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Virus-based reporter systems for monitoring transcriptional activity of hypoxia-inducible factor 1

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Abstract

Being key regulator of oxygen homeostasis hypoxia-inducible factor 1 (HIF-1) plays significant roles in cancer progression as well as in cardiovascular diseases. The modulation of HIF-1 α activity in vivo may represent a valuable therapeutic approach to these disorders [Hofer, T., Desbaillets, I., Hopfl, G., Wenger, R.H., Gassmann, M., 2002. Characterization of HIF-1 α overexpressing HeLa cells and implications for gene therapy. *Comp. Biochem. Physiol., Toxicol. Pharmacol.* 133, 475–481]. In order to monitor HIF-1 transcriptional activity, we have developed HIF-1 α -responsive reporter constructs, in which lacZ gene expression is driven by minimal Hsp70 gene promoter or minimal immediate early promoter of cytomegalovirus (CMV) and a combination of hypoxia response elements from regulatory regions of PGK1, ENO1 and LDHA genes. For the efficient delivery to a wide variety of cell types we chose retroviral and lentiviral vectors as carriers of the reporter cassette. We demonstrate that the obtained reporter system i) has a high inducibility in response to treatments leading to HIF-1 α activation, ii) shows upregulation in response to HIF-1 activation and downregulation following inhibition of HIF-1 α expression by small interfering RNA, iii) follows the dynamics of endogenous HIF-1 target gene expression. The retrovirus- and lentivirusbased reporters can be used for high-throughput screening of HIF-1 α modulators and for the study of crosstalk between HIF-1 and different related signal transduction pathways. Potential applications for the reporters are discussed.

Keywords

Hypoxia response element; h-galactosidase; HIF-1 α siRNA

1. Introduction

HIF-1 is the major transcription factor responsible for the induction of specific genes under conditions of low oxygen level, or hypoxia. In addition to hypoxic conditions HIF-1 can be activated through numerous signaling pathways by cytokines, hormones, reactive oxygen and nitrogen species, and in particular, under various conditions associated with tumor growth (Maxwell et al., 2001). HIF-1 is composed of α and β subunits. The constitutive HIF-1 β is present in the nucleus, while the expression, localization and functional activity of HIF-1 α subunit are subject of complex regulation. To activate the transcription of target genes, HIF-1 α

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forms complex with HIF-1 α and binds to a specific sequence (hypoxia response element, HRE) in regulatory regions of these genes. The genes that are upregulated through HRE include vascular endothelial growth factor (VEGF), erythropoietin, enzymes of glucose metabolism (glucose transporter-1, aldolase A, lactate dehydrogenase A (LDHA), phosphoglycerate kinase 1 (PGK1), enolase 1 (ENO1)) and many others (Semenza et al., 1996; Semenza, 2003). In normoxia, the activity of HIF-1 α is controlled by interactions with the Von Hippel Lindau protein (pVHL), which blocks its transcriptional activity and promotes rapid proteosomal degradation (reviewed in Semenza, 2001).

Deregulation of HIF-1 transcriptional activity is detected in variety of pathological conditions. Dramatic activation of HIF-1 is typical to majority of human cancers (Zhong et al., 1999; Maxwell et al., 2001). It is frequently associated with increased tumor vascularization (Zagzag et al., 2000; Giatromanolaki et al., 2001) leading to failures in the cancer treatment (Birner et al., 2000; Aebbersold et al., 2001). Hence, inhibition of HIF-1 activity represents a reasonable approach to the treatment of cancers with high levels of the HIF-1 α protein (reviewed in Semenza, 2002, 2003).

HIF-1 plays a key role in the pathogenesis of ischemic disorders. Under hypoxic conditions, HIF-1 triggers the transcriptional induction of a number of angiogenic growth factors, including VEGF (Semenza, 2001). Overexpression of the VEGF in ischemic tissues, has been successfully applied as angiogenic gene therapy (Schratzberger et al., 2000). The above observations suggest that HIF-1 α activating drugs would potentially elicit a more physiological induction of local angiogenesis, that, in turn, would increase the proportion of successful therapeutic angiogenesis applications (reviewed in Hofer et al., 2002).

