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

CHRISTINA WARNECKE,*,[1] ZANETA ZABOROWSKA,[†] JENS KURRECK,[†] VOLKER A. ERDMANN,[†] ULRICH FREI,* MICHAEL WIESENER,*,[2] AND KAI-UWE ECKARDT*,[2]

*Department of Nephrology and Medical Intensive Care, Virchow Clinic, Charité University Medicine, Berlin, Germany; and [†]Institute for Biochemistry, Free University Berlin, Germany

To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.04-1640fje; doi: 10.1096/fj.04-1640fje

SPECIFIC AIMS

Cellular adaptation to hypoxia is achieved by a transcriptional response system mediated by the hypoxia-inducible factor (HIF). Since the individual roles of its two alternative oxygen-regulated α subunits, HIF-1α and HIF-2α, are not fully understood, our aim was to determine functional differences between HIF-1α and HIF-2α in different human cell lines by using RNA interference (RNAi) and to compare the results with HIFα overexpression experiments.

PRINCIPAL FINDINGS

1. Specificity and efficiency of HIF-1α and HIF-2α siRNAs

The transfection of HIF-1α and HIF-2α siRNAs in HeLa and Hep3B cells selectively and reproducibly reduced mRNA expression of the respective HIFα isoform by >80%, whereas a luciferase control siRNA had no effect. Concordantly, knockdown of HIFα mRNA levels led to substantially diminished HIFα protein expression under inducing conditions.

2. Differential effects of HIF-1α and HIF-2α siRNAs on HIF target gene mRNA induction

RNase protection assays revealed that HIF-1α knockdown in HeLa cells reduced hypoxia- (0.5% oxygen) or iron chelator- (2,2 ́dipyridyl=DP) stimulated mRNA induction of glucose transporter 1 (GLUT-1), lactate dehydrogenase A (LDH-A), vascular endothelial growth factor (VEGF), carbonic anhydrase IX (CA IX), and HIF prolyl hydroxylase 2 (PHD2) by ~40–60% (**Fig. 1*A–F***); HIF-2α knockdown had no effect on these HIF target genes. The same applied for Hep3B cells, although the suppression of mRNA induction was less pronounced than in HeLa cells, possibly due to the lower abundance of HIF-1α in these cells and/or the contribution of other hypoxia-activated signaling pathways in regulating these genes (Fig. 1*G*).

3. Suppression of HIF-dependent reporter gene activation by siRNAs

In HeLa cells transfected with a luciferase reporter containing six copies of the phosphoglycerate kinase (PGK) hypoxia-responsive element (HRE), hypoxia- or DP-stimulated luciferase expression was reduced from ~11- to 2-fold by HIF-1α knockdown whereas HIF-2α knockdown had no effect. Identical results were achieved with Hep3B cells.

4. Erythropoietin is a HIF-2α target gene in Hep3B and Kelly cells

Surprisingly, mRNA induction of erythropoietin (EPO) after exposure of Hep3B cells to hypoxia or hypoxia mimetics was almost abolished by the HIF-2α siRNA whereas HIF-1α knockdown had no effect (**Fig. 2*A–C***). This result was confirmed in an unrelated cell line, neuroblastoma Kelly cells, which in hypoxia expressed EPO at high levels (Fig. 2*D*).

5. Transactivation of the EPO enhancer by HIF-2α requires *cis*-active elements adjacent to the HRE

Insights into the regulatory pathway underlying the HIF-2α–specific effect were obtained by the use of luciferase reporter assays. A reporter containing five copies of the EPO HRE was merely induced ~2- to 3-fold in HeLa and

[1] Correspondence: Nephrologische Forschungslaboratorien der Medizinischen Klinik IV, Universität Erlangen-Nürnberg, Loschgestrasse 8 1/2, D-91054 Erlangen, Germany. E-mail: christina.warnecke@med4.imed.uni-erlangen.de

[2] The two senior authors contributed equally to this work.

