Cell. Mol. Life Sci. 64 (2007) 989 – 998
1420-682X/07/070989-10
DOI 10.1007/s00018-007-6561-9

Cellular and Molecular Life Sciences

Research Article

Interleukin-1β inhibits the hypoxic inducibility of the erythropoietin enhancer by suppressing hepatocyte nuclear factor-4α

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Received 22 December 2006; received after revision 22 February 2007; accepted 1 March 2007
Online First 19 March 2007

Abstract. The suppression of hypoxia-induced erythropoietin (EPO) expression by inflammatory cytokines like interleukin-1 (IL-1) contributes to the development of the anemia of chronic disease (ACD). However, the precise mechanism of this suppression is unclear. The 3'-EPO enhancer mediates the transcriptional response to hypoxia by binding several transcription factors, including hypoxia-inducible factor, hepatocyte nuclear factor-4α (HNF-4α) and chicken ovalbumin upstream promoter transcription factor. We investigated whether IL-1β inhibits the activity of the 3'-EPO enhancer via HNF-4α. IL-1β inhibited HNF-4α mRNA expression and caused proteasome-dependent degradation of HNF-4α protein, which resulted in a strongly reduced DNA-binding activity of HNF-4α. Reporter gene assays revealed that IL-1β caused a complete suppression of the hypoxic inducibility of the 3′ enhancer via inhibition of HNF-4α. We conclude that IL-1β, at least partially, reduces hypoxia-induced EPO expression by down-regulation of HNF-4α.

Keywords. Erythropoietin, anemia of chronic disease, interleukin-1, hepatocyte nuclear factor-4, hypoxia inducible factor-1, chicken ovalbumin upstream promoter transcription factor.

Introduction

Anemia of chronic disease (ACD) frequently develops in patients with inflammation due to infection, autoimmune disease or malignancy. A shared attribute of inflammatory diseases is the increased production of cytokines like interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and interferons [1]. Several mechanisms have been proposed to explain as to how these cytokines might impair erythropoiesis. Cytokines can reduce the iron availability for erythrocytic precursors by promoting the retention of iron in macrophages and by lowering intestinal iron absorption [2, 3]. In addition, impaired erythropoietin (EPO) production is thought to contribute to the development of ACD [4]. TNF-α and IL-1β have been shown to suppress EPO formation in isolated perfused rat kidneys and to reduce EPO mRNA expression and EPO secretion in the human hepatoma cell lines HepG2 and Hep3B [5–7]. Moreover, clinical studies have shown that anemias associated with malignancy [8] or other chronic inflammatory diseases [1] can be reversed by treatment with recombinant human EPO, underlining the significance of insufficient EPO production in the pathogenesis of ACD.

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Expression of the EPO gene is maintained and regulated by the temporal binding of numerous transcription factors to specific promoter and enhancer regions [9, 10]. The EPO promoter is controlled by GATA transcription factors [11–13], whereby GATA-2 DNA binding prevents EPO transcription [11, 12, 14, 15]. The hypoxia-inducible enhancer, located 3' of the EPO gene, is a 50-base pair (bp) element containing two characterized transcription factor binding sites. The proximal site binds the heterodimeric transcriptional activators hypoxia-inducible factors-1 or -2 (HIF-1 or -2) [16, 17]. The distal site, located at the 3′ end of the enhancer, consists of a direct repeat of two nuclear hormone receptor half-sites separated by a 2-bp DNA sequence. It represents a DR2 site that functions as a DNA response element for nuclear hormone receptors (NHRs) such as for hepatocyte nuclear factor-4α (HNF-4α) and chicken ovalbumin upstream promoter transcription factor (COUP-TF) [18, 19].