In order to accurately monitor HIF-1 transcriptional activity under different conditions, we developed a series of virus-based reporter systems. We combined the existing knowledge about the HIF-1-mediated transcription of target genes with the advances in reporter gene technology to develop new powerful reporter system. We created a reporter cassette based on multiple HREs from the genes of glucose metabolism. This reporter cassette was cloned into retroviral and lentiviral vectors, allowing transduction of virtually any cell type within 24–48 h, as lentivirus can infect even non-dividing cell cultures with up to 100% efficiency. In addition, unlike plasmid transfection methods that frequently produce rearrangements and amplifications of the transgenes, the retro/lentivirus mediated gene transfer results in uniform integration into the genome of individual intact expression cassettes whose copy number can be easily controlled by variation in the multiplicity of infection. The obtained readout systems could be used to identify small molecules or genetic elements (including small interfering RNAs (siRNAs)) that affect signal transduction pathways modulating the activity of HIF-1. In addition, we expect our system to be useful in transgenic models for gene therapy, when the reporter gene is replaced by a gene of interest for targeted expression in hypoxic regions.

2. Materials and methods

2.1. Vectors

pSIP-mHsp70-lacZ and pUSTdS-mCMV-lacZ (Ivanov et al., in preparation) are self-inactivating (SIN) retroviral vectors (Yu et al., 1986) with full-length 5' long terminal repeat (LTR) and truncated 3' LTR devoid of viral enhancer. Upon infection of target cells, the 3' LTR replaces 5' LTR, leading to integration of the proviral DNA into the host genome as an insert with no functional viral promoters. Similarly, the pLV-mCMV-lacZ represents a SIN construct, though it includes a backbone of the HIV-1-based lentiviral vector (Pfeifer et al., 2002). The reporter constructs rely on minimal promoters from human Hsp70 gene (mHsp70) and from immediate early gene of cytomegalovirus (mCMV). The sequence of mHsp70 is: 5'-gcgggtctccgtgacgactataaaagccaggggcaagcggtccg; the sequence of mCMV is: 5'-

taggcgtgtacgggtgggaggtctatataagcagagctcgttagtgaaacgcgcagatcgctggagagccatccacgtgttttgac
ctccatagaagacacgggacgatccagcct.

2.2. Construction of HIF-1a-responsive reporter plasmids

2.2.1. HRE1 element—The element consists of HRE-containing portions from three hypoxia-inducible genes: PGK1 gene—5V-gacgtgacaaacgaagccgcacgtc, ENO1 gene—5V-agggccggacgtggggccccagagcgacgtgagtcggtgcgggactcggagtagctgacggagcccc and LDHA gene—5V-acacgtgggtcccgacgtccgc. An 88 nt long 5VHRE-XhoI (5V-agagactcgagacgtgacaaacgaagccgcacgtcagggccggacgtggggccccagagcgacgtgagtcggtgcgggactcggagt) and an 87 nt long 3VHRE-SphI (5V-ctctctgcatgcggacgtgcgggaacccacgtgtggggctccgtcacgtactccgagtcgccgcacgcactcagcgtcgtctgtgggc) partially complementary oligos were annealed and the single stranded regions were filled in using Klenow DNA polymerase. The resulting 137 bp double stranded DNA (dsDNA) fragment (HRE1, see Fig. 1A) was cleaved with XhoI and SphI restriction endonucleases and ligated into pSIP-mHsp70-lacZ plasmid according to standard ligation protocol (for map see Fig. 2A).

2.2.2. HRE12 element—Two self-complementary 79 nt oligos (5VHRE-XbaI: 5Vctagaggacgtgacaaacagaagccacacgtcctagggacgtggggagtcgctgaggagtagctgaggacacgtgggta and 3VHRE-SpeI: 5Vctagtagccacgtgtcctcactgactcctcagcactccccacgtccctaggacgtgtggcttctgtttgtcacgtcct) were annealed resulting in dsDNA fragment with XbaI and SpeI compatible cohesive ends. The self-ligation products of these duplexes could not be recleaved by either of these restriction endonucleases if ligated in a head-to-tail orientation. After selfligation and redigestion with XbaI and SpeI restriction enzymes, we obtained multimers in uniform head-to-tail orientation. The resulting ladder-like products were resolved on agarose gel and a band corresponding to 12 copies of the monomer (HRE12, see Fig. 1B for the monomer structure) was excised, purified and subcloned into pGEM-7Zf(+) vector (Promega U.S., Madison, WI) by XbaI restriction site. Proper orientation of the cloned fragment was verified to obtain a clone containing the HRE12 fragment with XbaI site being next to XhoI site and the XbaI/SpeI hybrid site close to SphI site. Then the HRE12 fragment cleaved with SphI and XhoI was cloned into pSIP-mHsp70-lacZ or pUSTdS-mCMV-lacZ plasmids (for maps see Fig. 2B,C).