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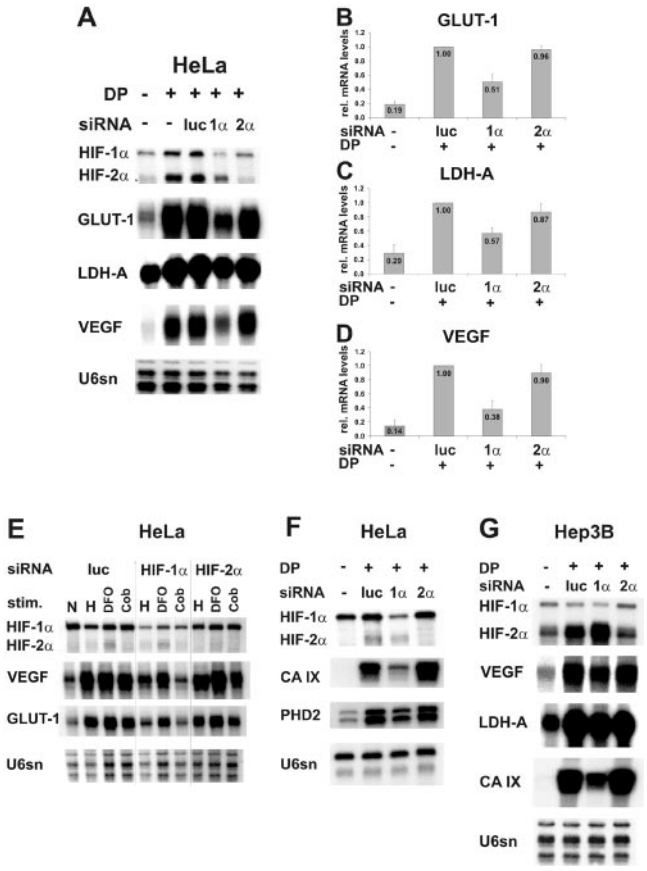

Figure 1. Reduction of HIF target gene mRNA induction by HIF-1α siRNA in HeLa and Hep3B cells. *A)* RNase protection assay demonstrating the influence of HIF-1α knockdown on GLUT-1, LDH-A, and VEGF mRNA expression in HeLa cells. U6 small nuclear RNA (U6sn) served as loading control. HIF-1α and -2α mRNA levels were shown to confirm HIFα knockdown; luc = luciferase control siRNA. *B–D)* Quantification of siRNA effects on GLUT-1, LDH-A, and VEGF mRNA levels in HeLa cells; data are means of 3–4 independent experiments ± SD. *E)* Effects of the siRNAs on VEGF and GLUT-1 were similar in hypoxic (H=0.5% O_2), desferrioxamine- (DFO), and cobalt chloride- (Cob) stimulated cells (each 100 μM). *F)* Induction of CA IX and PHD2 mRNA was reduced by the HIF-1α siRNA but not affected by HIF-2α siRNA. *G)* In Hep3B cells VEGF, LDH-A, and CA IX were dependent on HIF-1α.

Hep3B cells and responded only to HIF-1α knockdown. In contrast, luciferase expression from a reporter plasmid containing the full-length 223 bp EPO enhancer was activated in a cell type-dependent manner (2.9-fold in DP-stimulated or hypoxic HeLa cells and ~10-fold in Hep3B cells). In Hep3B cells, induction of luciferase activity was approximately halved by both HIF-1α and HIF-2α knockdown. These data indicate that reduction of the EPO enhancer to the 25 bp EPO HRE leads to a loss of the cell type-specific induction pattern and to a shift in response to the HIFα isoforms.

6. Target gene specificity can be overcome by forced expression of HIFα subunits

When wild-type human HIF-1α or HIF-2α expression plasmids were cotransfected with the 6× PGK HRE reporter in HeLa cells, luciferase expression was increased 10.1-fold by overexpression of HIF-2α even under baseline conditions; this increase responded to HIF-2α but not to HIF-1α knockdown. Similarly, the reporter could be induced by HIF-1α overexpression, although less efficiently. Thus, target gene specificity can be overcome by forced expression of the HIFα subunits.

To confirm this, we cotransfected stable mouse HIF-1α and HIF-2α mutants with inactivated prolyl and asparagyl hydroxylation sites together with a human VEGF promoter or a mouse LDH-A promoter construct in HeLa cells. We observed that both promoters were inducible by HIF-1α as well as by HIF-2α, albeit to different degrees.

Finally, we overexpressed the stable mouse HIFα mutants in Hep3B cells and determined endogenous HIF target gene mRNA levels. EPO mRNA levels were increased only 11.3-fold by HIF-1α- but 203.2-fold by HIF-2α-overexpression, supporting the concept that EPO is a HIF-2α target gene. HIF-2α overexpression resulted in mRNA induction of CA IX and VEGF but, in contrast to EPO, the amplitude of induction did not reach that achieved by activation of endogenous HIF.

