Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 312 (2003) 571–577
BBRC
www.elsevier.com/locate/ybbrc

RNA interference for HIF-1α inhibits its downstream signalling and affects cellular proliferation

Jörg Hänze,* Bastian G. Eul, Rajkumar Savai, Stefanie Krick, Parag Goyal, Friedrich Grimminger, Werner Seeger, and Frank Rose

Department of Internal Medicine 2, Klinikstr. 36, Medical School of the Justus-Liebig-University, Giessen D-35392, Germany

Received 8 October 2003

Abstract

Transcription factor HIF-1 is a key determinant of oxygen-dependent gene regulation. Suppression of HIF-1α is important for exploring HIF-1-dependent processes and for interfering with hypoxia-induced pathophysiological events. This study applied RNA-interference targeting HIF-1α to the human lung A549 cell line. Transfection of *HIF-1α-siRNA* reduced HIF-1α synthesis as measured on mRNA and protein level by realtime RT-PCR, Western blot, and immuncytochemistry. A time kinetic for hypoxic stabilization of HIF-1α protein and its inhibition by *HIF-1α-siRNA* is included. Hypoxic induction of HIF-1-controlled target genes as heme oxygenase I (HO-1), phosphoglycerate kinase (PGK), and vascular endothelial growth factor (VEGF) was markedly attenuated by *HIF-1α-siRNA* treatment. Correspondingly, gene activation via hypoxia-responsive-element, as shown by reporter gene assay, was inhibited by *HIF-1α-siRNA*. Moreover, this approach was found to suppress the shift from from S-phase to G_1-phase observed in A549 cells in response to hypoxia, supporting a role of HIF-1α in oxygen-dependent cell cycle regulation.

Keywords: HIF-1; RNA interference; siRNA; Cell cycle; Proliferation; Hypoxia

Transcription factor HIF-1 adapts cells to low oxygen partial pressure. HIF-1 is specifically activated in hypoxia [1] and induces target genes which have influence on energy metabolism [2], cell proliferation [3], hematopoiesis [4], vascular development (remodelling, angiogenesis) [5,6], and vasotone [7]. Regulation of HIF-1, which is a dimer of HIF-1α and HIF-1β, is mainly controlled by protein stabilization and transactivation of HIF-1α [8,9]. In normoxia, HIF-1α is instantaneously targeted to ubiquitination by hydroxylation of two specific proline residues and then degraded by proteasome pathway [10,11]. In hypoxia, hydroxylation and following proteasomal degradation is inhibited. This leads to a dramatic increase of HIF-1α protein in hypoxia. Transactivation of HIF-1α involves the negative regulation of hydroxylation of an asparagine-residue in hypoxia [12]. Subsequently, HIF-1α translocates to the nucleus, dimerizes with HIF-1β, and activates target genes by binding of HIF-1 to the *cis*-acting hypoxia-responsive-element (HRE). Related transcription factors of HIF-1α are HIF-2α and HIF-3α [13,14]. They are expressed in a more cell specific manner, also dimerize with HIF-1β, and activate target genes via HRE.

The regulation of cellular oxygen concentration is disturbed, e.g., in ischemic disease, cancer, and various lung diseases [15–17]. Whereas in ischemic disease (e.g., coronary heart disease, cerebrovascular disease) an up-regulation of HIF-1α is considered to have therapeutical benefit [18,19], the downregulation of HIF-1α may be beneficial in cancer for the inhibition of tumor angiogenesis or in hypoxic-triggered pulmonary hypertension [20]. Recent approaches for suppression of HIF-1 include HIF-1 transcription factor decoy [6], HIF-1α antisense [21], and overexpression of HIF dominant negative mutants [22,23].

In this study, a siRNA motif for suppression of HIF-1α was selected and applied to the human lung A549 cell line for RNA interference [24]. Suppression of HIF-1 pathway by *HIF-1α-siRNA* was monitored on regulatory

* Corresponding author. Fax: +49-641-9942429.
E-mail address: joerg.haenze@innere.med.uni-giessen.de (J. Hänze).

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.10.153

important levels as HIF-1α mRNA and protein expression as well as HRE activation. Additionally, effects on cell cycle control were analyzed. In essence, siRNA for HIF-1α was found to be an appropriate tool for specific interference with HIF-1 related signal transduction and hypoxia-driven downstream events.

Materials and methods

Cell culture. Culturing of the human pulmonary epithelial cell line A549 was performed according to the protocol given by the American Type Culture Collection.

Hypoxia treatment. Culturing of cells in hypoxia was performed in a chamber equilibrated with a water saturated gas mixture of 1% oxygen, 5% carbon dioxide, and 94% nitrogen at 37 °C. Culturing of normoxic control cells was performed using an incubator saturated with water in atmospheric condition and supplemented with 5% carbon dioxide at 37 °C.