To generate pLV-HRE12-mCMV-lacZ lentiviral construct, we cut the HRE12 repeats from the pUSTdSHRE12-mCMV-lacZ plasmid using XhoI and SpeI restriction enzymes and inserted them into the pLV-mCMV-lacZ plasmid (for map see Fig. 2D). All plasmid constructs were verified by nucleotide sequencing. Restriction endonucleases, Klenow enzyme and ligase were obtained from New England Biolabs, Beverly, MA.

2.3. Construction of HIF-1a siRNA expressing plasmid

HIF-1-siRNA-BamHI-direct (5V-gatccgtatggtctcacagatgatggtcctgtcaccatcatctgtgagaaccatattttg) and HIF-1-siRNA-EcoRI-reverse (5V-aattcaaaaataggttctcacagatgatggtgacaggaagccatcatctgtgagaaccatagc) (the regions corresponding to HIF-1a cDNA, GenBank accession no. NM 001530, are underlined) oligos were annealed and subcloned into BamHI and EcoRI restriction sites of a lentiviral pLSLG plasmid that contains H1 RNA gene promoter within right LTR for siRNA expression, and internal cassette for expression of EGFP driven by human histone H4 gene promoter. This resulted in the generation of pLSLG-HIF-1a-siRNA plasmid. The siRNA to firefly luciferase (GenBank accession no. U47122) was cloned into pLSLG plasmid according to the above procedure using the following oligos: luc-siRNA-BamHI-direct (5V-gatccgcacttacgtgacttctgacttctgtcatcgaagtactcagcgtaagtgttttg) and luc-siRNA-EcoRI-reverse (5V-aattcaaaaacacttacgtgacttctgacttctgtcatcgaagtactcagcgtaagtgcg).

2.4. Cell lines and retroviral/lentiviral infection

HCT116 cells (human colorectal carcinoma, ATCC CCL-247), HEF (human fibroblasts from 10-week embryo), 10(1) mouse embryo 3T3-like fibroblast cell line devoid of p53 expression (Harvey and Levine, 1991) and H1299 (human non-small cell lung carcinoma, ATCC CRL-5803) cells were grown in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum (HyClone, South Logan, UT), 2 mM l-glutamine, 100 u/ml penicillin, and 100 Ag/ml streptomycin. Retroviral stocks were prepared using Phoenix-Ampho packaging cells as described in (Grignani et al., 1998). Target cells were incubated with stocks for at least 12 h. Transduced cells were selected on 1 Ag/ml puromycin for 1 week. Similarly, lentiviral stocks were prepared using 293T packaging cells transduced with pLSLG-HIF-1a-siRNA or pLSLG-luciferase-siRNA constructs along with pCMVDR8.2 and pVSV-G helper plasmids as described in Kootstra et al. (2003).

2.5. b-Galactosidase (bGal) assays

2.5.1. o-Nitrophenyl b- d-galactopyranoside (ONPG) staining—Cells on 96-well plates were washed with PBS and lysed in 150 Al of staining solution (1 mM MgCl₂, 250 mM Tris HCl, pH 7.4, 0.02% NP-40, 2 mg/ml ONPG (Sigma- Aldrich, St. Louis, MO) in PBS) per well, following by the incubation at 37 °C. Optical density was assessed by spectrophotometry at 405 nm using Wallac Victor2 1420 Multilabel counter (Perkin Elmer life and analytical sciences, Boston, MA).

2.5.2. 5-Bromo-4-chloro-3-indolyl b- d-galactopyranoside (X-Gal) staining—Cells on 12-well plates were washed with PBS and fixed with ice-cold fixing solution (1 mM MgCl₂, 0.5% glutaraldehyde in PBS) for 10 min. Then cells were incubated with the staining solution (1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆, 3.3 mM K₃Fe(CN)₆, 0.02% NP-40, 0.2% XGal (Sigma-Aldrich) in PBS) at 37 °C.