7. In 786-0 renal carcinoma cells HIF-2α compensates for the loss of functional HIF-1α protein

In 786-0 renal carcinoma cells lacking functional von Hippel-Lindau protein (pVHL) and HIF-1α, HIF target gene expression is supposed to depend on HIF-2α. Indeed, knockdown of HIF-2α, but not HIF-1α, reduced the mRNA levels of GLUT-1, LDH-A, and VEGF

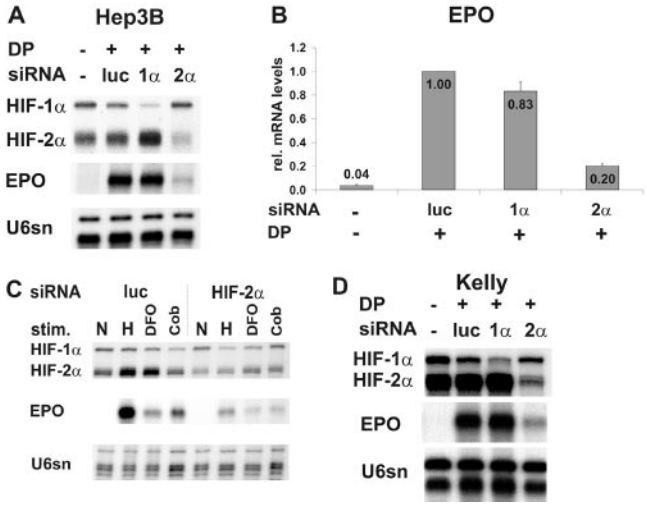

Figure 2. Reduction of EPO mRNA induction by the HIF-2α siRNA in Hep3B and Kelly cells. *A)* RNase protection assays revealed that EPO is a HIF-2α target gene in Hep3B cells (40 μg RNA/lane). *B)* Quantification of the siRNA effects on EPO mRNA levels in Hep3B cells (n=3). *C)* Effect of the HIF-2α siRNA on EPO mRNA expression in Hep3B cells was independent of the stimulus; N = normoxia, H = hypoxia (0.5% O_2) and the hypoxia mimetics desferrioxamine (DFO) and cobalt chloride (Cob). *D)* RNase protection assays with human neuroblastoma Kelly cells (80 μg RNA/lane) confirmed the HIF-2α dependency of EPO induction in an unrelated cell line.

in 786-0 cells and hypoxic induction of these mRNAs in 786-0 cells stably retransfected with functional pVHL. Thus, similar to HIFα overexpression, in 786-O cells target gene specificity is lost due to unknown mechanisms.

CONCLUSIONS AND SIGNIFICANCE

Until now, comparative studies aiming at a functional differentiation of the two HIFα subunits were hampered by the fact that in vitro most cell lines express both HIFα isoforms. In contrast, immunohistochemistry revealed differential expression of HIF-1α and HIF-2α in most cell types in vivo. Targeted deletion of the HIFα subunits in mice resulted in divergent—in most cases, lethal—phenotypes. On the other hand, forced expression of the α subunits in cell cultures suggested at least partial functional redundancy. In the present study, we show by use of the novel technique of RNAi that HIF-1α and HIF-2α have clearly defined and non-overlapping target gene specificities in different human cell lines, independent of the relative abundance of either HIFα isoform. Furthermore, we show for the first time that this specificity can be overcome by forced expression of the HIFα subunits from expression vectors and in tumor cells with severe perturbations in the HIF/pVHL axis. In **Fig. 3** we summarize and interpret our findings.

The fact that the genes involved in glucose metabolism, as well as CA IX and PHD2, were HIF-1α target genes was not entirely unexpected and agrees with other gene targeting studies. Concerning the previously proposed HIF-2α dependency of VEGF, we provide evidence that these conflicting results presumably were due to the experimental approach, i.e., overexpression of HIF-2α, which may override target gene specificity.

Thus, overexpression experiments do not seem to be a useful approach for the characterization of HIFα target gene specificities.