Treatment of cells with siRNA. Using HUSAR software package (Heidelberg Unix Sequence Analysis resources) a siRNA motif according to the AA-N_{19} rule was selected by applying find pattern program to the human HIF-1α cDNA sequence (GenBank Accession No. U22431). The target sequence was localized at position 146 bases downstream of the start codon from HIF-1α. The forward and reverse RNA strands (ugu gag uuc gca ucu uga u *dtdt*) and (auc aag aug cga acu cac a *dtdt*) with two 5′ deoxy-thymidine overhangs were commercially synthesized (Biospring, Frankfurt, Germany) and annealed at a final concentration of each 20 μM by heating at 95 °C for 1 min and incubating at 37 °C for 1 h in annealing buffer (20 mM potassium acetate, 6 mM Hepes–KOH, pH 7.4, and 0.4 mM magnesium acetate). Transfection of siRNA was performed at a concentration of 100 nM using Oligofectamin or Lipofectamin 2000 (Invitrogen). As a control for *HIF-1α-siRNA* we used either a corresponding random siRNA sequence (*control-siRNA*: uac acc guu agc aga cac c dtdt) or in case of luciferase reporter gene assay also *luciferase siRNA* (cuu acg cug agu acu ucg a dtdt) targeting firefly luciferase [24].

HIF-1α Western-blot analysis. Western-blot analysis of HIF-1α was performed using a previously described polyclonal HIF-1α antibody [25]. In brief, normoxic or hypoxic cells were scraped from dishes and cellular protein extracts were prepared by homogenization in an ice-cold buffer (8 M urea, 10% glycerol, 1% sodium dodecyl sulfate, 5 mM dithiothreitol, and 10 mM Tris–HCl, pH 6.8) and a protease inhibitor cocktail (Sigma–Aldrich Chemie GmbH, Deisenhofen, Germany). Twenty microgram protein was run per lane on a sodium dodecyl sulfate–polyacrylamide gel. After electroblot of the gel to a nylon membrane (PVDF, Pal), HIF-1α specific bands were visualized by chemiluminescence (ECL, Amersham, Freiburg, Germany) by using a second anti-chicken antibody coupled with horseradish peroxidase.

Immuncytochemistry of HIF-1α. A549 cells were grown on chamber slides and treated as indicated. Then cells were removed and fixed immediately in acetone and methanol (1:1). The fixed cells were incubated overnight at 4 °C with phosphate-buffered saline containing an anti-human HIF-1α monoclonal mouse antibody (BD Biosciences, Heidelberg, Germany) diluted 1:100. Indirect immunofluorescence was obtained by incubation with FITC-conjugated rabbit anti-mouse IgG antibodies (DAKO, Denmark) diluted 1:100 in phosphate-buffered saline. The coverslips were mounted on glass slides and subjected to microscopic analysis.

Construction of expression plasmid for HIF-1α. For isolation of full length cDNA fragments of HIF-1α, we employed a nested RT-PCR from RNA extracts of human lung cells. For maximal translation efficacy, we inserted the Kozak sequence (GCCGCCACCATGG) at the start codon of translation. The full length HIF-1α cDNA (Accession No. NM_001530) was ligated into expression verctor pMG (Invivogen, San Diego, USA) and verified by sequencing (ABI Prism 310, Applied Biosystems).

Reporter-gene-assay for HIF-1α-dependent gene transcription. A dual reporter gene assay for studying HIF-1α-dependent gene regulation was performed. One vector carrying a firefly luciferase gene, controlled by a three-tandem repeat of the HRE coupled to a thymidine-kinase minimal promoter (TK-MP) from *Herpes simplex*, was cotransfected with a second reporter vector carrying a *Renilla* luciferase gene controlled by the TK-MP promoter without HRE. The values represent the ratio of chemiluminescence measured for firefly and *Renilla*, respectively, thus giving the HRE specific luciferase activation. Hypoxic-responsive promoter constructs were prepared by inserting the consensus HRE element of the erythropoietin gene promoter (HIF-HRE: 5′-GCCCTACGTGCTGTCTCA-3′) as a three tandem repeat 5′ upstream of the herpes simplex thymidine kinase promoter into the pGL3-plasmid (Promega, Mannheim, Germany) containing the firefly luciferase. The insert was verified by sequencing. The HRE-TK-MP and pRL-TK-MP plasmids were transfected in a molar ratio of 3:1 into A549 cells by Lipofectamin 2000 according to the manufacturer's protocol (Invitrogen, California, USA) in a 48-well plate. Transfected cells were then incubated for another hour in FCS containing medium, after which medium was replaced by serum-free medium, and culturing in normoxic or hypoxic condition was conducted for 20 h. The cells were lysed in 4 °C luciferase lysis buffer (Promega, Mannheim, Germany), and firefly and *Renilla* luciferases activities were measured by a bioluminometer (TEKAN, Crailsheim, Germany).