HNF-4α is mainly expressed in liver and kidney, *i.e.*, at the sites of prominent EPO expression, and to a lesser extent in the intestine and pancreas [20], suggesting that HNF-4α may contribute to the tissue specificity of EPO expression. COUP-TF, an orphan member of the NHR superfamily, has been assigned a suppressive role in EPO gene expression as it competes with HNF-4α for the DNA-binding site [18]. Furthermore, CBP/P300 and SRC-1 are required as transcriptional co-activators that form a complex with HIF-1 and HNF-4α, recruit histone acetyltransferase activity and mediate the interaction between the 3′ EPO enhancer and the basal transcription apparatus of its promoter [21–24]. In hepatoma cells HNF-4 expression decreases at oxygen concentrations near anoxia, whereas moderate hypoxia (in the range of 2–3 % oxygen) has no effect on HNF-4 [25].

The molecular mechanisms underlying the inhibitory effect of inflammatory cytokines on the EPO gene are still poorly understood. Studies focusing on the EPO promoter have implicated GATA-2 and nuclear factor-κB (NF-κB) as mediators of the suppressive effect of IL-1β and TNF-α [14, 15]. Whether the EPO enhancer is blocked by the cytokines is unknown. Earlier studies from this laboratory have shown that the DNA-binding activity of HIF-1 is induced by IL-1β [26]. As HIF-1 is known to stimulate EPO transcription, this result did not provide an explanation for the suppressive effect of inflammatory cytokines on EPO production. The aim of the present study was to investigate whether the proinflammatory cytokine IL-1β reduces the activity of the EPO enhancer via reduced HNF-4α function despite HIF-1 activation.

Materials and methods

Cell culture. The human hepatoma cell line HepG2 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured in RPMI 1640 medium containing 10 % fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA). The human osteosarcoma cell line U2OS was a gift from Peter Ratcliffe (Oxford, UK). U2OS cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Karlsruhe, Germany) supplemented with 10 % FCS, 50 IU/mL penicillin and 50 μg/mL streptomycin sulfate (Sigma, Deisenhofen, Germany). Cells were grown in a humidified atmosphere of 95 % air and 5 % CO_2 at 37°C. For hypoxic treatment, low-density cultures were placed in a pO_2-controlled incubator (Heraeus, Hanau, Germany) with 3 % O_2, 5 % CO_2 and balanced N_2. Cells received fresh medium 16 h before nuclear extract preparation, RNA isolation or transient transfection.

Unless otherwise noted, recombinant IL-1β (Ciba-Geigy, Basel, Switzerland) and the proteasome inhibitor MG132 (Sigma) were added to final concentrations of 300 pg/mL and 1 μM, respectively, in serum- and antibiotic-free medium. Actinomycin D (Sigma) was used in a final concentration of 10 μM.

Nuclear extract preparation. Nuclear extracts of HepG2 cells were prepared as described earlier [26]. Cells were washed with ice-cold phosphate-buffered saline (PBS) and centrifuged at 800 *g* and 4°C for 5 min. The cell pellets were washed with 4 mL ice-cold buffer A (10 mM Tris, pH 7.8, 1.5 mM $MgCl_2$, and 10 mM KCl), resuspended in this buffer, and then kept on ice for 10 min. Cell lysis was confirmed by trypan blue staining. Nuclei were pelleted at 3500 *g* and 4°C for 5 min, resuspended in 100 μL ice-cold buffer C (420 mM KCl, 20 mM Tris, pH 7.8, 1.5 mM $MgCl_2$, and 20 % glycerol) and incubated on ice for 30 min with occasional flicking of the tubes. Just prior to use, buffers A and C were supplemented with 2 μg/mL aprotinin, 10 μg/mL leupeptin, 20 μg/mL pepstatin, 1 mM Na_3VO_4, 0.5 mM benzamidine, 2 mM levamisole, 10 mM β-glycerophosphate, 0.5 mM dithiothreitol (DTT), and 0.4 mM phenylmethylsulfonyl fluoride (PMSF). Nuclear extracts were centrifuged at 13 000 *g* and 4°C for 30 min. The supernatants were stored at –80°C. Protein concentrations were determined using the Bradford method with bovine serum albumin (BSA) as standard.