2.6. RT-PCR

Total RNA from cells was extracted using the Trizol reagent according to manufacturer's protocol (Invitrogen Carlsbad, CA). First cDNA strand was synthesized from oligo dT12–18 primer using SuperScript First-strand Synthesis System for RT-PCR (Invitrogen). RT-PCR was performed using primers specific to LDHA (LDHA-forward: 5V-tggcaactctaaaggatcag and LDHA-reverse: 5V-accaaattaagacggcttc), PGK1 (PGK1-forward: 5V-tttctaacaagctgacgctg and PGK1-reverse: 5V-ttctctccacatgaaagc) and cyclophilin (PPIA-forward: 5V-cttcacagccataatggc and PPIA-reverse: 5V-gtgatcttctgctgtcttg) genes.

3. Results

3.1. Design of HIF-1a-dependent reporter plasmids and their induction in response to desferrioxamine mesylate (DFO) treatment

For the generation of HIF-1-dependent reporter constructs we used small fragments from regulatory regions of ENO1, PGK1 and LDHA genes, containing previously identified HIF-1 binding sites along with their flanking regions. First, we obtained pSIP-HRE1-mHsp70-lacZ reporter construct. The HRE containing promoter sequences of the three selected HIF-1-responsive genes were cloned side by side (Fig. 1A, also see Section 2.2) upstream of mHsp70 promoter and lacZ reporter gene. This reporter construct was introduced into H1299 and HEF cell lines by retroviral infection, and induction of the reporter was monitored following DFO treatment. DFO chelates iron, which is an essential cofactor in prolyl hydroxylation of HIF-1a, leading to pVHL binding, HIF-1a ubiquitination and proteosomal degradation (Jaakkola et al., 2001). Thus, DFO can mimic some aspects of hypoxic stress, causing the HIF-1a stabilization (Vincent et al., 2002).

Treatment with DFO produced a rather weak effect on the lacZ gene expression when it was measured by ONPG assay in H1299 and HEF cells with the pSIP-HRE1- mHsp70-lacZ construct (1.5- and 2.0-fold induction, respectively, data not shown). Moreover, such induction was achieved only at maximal concentrations of DFO (450 AM), which were associated with signs of toxicity suggesting poor sensitivity of the reporter system.

In order to improve the sensitivity, we made several modifications within the original pSIP-HRE1-mHsp70-lacZ (Fig. 2A). First, we reorganized the HRE1 cassette in such a way that the HIF-1 binding sites (HRE consensus sequences 5VCGTG3V or 5VCACG3V, in reverse order) became exposed on the same side of DNA double helix. We designed the new HIF-1-responsive cassette to make sure that the distance between the consensus sites corresponds to one or more turns of the double helix (or equivalent to approximately 11 base pairs, which is marked by (I) symbols on Fig. 1B). The resulting HRE was repeated 12 times to increase the HIF-1 binding capacity (see Section 2.2). In addition, we compared two different minimal promoters (mHsp70 and mCMV) to choose the one most suitable for reporter constructs. These modifications are represented in the constructs pSIP-HRE12-mHsp70-lacZ, pUSTdSHRE12- mCMV-lacZ and pLV-HRE12-mCMV-lacZ (Fig. 2B,C,D).

We introduced pSIP-HRE12-mHsp70-lacZ and pUSTdSHRE12- mCMV-lacZ reporter constructs into a set of cell lines by retroviral infection and treated cells with DFO in 300 AM concentration for 12 h to induce the hypoxic response. As shown in Fig. 3, both reporter constructs showed a good lacZ induction in response to DFO treatment. As a control for possible leakage of minimal Hsp70/CMV promoters (HRE-independent induction of lacZ gene expression), we used constructs without HRE12 (minimal promoter only). With these control constructs the reporter gene was not inducible in response to DFO (data not shown).