Realtime RT-PCR. RNA was extracted from human cells using guanidine thiocyanate-acid phenol (RNAzol B, WAK-Chemie, Germany). Two microgram RNA per sample was copied to cDNA using reverse transcriptase (MMLV-RT) (Gibco-BRL, Karlsruhe, Germany) in a standard protocol with random hexamer primers. For the negative control, MMLV was omitted. Real-time PCR was performed using the ABI Prism 7700 Detection system (Applied Biosystem, Weiterstadt, Germany) with SYBR-Green as fluorescent dye enabling real time detection of PCR products according to the manufacturer's protocol. The cDNA was submitted to real-time PCR using the primer pairs as given below. Cycling conditions were 95 °C for 15 min, followed by 50 cycles of 94 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s. For quantification, the target gene was normalized to the internal standard gene β-actin or hypoxanthine phosphoribosyltransferase 1 (HPRT) using the following equation: $T_0/R_0 = K \cdot (1+E)^{\mathrm{CT,R\text{-}CT,T}}$ with T_0 is the initial number of target gene mRNA copies, R_0 is the initial number of standard gene mRNA copies, E is the efficiency of amplification, CT,T is the threshold cycle of target gene, CT,R, is the threshold cycle of standard gene, and K is the constant. The efficiencies were shown to be in the range $0.90 < E < 1.0$. The following primer sets (+, sense; −, antisense) were derived from the GenBank sequences: heme oxygenase 1 (HO-1) (Accession No. NM_002133) HO-1+: 5′ ATG ACA CCA AGG ACC AGA GC 3′ HO-1−: 5′ AGA CAG CTG CCA CAT TAG GG3′, HPRT (Accession No. NM_000194) HPRT+: 5′ TCG AGA TGT GAT GAA GGA GAT GGG A 3′ HPRT−: 5′ TCA AAT CCA ACA AAG TCT GGC TTA T 3′, β-actin (Accession No. NM_001101) β-actin+: 5′ GAT CAT TGC TCC TCC TGA GC 3′, β-actin−: 5′ TGT GGA CTT GGG AGA GGA CT 3′, PGK (Accession No. NM_000291) PGK+: 5′ CCC TGG CAA ATG CTT CCC ATT CAA ATA CC 3′, PGK−: 5′ TCA TCC TCC TGG AGA ACC TCC GCT TTC AT 3′, HIF-1α (Accession No. NM_001530) HIF-1α+: 5′ CCA TTA GAA AGC AGT TCC GC 3′ HIF-1α−: 5′ TGG GTA GGA GAT GGA GAT GC 3′, and VEGF (Accession No. NM_003376) VEGF+: 5′ GGA GTG TGT GCC CAC CGA GGA GTC CAA C 3′ VEGF−: 5′ GGT TCC CGA AAC CCT GAG GGA GGC T 3′.

Cell cycle analysis. As much as 1×10^6 growth arrested cells (serum-depleted medium for 24 h) were set under hypoxic conditions. After 24 h, cells were washed in ice-cold PBS before trypsinization. After fixation with 75% ethanol, cells were digested with DNAse-free RNAse in PBS containing 5 μg/ml propidium iodide for DNA-staining

(45 min at 37 °C). PI and forward light scattering was detected by using a flow cytometer of the type FACSCalibur (Beckton–Dickinson USA) equipped with Cellquest software.

BrdU incorporation. Proliferation was assessed by incorporation of the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) into the DNA of replicating cells using a commercially available colorimetric immunoassay according to the recommended protocol of the company (Promega). After addition of BrdU to cells they were fixed and incorporated BrdU was measured by ELISA using a specific BrdU antibody. The values given in the figures represent the raw data obtained by photometric measurement at 450 nm.

Results and discussion

RNA interference for HIF-1α was employed by transfection of a siRNA motif for HIF-1α (*HIF-1α-siRNA*) to A549 cells. Effects were analyzed and compared with those of a non-HIF-1α related siRNA (*control-siRNA*). Measuring HIF-1α mRNA concentration in *HIF-1α-siRNA* transfected cells, we found reduction of HIF-1α mRNA by approximately 70%, when compared to *control-siRNA* treated cells in both normoxia and hypoxia (Fig. 1A). Since primer pairs selected for HIF-1α realtime RT-PCR did not span the *HIF-1α-siRNA* target region, reduction of full length HIF-1α mRNA (at least cleaved within the siRNA target region) is expected to be even stronger than that measured in *HIF-1α-siRNA* treated cells. We next investigated hypoxic induction of HIF-1α protein by Western-blot analysis in a time-dependent manner (Fig. 1B). HIF-1α protein was detected at all time points within the studied time period from 10 to 72 h of hypoxic treatment in *control-siRNA* treated cells. Maximal induction was observed already after 10 h of hypoxia. This induction was strongly reduced in *HIF-1α-siRNA* treated cells.

Additionally, we investigated HIF-1α expression by immuncytochemistry for controlling *HIF-1α-siRNA* effects on the single cellular level (Fig. 2A). In *HIF-1α-siRNA* treated cells, but not in those treated with *control-siRNA*, hypoxic induction of HIF-1α was abrogated in the majority of cells reflecting the high transfection efficacy of siRNA, whereas in normoxia almost no HIF-1α staining was visible, as expected. Employing samples transfected by a HIF-1α expression-plasmid for HIF-1α overexpression, *HIF-1α-siRNA* treatment was again potent enough to drastically diminish HIF-1α protein. Interestingly, in these HIF-1α overexpressing cells, HIF-1α was detectable in a small cell population even in normoxia, indicating that the normal normoxic proteasomal degradation capacity for HIF-1α is overridden under these conditions. This normoxic HIF-1α expression was also inhibited by *HIF-1α-siRNA* treatment. Samples investigated by HIF-1α immuncytochemistry were additionally controlled by HIF-1α Western blot (Fig. 2B) showing concordant results.