Western blot analyses. For determination of HNF-4α and HIF-1α protein, nuclear extracts were subjected to Western blot analysis as described previously [26]. For detection of HNF-4α, 6 μg nuclear extract protein was run on sodium dodecyl sulfate (SDS)/7.5 % polyacrylamide gels (PAG) and transferred electrophoretically (Trans-Blot SD; Bio-Rad, München, Germany) onto PVDF membranes (Roti-PVDF; Carl Roth, Karlsruhe, Germany). For detection of HIF-1α, 20 μg nuclear extract protein was loaded. Loading precision and transfer efficiency were verified by staining with 2 % Ponceau S. Membranes were blocked with PBS/5 % fat-free skim milk. The primary antibodies used were polyclonal goat antibodies raised against human HNF-4α (diluted 1:500 in PBS/3 % skim milk; Santa Cruz, Heidelberg, Germany) and monoclonal mouse antibodies against human HIF-1α (diluted 1:1000; BD Biosciences, Heidelberg, Germany). Sp1 antibodies (diluted 1:1000; Santa Cruz) were applied to monitor constitutively expressed Sp1 as loading control. For detection, horseradish peroxidase-linked anti-goat or anti-mouse antibodies (diluted 1:2000; Santa Cruz) and enhanced chemiluminescence substrate (Amersham, Freiburg, Germany) were used.

Electrophoretic mobility shift assays. Oligonucleotides containing the DR2 sequences of the EPO enhancer (DR2), and a synthetic thyroid hormone response element (TRE) [27] were synthesized by MWG Biotech (Ebersberg, Germany). Sequences were: DR2 sequence: 5'-CGG TAG GTC GAG AGG TCA GAC AGG CTG-3'; TRE sequence: 5'-CCT GTC AGG TCA TGA CCT ACC G-3'. [γ-^{32}P]ATP was obtained from New England Nuclear (Köln, Germany). The COUP-TF antiserum was kindly provided by Ming-Jer-Tsai (Baylor College of Medicine, Houston, TX, USA). For the electrophoretic mobility shift assays (EMSAs), 4 μg HepG2 nuclear extract was added to each gel shift reaction. The reaction

mixture (20 μL) contained 10^4 cpm of the end-labeled double-stranded oligonucleotide, 5 mM Tris pH 7.5, 50 mM NaCl, 0.375 mM $MgCl_2$, 105 mM KCl, 1 mM EDTA, 1 mM DTT, 1 μg poly(dI-dC), 3.75 μg BSA and 5 % (vol/vol) glycerol. After 15 min of incubation, samples were resolved by electrophoresis on native 6 % polyacrylamide gels at room temperature. Gels were dried and analyzed by phosphorimaging (BAS 1000; Fujifilm Europe, Düsseldorf, Germany). For competition experiments, a 250-fold molar excess of unlabeled annealed oligonucleotides was provided before addition of the labeled probes. For supershift experiments, the binding reaction was carried out as described above, but after incubation of the probes, 1 μL antiserum against HNF-4α or COUP-TF was added for 45 min incubation at room temperature.

mRNA quantification. Total RNA of HepG2 cells was isolated with the ABI Prism™ 6100 NucleicAcid PrepStation (Applied Biosystems, Darmstadt, Germany). Total RNA (1 μg) was reverse transcribed in a total volume of 25 μL using Transcriptor Reverse Transcriptase (Roche, Mannheim, Germany). For quantification of HNF-4α cDNA, a PCR was set up in a total volume of 25 μL with 5 μL cDNA as template, commercially available Assay-on-Demand gene expression reagents containing the TaqMan probe and primers (HS00230853_m1; Applied Biosystems) and TaqMan Universal PCR Mastermix (Applied Biosystems). A two-step real-time PCR with denaturation at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 60 s was applied using an ABI 7000 Sequence detection system (Applied Biosystems). Relative mRNA expression levels were calculated with the $\Delta\Delta C_T$ method, normalized to ribosomal protein L28 cDNA and related to the normoxic control. Specificity was verified by agarose gel electrophoresis.