3.2. Reporter constructs are specifically activated by HIF-1

To show the HIF-1a specificity of obtained constructs, a HIF-1a siRNA was designed as a stem-loop structure and cloned into the lentiviral pLSLG plasmid to be driven by the H1 RNA promoter (see Section 2.3). H1299 and HCT116 cell lines stably transduced with the pLV-HRE12-mCMVlacZ reporter construct were infected with pLSLG-HIF-1asiRNA or pLSLG-luciferase-siRNA (a control for possible non-specific siRNA effects in target cells) lentiviruses. Forty-eight hours after lentiviral infection with siRNA expressing plasmids the H1299/pLV-HRE12-mCMV-lacZ cells were treated with 300 AM DFO for 12 h and stained with X-Gal. As HCT116/pLV-HRE12-mCMV-lacZ cells demonstrated in reporter assay a high level of HIF-1 activity without any treatment (Figs. 3 and 4), we left them untreated. The difference in activity of hGal in the cells infected with HIF-1a siRNA and the control cells infected with non-specific luciferase siRNA is shown in Fig. 4. As HIF-1a siRNA acts post-transcriptionally leading to HIF-1a mRNA degradation, the HIF-1a protein level decreases, which correlates with the inhibition of HIF-1-dependent transcription of the reporter gene. The results of this experiment indicate that in H1299 cells the reporter induction after DFO treatment depends on the activation of HIF-1a protein. In case of untreated HCT116 reporter cell line the level of hGal activity was reduced 5-fold upon the HIF-1a siRNA introduction (data not shown). We then subjected HCT116 cell line to treatment with camptothecin, which is a well-known HIF-1 inhibitor (Semenza, 2003). This treatment, like in case of HIF-1a siRNA infection, resulted in a 3.5-fold decrease in hGal expression (data not shown).

3.3. HIF-1 transcriptional activation is time- and dose-dependent

In order to estimate dynamics of HIF-1 activation H1299/, HEF/and HCT116/pSIP-HRE12-mHsp70-lacZ reporter cell lines were treated for varying time intervals with different concentrations of DFO (Fig. 5). There was up to 9-fold difference in lacZ expression for the H1299 cell line treated with maximal DFO concentration (450 AM) for 12 h. For HEF the

difference in reporter gene expression was even larger (20- fold). In the HCT116 cell line the level of HIF-1 activity was initially high in untreated cells (see Figs. 3–5), while treatment with DFO did not cause visible induction of the reporter.

When pUSTdS-HRE12-mCMV-lacZ reporter construct was introduced into the H1299 and HEF cell lines, we observed 2- to 3-fold higher background level of lacZ gene expression in untreated cells compared to a construct with mHsp70 promoter. However, the level of hGal induced in response to DFO treatment was similarly (2- to 3-fold) higher with this construct. There was no difference in folds of lacZ induction between the two constructs, suggesting that the mCMV construct is up to two-fold more sensitive (data not shown).

Comparison of H1299, HEF and HCT116 reporter cell lines suggested that the pattern of lacZ expression is clearly cell type-specific and depends on the dose of DFO and the duration of treatment. Indeed, there is a considerable difference in HIF-1 activation pattern between H1299, HEF and HCT116 cell lines (Fig. 5). In H1299 cells the maximum of lacZ expression corresponds to a 12 h time point, after which the level of hGal decreases and drops down to background level at a 48 h time point. Also, the intensity of ONPG staining is strictly dependent on the DFO concentration. In HEF cells, the maximum of lacZ expression corresponds to a 24 h time point, and then the level of hGal decreases slightly by the 48 h time point. Surprisingly, we did not observe substantial dose-dependence, as concentrations of DFO as low as 7 AM increased the lacZ expression to levels comparable to those seen at the much higher doses, at 24 and 48 h time points. In HCT116 cells the maximum of lacZ expression corresponds to a 12 h time point when high DFO concentrations are used and to a 24 h time point when cells are treated with lower DFO concentrations.

3.4. The dynamics of lacZ expression in response to DFO correlates with the expression of HIF-1-dependent genes

We further addressed the question of whether dynamics of reporter gene expression in response to DFO treatment correlates with the one of endogenous HIF-1 targets. For this purpose H1299 and HCT116 cells were treated with 300 AM DFO for different time periods. The expression of HIF-1 target genes, LDHA and PGK1, and unrelated gene, cyclophilin, was assessed by RT-PCR. We observed that DFO treatment leads to expression of HIF-1-dependent target genes along with the induction of the lacZ reporter gene expression (compare Figs. 5 and 6).