We then analyzed endogenous HIF-1α-dependent target genes which are controlled via a hypoxia-responsive-element (HRE). These included heme oxygenase I (HO-1) [26], phosphoglycerate kinase (PGK) [2], and the vascular endothelial growth factor (VEGF) [5] and were analysed in relation to the non-hypoxic controlled housekeeping genes HPRT or β-actin by realtime RT-PCR (Fig. 3). We found a typical upregulation of these target genes in hypoxia, which was abrogated in *HIF-1α-siRNA* treated cells. Since the investigated target genes are additionally controlled by factors beside HIF [27], we specifically analyzed activation of the solely HIF dependent hypoxia-responsive element (HRE) by reporter-gene-assay (Fig. 4). In these experiments, HRE was coupled to the thymidine-kinase minimal promoter (TK-MP). This construct shows most specific HIF-1-dependent gene induction [28]. According to the HIF-1α protein data, we found abrogation of HRE induction in hypoxia by *HIF-1α-siRNA* treatment. As an additional control, we used *luciferase-siRNA* targeted against firefly luciferase [24]. *Luciferase-siRNA* inhibited

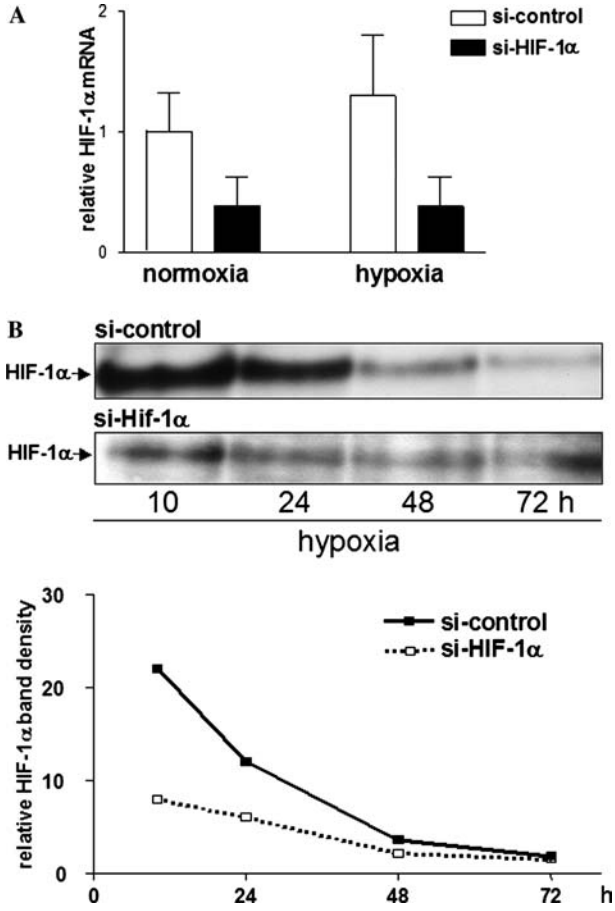


Fig. 1. (A) Relative HIF-1α mRNA quantification, related to HPRT mRNA by realtime RT-PCR. A549 cells were transfected by *control-siRNA* (si-control) or *HIF-1α-siRNA* (si-HIF-1α) and cultured for 24 h in normoxia or hypoxia (mean, SEM, $n = 3$). (B) Top: HIF-1α Western-blot analysis of protein extracts from A549 cells treated by *control-siRNA* or *HIF-1α-siRNA* and cultured for the given time periods (h) in hypoxia. Bottom: densitometric quantification of HIF-1α Western blot.

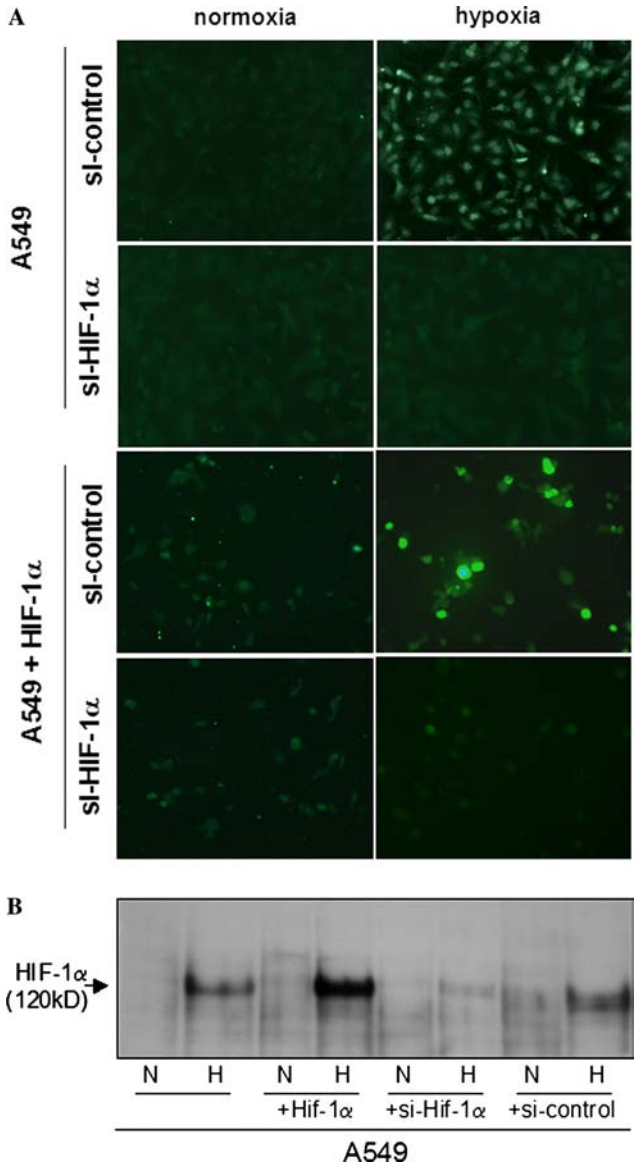


Fig. 2. (A) Immuncytochemistry for HIF-1α in normoxia (N) and hypoxia (H). A549 cells treated by *control-siRNA* (si-control) or *HIF-1α-siRNA* (si-HIF-1α), and A549 cells transfected by HIF-1α expression plasmid (+HIF-1α) and then treated by *control-siRNA* or *HIF-1α-siRNA* were analyzed. (B) The corresponding HIF-1α Western-blot analysis of samples from (A) is shown.