For quantification of L28 mRNA, the PCR reaction was set up in a total volume of 25 μL with 5 μL of a 1:5000 dilution of the cDNA as template and a commercially available SYBR green PCR kit (Eurogentech, Seraing, Belgium). Primer sequences were 5'-ATG GTC GTG CGG AAC TGC T-3' (sense) and 5'-TTG TAG CGG AAG GAA TTG CG-3' (antisense). The amplification process was performed as described above for HNF-4α. Specificity was verified by agarose gel electrophoresis and melting point curve analysis. To determine relative HNF-4α mRNA amounts in time course experiments after treatment with IL-1β or actinomycin D, the mean HNF-4α/L28 ratios at time point 0 h were set to 1 in each group. All later time points were related to 0 h.

Plasmid construction. EPO enhancer reporter plasmids were constructed by ligating synthetic oligonucleotides (sequence derived from the EPO enhancer) downstream of the luciferase gene between the *Sal*I and *Bam*HI restriction sites within the pGL3-Promoter vector (Promega, Mannheim, Germany). For the construction of the EPO-Enhancer-wt plasmid, the sequence of the EPO enhancer was used as an insert (5'-GAT CCC TAC GTG CTG TCT CAC ACA GCC TGT CTG ACC TCT CGA CCT ACC G-3'). To construct the EPO-Enhancer-dHIF plasmid, the same sequence was used except that the HIF binding site was deleted (5'-GAT CCC TAC GTG CTG TCT CAC ACA GCC TGT CAC C–3'). The oligonucleotides were synthesized by MWG Biotech.

An expression plasmid for the fusion protein HNF-4α-dsRed2 was constructed as follows: human HNF-4α was amplified by PCR with a 5' primer containing a *Kpn*I site and a 3' primer containing a *Bam*HI site. After *Kpn*I/*Bam*HI double digestion, the PCR product was inserted into the equally treated pDsRed2-N1 plasmid (BD Biosciences, Clonetech, Heidelberg) and the resulting plasmid was designated pHNF-4α-dsRed2. All plasmids were verified by DNA-sequence analysis.

Transient transfection and luciferase assay. All transfections were performed using FuGENE 6 transfection reagent (Roche). Cells were equally seeded into six-well plates and grown to 50 % confluence. To each well, 3 μL FuGENE reagent and 1 μg plasmid DNA were added. After 12 h of incubation, cells received fresh medium with or without cytokines. Empty pcDNA3 vector (Invitrogen) was used as control. HepG2 cells were cotransfected with a SV40-Renilla-luciferase plasmid as a measure for transfection efficiency. In U2OS transfections, the Renilla luciferase plasmid was omitted to avoid three-plasmid transfections. Transfected cells were then incubated under normoxic (20 % O_2) or hypoxic conditions (3 % O_2) for 24 h.

After incubation, cells were washed twice with PBS and lysed with passive buffer (Promega). Luminescence was measured using a MicroLumat LB 96P (Berthold EG & G, Bad Wildbach, Germany). Firefly luciferase-induced luminescence in cell lysates of HepG2 cells was normalized to Renilla luciferase-induced luminescence. In U2OS cells, luminescence of each sample was normalized to total cellular protein. Protein concentrations in cell lysates were determined using the Bradford method and BSA as standard.

Fluorescence visualization of HNF-4α-dsRed2 fusion proteins by microscopy. U2OS cells were cotransfected with pEPO-Enhancer-wt and pHNF-4α-dsRed2 plasmids as described, and were seeded on cover slips in six-well plates. After 12 h of incubation, cells received fresh medium with or without IL-1β and were incubated for further 4 h under normoxia (20 % O_2) or hypoxia (3 % O_2). Cells were then fixed in 3.7 % formaldehyde in PBS, washed once in PBS and mounted on glass slides using Mowiol. Digital images were taken from a Zeiss Axioplan 2 fluorescence microscope with 200× magnification. Difference interference contrasted pictures were taken with 0.7-s exposure time and fluorescence pictures were taken with an excitation wavelength of 546 nm and 6-s exposure time.