In order to confirm that the LDHA and PGK1 gene induction after DFO treatment is HIF-1-dependent HIF-1a siRNA was introduced into the cells to downregulate HIF-1a expression. Luciferase siRNA was used as a control for non-specific effects of siRNA. The results obtained for cells not expressing any siRNA are not included as they were similar to those for the cells expressing luciferase siRNA. As expected, when HIF-1a expression was inhibited by the siRNA, the expression of target genes remained unchanged in response to DFO treatment (Fig. 6). At the same time the induction of LDHA, PGK1 and lacZ genes peaked at 12 h after DFO treatment in H1299 and HCT116 cell lines infected with control luciferase siRNA (Figs. 5 and 6).

4. Discussion

Transcription of HIF-1-dependent genes is activated in cells by a common molecular mechanism mediated by the binding of HIF-1 heterodimer to HREs (5VCGTG3V consensus sequence or 5VCACG3V (its complement)), lying in regulatory regions of target genes (reviewed in Wang and Semenza, 1996). It was shown that these sequences are necessary, but not sufficient for HIF-1 mediated transcription (Semenza et al., 1996) as surrounding nucleotides are also important. Several HIF-1-dependent reporter systems, that used the HRE-containing portions of promoters/enhancers or multimerized HIF-1 binding sites, were

previously described (Forsythe et al., 1996; Shibata et al., 1998, 2000; Koshikawa et al., 2000; Post and Van Meir, 2001; Rapisarda et al., 2002; Su et al., 2002; Burroughs et al., 2003; Hanze et al., 2003; Linden et al., 2003). However it was noticed that a large portion of upstream region from HIF-1-responsive gene can be responsible for HIF-1- independent activation of a reporter gene expression (Burroughs et al., 2003). To avoid these effects we decided to use short sequences from upstream regions of three HIF- 1-responsive genes, containing HRE consensus sequences and immediately adjacent nucleotides. We combined HRE elements from three different hypoxia-inducible genes PGK1, ENO1 and LDHA, as we wanted to create the reporter construct which reflects HIF-1 activity independently on the affinity to one particular response element, which can vary from gene to gene and limit the range of treatments which can be monitored as potential HIF-1 modulators. It has previously been shown that multiple copies of the HRE enhancer improved the hypoxic response (Firth et al., 1994); thus, we fused multiple copies of HREs to the minimal promoter. Also, we arranged the HIF-1 binding sites to provide them with the bcooperativeQ orientation for the recognition and binding by HIF-1. We established the suitability of HREs from genes of glucose metabolism for design of reporter constructs. Using these elements we achieved high levels of reporter induction in response to DFO treatment. The obtained results correlate well with the fact that genes of glucose metabolism are strongly inducible in response to hypoxia and can be induced in response to a simple overexpression of HIF-1a (Semenza et al., 1996). We did not compare activities of HREs from individual genes, though it might potentially lead to the development of reporter constructs with even higher performance under certain conditions.

Rapisarda and coworkers had successfully applied the reporter gene technology for the identification of small molecule inhibitors of HIF-1 (Rapisarda et al., 2002). They used three copies of HRE from nitric oxide synthase gene for reporter construct design and performed screening for HIF-1 inhibitors under hypoxic conditions using a cell line with undisturbed HIF-1 pathway (Rapisarda et al., 2002). Although the study resulted in identification of several useful compounds, their reporter system had certain limitations, which are common for those relying on plasmid transfection of reporter cassettes. As a rule, the expression of a reporter protein following induction is low in stablytransfected cells, compared to the transiently transfected cultures. Also, the type of cells suitable for the assay depends significantly upon the efficiency of DNA transfection, which may vary substantially between different cell lines. We solved these limitations by using an alternative design of reporter cassette placed in the context of retroviral and lentiviral SIN vectors (see Section 2.1). The retroviral and lentiviral gene transfer into target cells is highly efficient (routinely 100% of infected cells for lentivirus) and allows standard introduction of genetic material into a wide variety of cell lines. Using the puromycin resistance gene, present in all designed constructs, we were able to obtain stably infected target cells thus avoiding loss of DFO inducibility following multiple passages in culture.