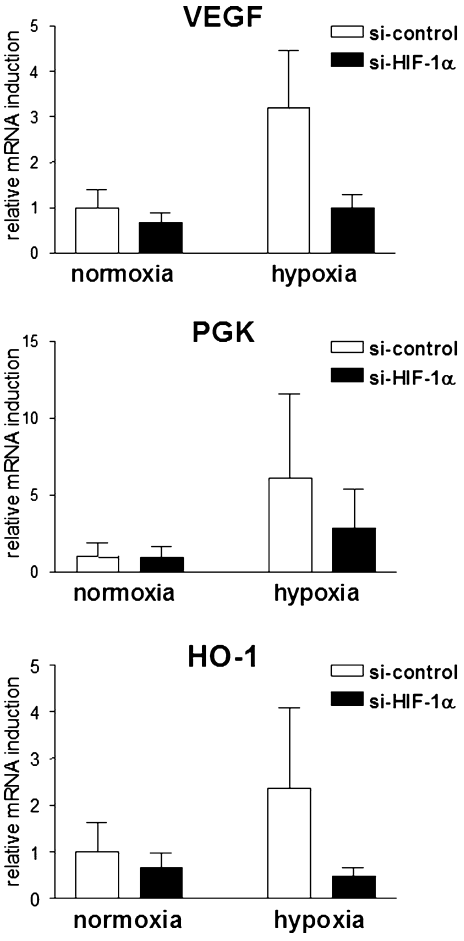


Fig. 3. Relative quantification of HIF-1-dependent target gene expression. mRNAs encoding VEGF, PGK, and HO-1 related to housekeeping genes β-actin or HPRT are analyzed by realtime RT-PCR. A549 cells were transfected by *control-siRNA* (si-control) or *HIF-1α-siRNA* (si-HIF-1α) and cultured for 24 h in normoxia or hypoxia (mean, SEM, $n = 3$).

firefly-luciferase reporter gene expression more strongly and independent of normoxia or hypoxia when compared to the effects of *HIF-1α-siRN*A, as expected.

Considering hypoxia-induced changes in cell growth [3,29], we studied proliferation of A549 cells in normoxia and hypoxia by cell cycle analysis. We employed flow cytometry and DNA staining by propidium iodide for discrimination of cells in G_1/G_0 phase or S/G_2 phase. In hypoxia we observed an increase of G_1/G_0 phase and a decrease of S/G_2 phase when compared to normoxia (Fig. 5A). Additionally, we used BrdU incorporation to measure S-phase directly. We found a decrease of BrdU incorporation in hypoxia versus normoxia performed at different fetal bovine serum concentrations (Fig. 5B). These findings reflect a hypoxia-induced growth arrest in A549 cells, which has also been observed in other cell types [3]. We then analyzed proliferation in *siRNA* treated cells. Transfection of cells by *control-siRNA* transfection again revealed a decrease of proliferation when comparing normoxia and hypoxia using either cell cycle analysis by propidium iodide staining (Fig. 6A) or BrdU incorporation method (Fig. 6B). However, in case of *HIF-1α-siRNA* transfection the situation was different. We did not further observe a growth arrest when changing from normoxia to hypoxia. In hypoxia we rather observed a decrease of G_1/G_0 phase and an increase of S/G_2 phase when compared to normoxia. Also, analysis of BrdU incorporation in *HIF-1α-siRNA* transfected cells was significantly higher than that of *control-siRNA* treated cells in hypoxia.

