2002; 16:1151–62.
3. Cockman ME, Masson N, Mole DR, et al. Hypoxia inducible factor-α binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem 2000;275:25733–41.
4. Huang LE, Gu J, Schau M, et al. Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 1998;95:7987–92.
5. Kamura T, Sato S, Iwai K, et al. Activation of HIF1α ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. Proc Natl Acad Sci U S A 2000;97:10430–5.
6. Maxwell PH, Wiesener MS, Chang GW, et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399:271–5.
7. Ohh M, Park CW, Ivan M, et al. Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel-Lindau protein. Nat Cell Biol 2000;2:423–7.
8. Tanimoto K, Makino Y, Pereira T, et al. Mechanism of regulation of the hypoxia-inducible factor-1α by the von Hippel-Lindau tumor suppressor protein. EMBO J 2000; 19:4298–309.
9. Jaakkola P, Mole DR, Tian YM, et al. Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 2001;292:468–72.
10. Epstein AC, Gleadle JM, McNeill LA, et al. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 2001;107:43–54.
11. Masson N, Willam C, Maxwell PH, et al. Independent function of two destruction domains in hypoxia-inducible factor-α chains activated by prolyl hydroxylation. EMBO J 2001;20:5197–206.
12. Ivan M, Kondo K, Yang H, et al. HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science 2001;292:464–8.
13. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 2001;294: 1337–40.
14. Iyer NV, Kotch LE, Agani F, et al. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1α. Genes Dev 1998;12:149–62.
15. Kotch LE, Iyer NV, Laughner E, et al. Defective vascularization of HIF-1α-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Dev Biol 1999;209:254–67.
16. Ryan HE, Lo J, Johnson RS. HIF-1α is required for solid tumor formation and embryonic vascularization. EMBO J 1998;17:3005–15.
17. Tian H, Hammer RE, Matsumoto AM, et al. The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev 1998;12:3320–4.
18. Peng J, Zhang L, Drysdale L, et al. The transcription factor EPAS-1/hypoxia-inducible factor 2α plays an important role in vascular remodeling. Proc Natl Acad Sci U S A 2000;97:8386–91.
19. Compernolle V, Brusselmans K, Acker T, et al. Loss of HIF-2α and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med 2002;8:702–10.
20. Talks KL, Turley H, Gatter KC, et al. The expression and distribution of the hypoxia-inducible factors HIF-1α and HIF-2α in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol 2000;157: 411–21.
21. Semenza GL. HIF-1 and tumor progression: pathophysiology and therapeutics. Trends Mol Med 2002;8: S62–7.
22. Goonewardene TI, Sowter HM, Harris AL. Hypoxia-induced pathways in breast cancer. Microsc Res Tech 2002;59:41–8.
23. Yoshimura H, Dhar DK, Kohno H, et al. Prognostic impact of hypoxia-inducible factors 1α and 2α in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. Clin Cancer Res 2004;10:8554–60.
24. Aprelikova O, Chandramouli GV, Wood M, et al. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J Cell Biochem 2004;92:491–501.
25. Jiang Y, Zhang W, Kondo K, et al. Gene expression profiling in a renal cell carcinoma cell line: dissecting VHL and hypoxia-dependent pathways. Mol Cancer Res 2003;1:453–62.
26. Hu CJ, Wang LY, Chodosh LA, et al. Differential roles of hypoxia-inducible factor 1α (HIF-1α) and HIF-2α in hypoxic gene regulation. Mol Cell Biol 2003;23:9361–74.
27. Wykoff CC, Sotiriou C, Cockman ME, et al. Gene array of VHL mutation and hypoxia shows novel hypoxia-induced genes and that cyclin D1 is a VHL target gene. Br J Cancer 2004;90:1235–43.
28. Wang V, Davis DA, Haque M, et al. Differential gene up-regulation by hypoxia-inducible factor-1α and hypoxia-inducible factor-2α in HEK293T cells. Cancer Res 2005;65:3299–306.
29. Salnikow K, Davidson T, Zhang Q, et al. The involvement of hypoxia-inducible transcription factor-1-dependent pathway in nickel carcinogenesis. Cancer Res 2003;63:3524–30.
30. Vengellur A, Woods BG, Ryan HE, et al. Gene expression profiling of the hypoxia signaling pathway in hypoxia-inducible factor 1α null mouse embryonic fibroblasts. Gene Expr 2003;11:181–97.
31. Sowter HM, Raval RR, Moore JW, et al. Predominant role of hypoxia-inducible transcription factor Hif-1α versus Hif-2α in regulation of the transcriptional response to hypoxia. Cancer Res 2003;63:6130–4.
32. Warnecke C, Zaborowska Z, Kurreck J, et al. Differentiating the functional role of hypoxia-inducible factor HIF-1α and HIF-2α (EPAS-1) by the use of RNA interference: erythropoietin is a HIF-2α target gene in Hep3B and Kelly cells. FASEB J 2004;18:1462–4.
33. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via *de novo* protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992;12: 5447–54.
34. Elvert G, Kappel A, Heidenreich R, et al. Cooperative interaction of hypoxia-inducible factor-2α (HIF-2α) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). J Biol Chem 2003;278:7520–30.
35. Bhattacharya S, Michels CL, Leung MK, et al. Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. Genes Dev 1999;13:64–75.
36. Kirschmann DA, Seftor EA, Fong SF, et al. A molecular role for lysyl oxidase in breast cancer invasion. Cancer Res 2002;62:4478–83.
37. Akiri G, Sabo E, Dafni H, et al. Lysyl oxidase-related protein-1 promotes tumor fibrosis and tumor progression *in vivo*. Cancer Res 2003;63:1657–66.
38. Saxena N, Banerjee S, Sengupta K, et al. Differential expression of WISP-1 and WISP-2 genes in normal and transformed human breast cell lines. Mol Cell Biochem 2001;228:99–104.
39. Banerjee S, Saxena N, Sengupta K, et al. WISP-2 gene in human breast cancer: estrogen and progesterone inducible expression and regulation of tumor cell proliferation. Neoplasia 2003;5:63–73.
40. Galson DL, Tsuchiya T, Tendler DS, et al. The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. Mol Cell Biol 1995;15: 2135–44.
41. Hsu T, Trojanowska M, Watson DK. Ets proteins in biological control and cancer. J Cell Biochem 2004;91: 896–903.
42. Muller JM, Krauss B, Kaltschmidt C, et al. Hypoxia induces c-*fos* transcription via a mitogen-activated protein kinase-dependent pathway. J Biol Chem 1997; 272:23435–9.
43. Yan SF, Lu J, Zou YS, et al. Hypoxia-associated induction of early growth response-1 gene expression. J Biol Chem 1999;274:15030–40.