We successfully tested our reporters in a variety of cell types including human epithelial tumor-derived H1299, primary fibroblasts HEF and 3T3-like mouse cells 10(1). In these cell lines the HIF-1 pathway is intact, allowing usage of these reporter cultures as readouts for screening of chemical compounds that activate HIF-1 at normoxic conditions, or inhibit—under conditions of hypoxia. Our readout system allowed us to detect an abnormally high level of HIF-1 activity in HCT116 human colorectal carcinoma cell line, which can be used for screening of HIF-1 inhibitors under normoxic conditions. When we compared the kinetics of HIF-1 transcriptional activity in different cell lines, we concluded that our reporter plasmids can be used not only for drug screening purposes, but also for monitoring of cell typespecific activity of HIF-1 including crosstalks between different signal transduction pathways and HIF-1.

The specificity of the reporter system and its strict dependence on the HIF-1 α activity was confirmed in experiments with knock-down of HIF-1 α by siRNA and by treatment of cells with a HIF-1 inhibitor camptothecin. The HIF-1 α siRNA experiments have confirmed the predominant role of HIF-1 α over other members of HIF family of genes (HIF-2 α , HIF-3 α) in HRE-dependent gene expression in response to DFO treatment in H1299 and HCT116 cell lines.

Our HIF-1-responsive constructs could also be used in experimental tumor-specific gene therapy, provided the lacZ gene is replaced by a suicide gene (tumor suppressor or apoptosis promoting gene). In this case, the suicide gene will be expressed in hypoxic regions of solid tumors but not in surrounding normal tissues. Dachs et al. have demonstrated that heterologous gene expression driven by HREs from the mouse PGK1 gene could be activated in hypoxic tumor cells (Dachs et al., 1997). As the inducibility of our reporter constructs in response to a HIF-1 activating stimulus is much higher, we expect our reporters to be effective in transgenic studies.

In summary, in this study we were able to obtain a reliable cell-based system for detection of HIF-1 transcriptional activity. Rational design improved the sensitivity of the readout system. Our reporter constructs can be efficiently transduced into a wide variety of target cells as they are retro/lentiviruses. The described reporter plasmids can be used for drug screening purposes, for testing the effects of genetic elements affecting HIF-1 (such as siRNAs) as well as for the monitoring of HIF-1 activity during modulation of various signal transduction pathways. In addition, the developed HIF-1-dependent expression system could find application in experimental cancer gene therapy.

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Abbreviations

HIF-1	hypoxia-inducible factor 1
HRE	hypoxia response element
VEGF	vascular endothelial growth factor
ENO1	enolase 1
LDHA	lactate dehydrogenase A
PGK1	phosphoglycerate kinase 1
pVHL	Von Hippel Lindau protein
LTR	long terminal repeat
SIN	self-inactivating vector
dsDNA	double stranded DNA
HIV-1	human immunodeficiency virus type 1
mHsp70	minimal Hsp70 gene promoter
mCMV	minimal immediate early promoter of cytomegalovirus

DFO	desferrioxamine mesylate
lacZ	h-galactosidase gene
hGal	hgalactosidase protein
X-Gal	5-bromo-4-chloro-3-indolyl h-d-galactopyranoside
ONPG	o-nitrophenyl h-d-galactopyranoside
siRNA	small interfering RNA
u	unit(s)
bp	base pair(s)
nt	nucleotide(s)
h	hour(s)

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Fig. 1.

Design of HRE1 and HRE12 hypoxia-inducible factor 1 binding elements. (A) The sequence of HRE1 in pSIP-HRE1-mHsp70-lacZ reporter construct is shown. The HRE containing promoter regions (shown by arrows) of three different HIF-1-responsive genes (PGK1, ENO1 and LDHA) were inserted side by side into pSIP-mHsp70-lacZ vector. *XhoI* and *SphI* restriction endonucleases were used for cloning procedures. (B) The sequence of monomer of HIF-1 binding element HRE12 in pSIP-HRE12-mHsp70-lacZ, pUSTdS-HRE12-mCMV-lacZ and pLV-HRE12-mCMV-lacZ reporter constructs is shown. The HRE containing regions (shown by arrows) from PGK1, ENO1 and LDHA genes were cloned side by side in such a way that the HIF-1 binding sites are exposed to the same side of DNA double helix, allowing their cooperation in HIF-1 binding. The monomer was multimerized to 12 copies to obtain a strong HIF-1 binding element HRE12 in the resulting reporter constructs. *XbaI* and *SpeI* restriction endonucleases were used for cloning procedures. HIF-1 consensus binding sites are shown in bold. |— a symbol showing one turn of DNA double helix (see Sections 2.2 and 3.1 for details). Sequences are shown in 5'3' orientation.