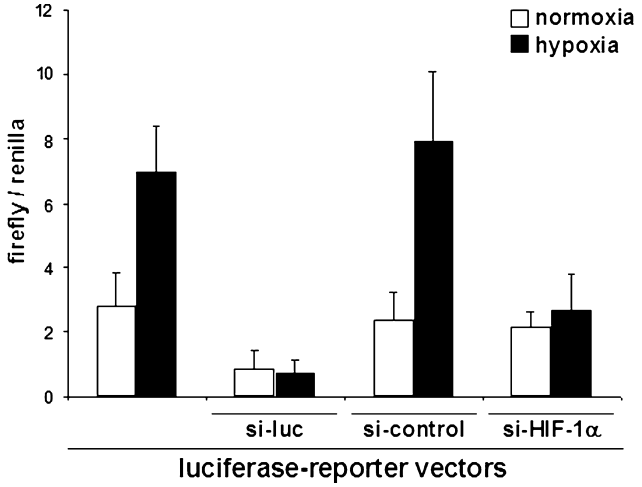
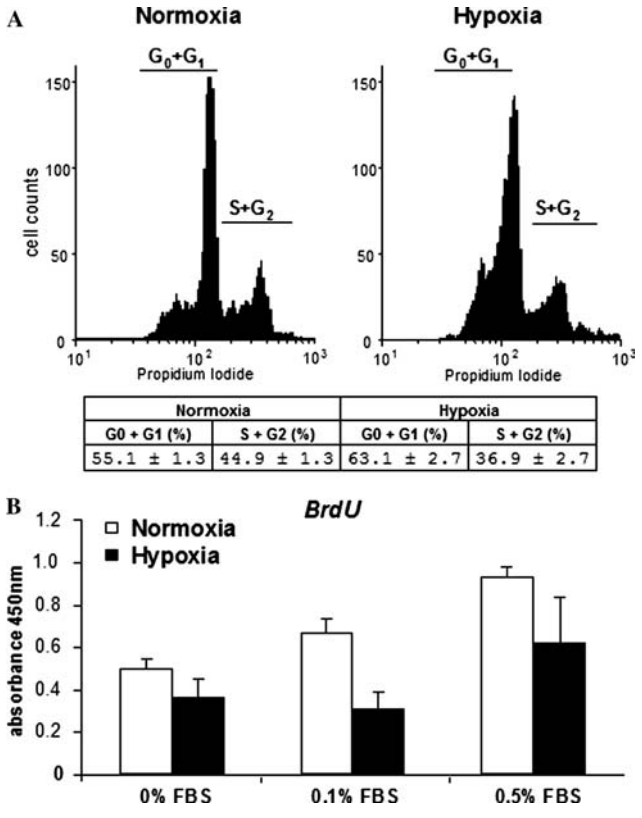
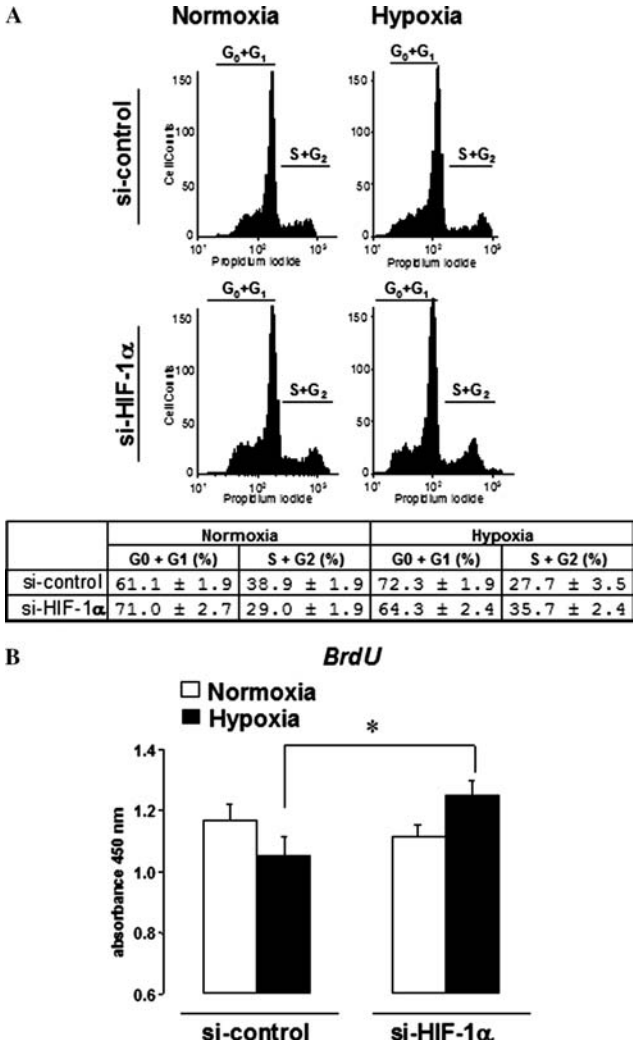


Fig. 4. Hypoxia-responsive-element (HRE) reporter gene assay performed on A549 cells, without any treatment, or treated with *luciferase-siRNA* (si-luc), *control-siRNA* (si-control), or *HIF-1α-siRNA* (si-HIF-1α). Shown is the ratio of hypoxia-dependent firefly-luciferase and hypoxia-independent *Renilla* luciferase values (see Methods) (mean, SEM, $n = 5$).



Normoxia		Hypoxia	
G0 + G1 (%)	S + G2 (%)	G0 + G1 (%)	S + G2 (%)
55.1 ± 1.3	44.9 ± 1.3	63.1 ± 2.7	36.9 ± 2.7

Fig. 5. Proliferation and cell cycle analysis of A549 cells cultured in normoxia or hypoxia (1% O_2) after serum deprivation for 24 h. (A) Representative flow cytometric analysis of cell cycle using propidium iodide for DNA staining: shown is the pattern of A549 cells being in $G_0 + G_1$ phase or $G_2 + S$ phase during normoxia and hypoxia. The quantitative analysis of cells in $G_0 + G_1$ and $G_2 + S$ phase is given in the table below ($n = 3$, ±SEM). (B) BrdU incorporation of A549 cells cultured at various concentrations of fetal bovine serum (FBS) in normoxia and hypoxia (mean ± SEM).