Statistics. Results are shown as means + standard deviations (SDs) of separate experiments. Student's *t*-test was used to show statistical significance.

Results

Basal HNF-4α levels in HepG2 and U2OS cells. HNF-4α protein was detectable in extracts from untreated HepG2 (Fig. 1a, lane 1), but not from U2OS cultures (Fig. 1a, lane 2). In U2OS cells transiently transfected with the pHNF-4α-dsRed2 expression plasmid, immunoreactive HNF-4α-dsRed2 fusion protein became visible (Fig. 1a, lane 3).

Effects of IL-1β and hypoxia on HNF-4α and HIF-1α levels in HepG2 cells. There was a dose-dependent decrease in the amount of HNF-4α protein in nuclear extracts from hypoxic HepG2 cells treated with IL-1β for 4 h (Fig. 1b, lanes 3–5). Likewise, decreased levels of HNF-4α protein were detected in cells cultured under normoxic conditions (Fig. 1c, lanes 3–9). HNF-4α levels were not affected by hypoxia in HepG2 cultures (3 % O_2; Fig. 1b and c, lanes 2 vs. respective lanes 1). In contrast, nuclear HIF-1α protein levels were increased in IL-1β-treated cells, as determined in the same extracts used to study normoxic HNF-4α expression (Fig. 1d, lanes 3–9). In addition, strongly increased amounts of HIF-1α protein were detected in extracts from cells exposed to hypoxia (Fig. 1d, lane 2 vs. lane 1).

Effect of MG132 on HNF-4α levels in IL-1β-treated HepG2 cells. To test whether HNF-4α is degraded by the proteasome and if this process is accelerated by IL-1β, normoxic HepG2 cells were incubated with the proteasome inhibitor MG132. After 8 h there was