Fig. 2.

Maps of HIF-1 reporter plasmids. pSIP-HRE1-mHsp70-lacZ (A) and pSIP-HRE12-mHsp70-lacZ (B) are retrovirus-based reporter plasmids with lacZ expression driven by the mHsp70 promoter and HIF-1 binding elements (HRE1 and HRE12, respectively). pUSTdS-HRE12-mCMV-lacZ (C) is a retrovirusbased vector with lacZ expression driven by the mCMV promoter and the HIF-1 binding element HRE12. pLV-HRE12-mCMV-lacZ (D) is a lentiviral equivalent to pUSTdS-HRE12-mCMV-lacZ reporter. 5V, 3V—5VLTR and 3VLTR, respectively. HRE1, HRE12—HIF-1 binding elements (see Fig. 1 for the structure). lacZ—*galactosidase* gene, H4—histone H4 gene promoter, PuroR—puromycin resistance gene. ApR—ampicillin resistance gene. Restriction enzymes used for cloning procedures are indicated. See Sections 2.1 and 2.2 for details.

Fig. 3.

HIF-1-dependent reporter activity in different cell lines treated with DFO. The cell lines with introduced pSIP-HRE12-mHsp70-lacZ or pUSTdSHRE12- mCMV-lacZ reporter constructs were treated (as indicated) with 300 AM of hypoxia mimicking compound DFO for 12 h and subjected to X-Gal staining. The comparison between DFO treated and untreated cells indicates that DFO activates the HIF-1 transcription factor, which, in turn, induces the reporter gene expression.

Fig. 4.

Inhibition of HIF-1-responsive reporter activity by HIF-1α siRNA. HIF-1 specificity of lacZ gene expression in H1299/pLV-HRE12-mCMV-lacZ and HCT116/pLV-HRE12-mCMV-lacZ cell lines was confirmed by knocking down of HIF-1α expression with siRNA. Luciferase siRNA was used as a control. In HCT116 cell line there is a high level of HIF-1 activity under normoxia, judging by the high level of hGal protein (X-Gal staining). In H1299 cells the HIF-1 activity is detectable only upon DFO treatment. Inhibition of the HIF-1α by siRNA leads to a nearly complete inhibition of reporter gene expression both in untreated HCT116 and DFO treated H1299 cells.

Fig. 5.

Dynamics of HIF-1 transcriptional activity following treatment with different concentrations of DFO. HIF-1-responsive retroviral reporter construct pSIP-HRE12-mHsp70-lacZ was introduced into H1299, HEF and HCT116 cell lines. The *lacZ* gene expression was measured by ONPG assay after treatment with DFO at different concentrations for different periods of time. Folds of increase in *lacZ* gene expression in response to DFO treatment are shown. Dynamics of HIF-1 activation is cell type-specific when comparing H1299 (A), HEF (B) and HCT116 (C) cell lines. Error bars show the standard deviation of three identical experiments.

Fig. 6.

Dynamics of transactivation of HIF-1-dependent genes after DFO treatment. The expression of LDHA, PGK1 and cyclophilin genes was assessed by RT-PCR in H1299 (A) and HCT116 (B) cells treated with 300 AM DFO for different periods of time (X-axis, hours). Y-axis shows folds of increase in LDHA and PGK1 gene expression normalized to control (expression of cyclophilin gene). In order to show the HIF-1 dependence of LDHA and PGK1 gene expression after DFO treatment, HIF-1 α was inhibited by siRNA. Luciferase siRNA was used as a control. Dynamics of expression of HIF-1 target genes is similar to that of lacZ reporter gene expression (compare with Fig. 5). The maximum level of expression of LDHA, PGK1 and lacZ genes is induced after 12 h of DFO treatment in both cell lines. Error bars show the standard deviation of two identical experiments.