	Normoxia		Hypoxia	
	G0 + G1 (%)	S + G2 (%)	G0 + G1 (%)	S + G2 (%)
si-control	61.1 ± 1.9	38.9 ± 1.9	72.3 ± 1.9	27.7 ± 3.5
si-HIF-1α	71.0 ± 2.7	29.0 ± 1.9	64.3 ± 2.4	35.7 ± 2.4

Fig. 6. Proliferation and cell cycle analysis of A549 cells transfected by *control-siRNA* (si-control) or by *HIF-1α-siRNA* (si-HIF-1α), after serum deprivation for 24 h. (A) Representative flow cytometric analysis of cell cycle using propidium iodide for DNA staining. Shown is the pattern of cells being in $G_0 + G_1$ phase or $G_2 + S$ phase during normoxia and hypoxia (1% O_2). The quantitative analysis of cells in $G_0 + G_1$ and $G_2 + S$ phase is given in the table below ($n = 3$, ±SEM). (B) BrdU incorporation of A549 cells transfected by *control-siRNA* (si-control) or by *HIF-1α-siRNA* (si-HIF-1α) in normoxia and hypoxia (mean ± SEM, $*p < 0.05$).

This shows that in hypoxia *HIF-1α-siRNA* treatment abrogates hypoxia-induced cell growth arrest. The effects of HIF-1α on cell cycle may depend on the interaction of HIF-1 with cell cycle regulatory proteins. There are data which demonstrate that hypoxia-induced cell growth arrest depends on activation of P^{27} and P^{21} in hypoxia [30,31]. Also hypoxia-dependent hypophosphorylation of retinoblastoma protein has been shown to be a cause of growth arrest in hypoxia [32,33]. Our study performed on A549 cells demonstrates that HIF-1 is directly involved in hypoxia-induced cell growth arrest, even if we did not intend to identify the

relevant cell cycle regulatory factors downstream of HIF-1 activation.

Taken together this study shows the potency of RNA-interference for HIF-1α suppression. This approach thus offers a powerful tool for studying HIF-1α-dependent processes under physiological and pathophysiological conditions. It may be considered as new therapeutic strategy for hypoxia related diseases.

Acknowledgments

We thank Gudrun Albohn, Christiane Hild, Jessica Lange, and Annette Tschuschner for excellent technical assistance. The HIF-1α antibody was kindly provided by Professor Max Gassmann (University of Zürich, Switzerland). B.G. Eul, S. Krick, and P. Goyal are supported by predoctoral fellowships from ALTANA Pharma. We thank Aparna Kudumu for proof reading of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft SFB547-B9.

References

[1] G.L. Wang, G.L. Semenza, General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia, Proc. Natl. Acad. Sci. USA 90 (1993) 4304–4308.

[2] G.L. Semenza, P.H. Roth, H.M. Fang, G.L. Wang, Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1, J. Biol. Chem. 269 (1994) 23757–23763.

[3] P. Carmeliet, Y. Dor, J.M. Herbert, D. Fukumura, K. Brusselmans, M. Dewerchin, M. Neeman, F. Bono, R. Abramovitch, P. Maxwell, C.J. Koch, P. Ratcliffe, L. Moons, R.K. Jain, D. Collen, E. Keshert, E. Keshet, Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis, Nature 30 (1998) 485–490.

[4] C. Lacombe, P. Mayeux, Biology of erythropoietin, Haematologica 83 (1998) 724–732.

[5] Y. Liu, S.R. Cox, T. Morita, S. Kourembanas, Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5′ enhancer, Circ. Res. 77 (1995) 638–643.

[6] F. Rose, F. Grimminger, J. Appel, M. Heller, V. Pies, N. Weissmann, L. Fink, S. Schmidt, S. Krick, G. Camenisch, M. Gassmann, W. Seeger, J. Hanze, Hypoxic pulmonary artery fibroblasts trigger proliferation of vascular smooth muscle cells: role of hypoxia-inducible transcription factors, FASEB J. 16 (2002) 1660–1661.

[7] S. Kourembanas, T. Morita, H. Christou, Y. Liu, H. Koike, D. Brodsky, V. Arthur, S.A. Mitsial, Hypoxic responses of vascular cells, Chest. 114 (1998) 25–28, Review.

[8] S. Salceda, J. Caro, Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin–proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes, J. Biol. Chem. 272 (1997) 22642–22647.

[9] B.H. Jiang, E. Rue, G.L. Wang, R. Roe, G.L. Semenza, Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1, J. Biol. Chem. 271 (1996) 17771–177718.

[10] P. Jaakkola, D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A.V. Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Targeting of HIF-alpha to the von Hippel–Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation, Science 292 (2001) 468–472.

[11] R.K. Bruick, S.L. McKnight, A conserved family of prolyl-4-hydroxylases that modify HIF, Science 294 (2001) 1337–1340.

[12] D. Lando, D.J. Peet, D.A. Whelan, J.J. Gorman, M.L. Whitelaw, Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch, Science 295 (2002) 858–861.

[13] Flamme, T. Frohlich, M. von Reutern, A. Kappel, A. Damert, W. Risau, HRF, a, putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels, Mech Dev. 63 (1997) 51–60.

[14] Y.Z. Gu, S.M. Moran, J.B. Hogenesch, L. Wartman, C.A. Bradfield, Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha, Gene Expr. 7 (1998) 205–213.