The most significant finding of the present study was the exclusive and unequivocal HIF-2α dependency of the hypoxic EPO mRNA induction in Hep3B and Kelly cells. This finding was surprising, since HIF-1α was identified as the nuclear factor that bound to the EPO HRE. On the other hand, immunohistochemical studies provided the first evidence for EPO as an HIF-2α target: renal peritubular fibroblasts, known to produce EPO, express HIF-2α but not HIF-1α. Finally, recent HIF-2α gene targeting studies have supported our finding.

We also provide insight into the regulatory mechanism underlying the specificity of the endogenous EPO gene for transactivation by HIF-2α. Reporter assays revealed that HIF-2α activates the EPO enhancer only in conjunction with other regulatory sequences in the vicinity of the HRE; the nuclear factors presumably binding to these *cis*-active elements remain to be determined. Since the 25 bp EPO HRE oligonucleotide has often been used as a probe in gel shift experiments, the difficulties encountered with HIF-2α gel shifts are now comprehensible.

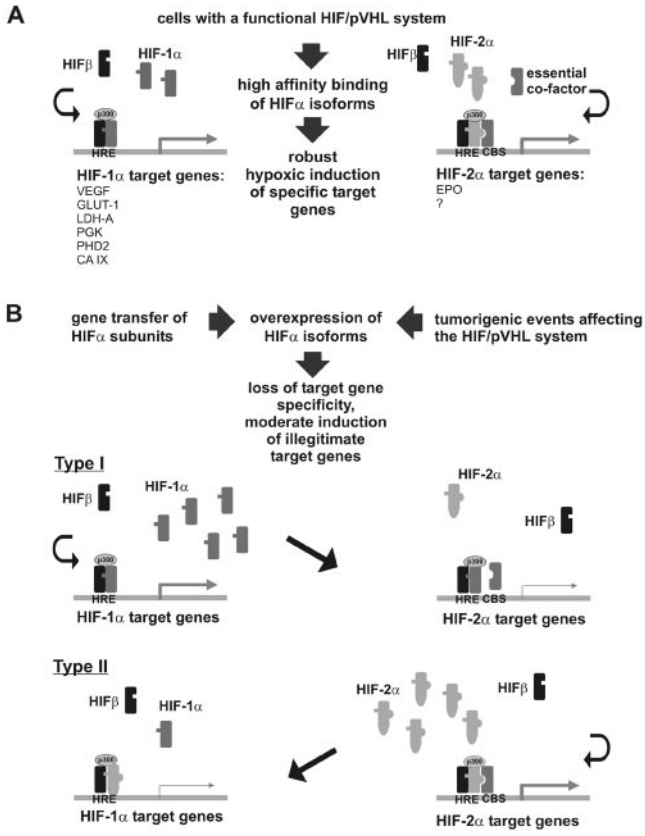

Figure 3. Predicted model of the molecular mechanism of HIFα isoform specificity and its loss by HIFα overexpression. *A)* In mammalian cells with a functional HIF/pVHL system, each HIFα isoform exhibits high affinity binding only to the HREs of its specific target genes, resulting in maximum hypoxic gene induction. Of course, HIF-1α also acts in cooperation with other transcription factors and transcriptional coactivators (not depicted) beyond CBP/p300, but for binding of HIF-2α to the EPO enhancer and full transactivation of the EPO gene the interaction with another transcription factor that binds to *cis*-active elements in the vicinity of the HRE is an absolute requirement (CBS=cofactor binding site). A candidate transcription factor for this interaction is hepatocyte nuclear factor 4 (HNF-4), which binds to a direct repeat of a steroid/thyroid nuclear hormone receptor response element half-site separated by a 2 bp spacer and was shown to be essential for the full hypoxic induction of the EPO gene. Formation of the stable DNA binding complex is possibly subject to redox control. *B)* HIFα isoform overexpression induced by gene transfer or tumorigenic events affecting the HIF/pVHL system may lead to a loss of target gene specificity in that HIF-1α is now capable of binding to the HREs of HIF-2α targets, and vice versa. However, this promiscuous binding may be of lower affinity and the resulting gene induction less robust than the induction by the respective legitimate HIFα isoform.

In light of the increasing significance of recombinant EPO as a therapeutic drug not only in treating anemia, but possibly in the therapy of stroke and heart failure, the knowledge that EPO is a physiological HIF-2α target gene and that HIF-1α and HIF-2α signaling differs will be an important prerequisite for attempts to design specific therapeutic approaches to stimulate endogenous EPO production. FJ