[15] G.L. Semenza, Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology, Trends Mol. Med. 7 (2001) 345–350.

[16] G.L. Semenza, Oxygen-regulated transcription factors and their role in pulmonary disease, Respir. Res. 1 (2000) 159–162.

[17] G.L. Semenza, Involvement of hypoxia-inducible factor 1 in human cancer, Intern. Med. 41 (2002) 79–83.

[18] C. Warnecke, W. Griethe, A. Weidemann, J.S. Jurgensen, C. Willam, S. Bachmann, Y. Ivashchenko, I. Wagner, U. Frei, M. Wiesener, K.U. Eckardt, Activation of the hypoxia-inducible factor-pathway and stimulation of angiogenesis by application of prolyl hydroxylase inhibitors, FASEB J. 17 (2003) 1186–1188.

[19] C. Willam, N. Masson, Y.M. Tian, S.A. Mahmood, M.I. Wilson, R. Bicknell, K.U. Eckardt, P.H. Maxwell, P.J. Ratcliffe, C.W. Pugh, Peptide blockade of HIFalpha degradation modulates cellular metabolism and angiogenesis, Proc. Natl. Acad. Sci. USA 99 (2002) 10423–10428.

[20] A.Y. Yu, L.A. Shimoda, N.V. Iyer, D.L. Huso, X. Sun, R. McWilliams, T. Beaty, J.S. Sham, C.M. Wiener, J.T. Sylvester, G.L. Semenza, Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha, J. Clin. Invest. 103 (1999) 691–696.

[21] X. Sun, J.R. Kanwar, E. Leung, K. Lehnert, D. Wang, G.W. Krissansen, Gene transfer of antisense hypoxia inducible factor-1 alpha enhances the therapeutic efficacy of cancer immunotherapy, Gene Ther. 8 (2001) 638–645.

[22] J. Chen, S. Zhao, K. Nakada, Y. Kuge, N. Tamaki, F. Okada, J. Wang, M. Shindo, F. Higashino, K. Takeda, M. Asaka, H. Katoh, T. Sugiyama, M. Hosokawa, M. Kobayashi, Dominant-negative hypoxia-inducible factor-1 alpha reduces tumorigenicity of pancreatic cancer cells through the suppression of glucose metabolism, Am. J. Pathol. 162 (2003) 1283–1291.

[23] K. Maemura, C.M. Hsieh, M.K. Jain, S. Fukumoto, M.D. Layne, Y. Liu, S. Kourembanas, S.F. Yet, M.A. Perrella, M.E. Lee, Generation of a dominant-negative mutant of endothelial PAS domain protein 1 by deletion of a potent C-terminal transactivation domain, J. Biol. Chem. 274 (1999) 31565–31570.

[24] S.M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, Nature 411 (2001) 494–498.

[25] G. Camenisch, M. Tini, D. Chilov, I. Kvietikova, V. Srinivas, J. Caro, P. Spielmann, R.H. Wenger, M. Gassmann, General applicability of chicken egg yolk antibodies: the performance of IgY immunoglobulins raised against the hypoxia-inducible factor 1alpha, FASEB J. 13 (1999) 81–88.

[26] P.J. Lee, B.H. Jiang, B.Y. Chin, N.V. Iyer, J. Alam, G.L. Semenza, A.M. Choi, Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia, J. Biol. Chem. 272 (1997) 5375–5381.

[27] J. Li, M.A. Perrella, J.C. Tsai, S.F. Yet, C.M. Hsieh, M. Yoshizumi, C. Patterson, W.O. Endege, F. Zhou, M.E. Lee,

Induction of vascular endothelial growth factor gene expression by interleukin-1 beta in rat aortic smooth muscle cells, J. Biol. Chem. 270 (1995) 308–312.

[28] E.C. Vaux, S.M. Wood, M.E. Cockman, L.G. Nicholls, K.M. Yeates, C.W. Pugh, P.H. Maxwell, P.J. Ratcliffe, Selection of mutant CHO cells with constitutive activation of the HIF system and inactivation of the von Hippel–Lindau tumor suppressor, J. Biol. Chem. 276 (2001) 44323–44330.

[29] N. Goda, H.E. Ryan, B. Khadivi, W. McNulty, R.C. Rickert, R.S. Johnson, Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia, Mol. Cell. Biol. 23 (2003) 359–369.

[30] L.B. Gardner, Q. Li, M.S. Park, W.M. Flanagan, G.L. Semenza, C.V. Dang, Hypoxia inhibits G1/S transition through regulation of p27 expression, J. Biol. Chem. 276 (2001) 7919–7926.

[31] S.L. Green, R.A. Freiberg, A.J. Giaccia, p21(Cip1) and p27(Kip1) regulate cell cycle reentry after hypoxic stress but are not necessary for hypoxia-induced arrest, Mol. Cell. Biol. 21 (2001) 1196–1206.

[32] Krtolica, N.A. Krucher, J.W. Ludlow, Molecular analysis of selected cell cycle regulatory proteins during aerobic and hypoxic maintenance of human ovarian carcinoma cells, Br. J. Cancer 80 (1999) 1875–1883.

[33] J.W. Ludlow, R.L. Howell, H.C. Smith, Hypoxic stress induces reversible hypophosphorylation of pRB and reduction in cyclin A abundance independent of cell cycle progression, Oncogene 8 (1993) 331–339.