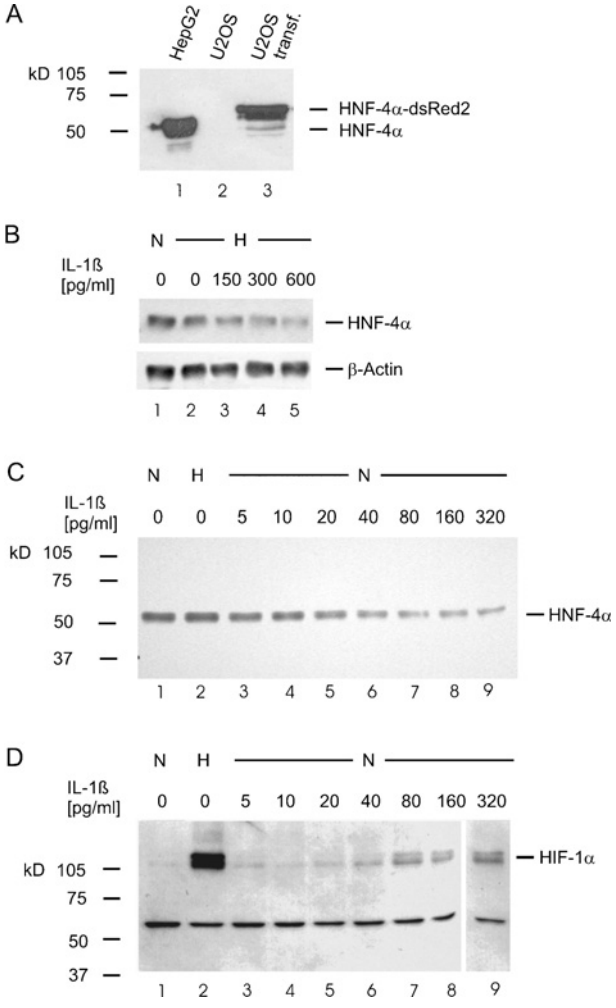

Figure 1. Hepatocyte nuclear factor (HNF)-4α and hypoxia-inducible factor (HIF)-1α protein levels are reciprocally regulated by interleukin (IL)-1β. (*a*) Western blot analysis of HNF-4α in nuclear extracts of HepG2 cells (lane 1), U2OS cells (lane 2) and U2OS cells transiently transfected with the dsRed2-HNF-4α expression plasmid (lane 3). (*b*) Western blot analysis of HNF-4α in nuclear extracts from hypoxic (3 % O_2) (H) HepG2 cells. Cells were incubated with increasing concentrations of IL-1β. Untreated control cells were kept in normoxia (20 % O_2) (N) or hypoxia. (*c*) Western blot analysis of HNF-4α in nuclear extracts from HepG2 cells. Cells were incubated with increasing concentrations of IL-1β under normoxic conditions (N). Untreated control cells were kept in normoxia (N) or hypoxia (H). (*d*) Western blot analysis of HIF-1α in the same nuclear extracts as used for (*c*). The 55-kDa signal, unspecifically raised by the secondary antibody, served as loading control since the signal intensities corresponded to immunodetected Sp1 (not shown).

moderate increase in HNF-4α protein compared to control cells (Fig. 2, lanes 5 vs. 7). IL-1β reduced HNF-4α protein as in the previous experiments and this effect could be reversed by simultaneous administration of MG132 (Fig. 2, lane 2 vs. lane 4 and lane 6 vs. lane 8). To prove effective proteasome inhibition HIF-1α protein, which is known to be degraded by the proteasome under normoxic conditions, was studied.

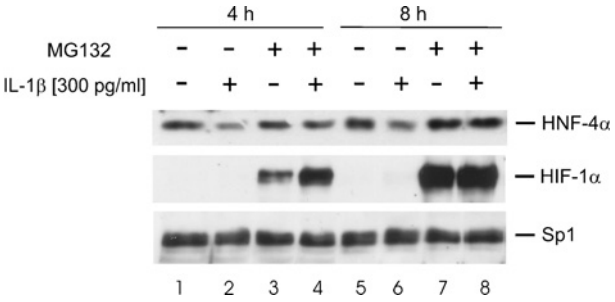

Figure 2. The proteasome inhibitor MG132 prevents IL-1β-induced HNF-4α reduction. Western blot analysis of HNF-4α and HIF-1α in nuclear extracts from HepG2 cells. Cells were normoxically incubated in the absence or presence of IL-1β, MG132 or a combination of both for 4 or 8 h. Constitutively expressed Sp1 served as loading control.

The amount of HIF-1α was raised in cells treated with MG132 and simultaneous incubation with IL-1β had an additional effect (Fig. 2). Normoxically IL-1β-induced HIF-1α became detectable on prolonged exposure of the blots (not shown).

Effect of IL-1β on HNF-4α mRNA levels. HNF-4α mRNA levels were reduced in normoxic HepG2 cells exposed to IL-1β within 2 h (earlier time points were not tested; Fig. 3). HNF-4α mRNA levels remained low for at least another 6 h. Interestingly, actinomycin D reduced HNF-4α mRNA levels in a similar manner with respect to magnitude and time course (Fig. 3).

Identification of HNF-4α-protein-DNA complexes. EMSAs were carried out with labeled oligonucleotide probes containing the DR2 site of the EPO enhancer. On incubation with nuclear extracts from untreated HepG2 cells, two DNA/protein complexes migrating with differing electrophoretic mobility were formed (Fig. 4a). Since Galson and co-workers [18] have shown that HNF-4α and COUP-TF compete for binding the DR2 element of the EPO enhancer, the likely possibility is that the two complexes we observed in EMSA experiments were formed by these two transcription factors. Indeed, supershift experiments identified the slower migrating complex to contain COUP-TF and the faster migrating complex to contain HNF-4α (Fig. 4a, lanes 2 and 3). It has been shown earlier that COUP-TF, but not HNF-4α, binds specifically to a TRE [27]. When the TRE-oligonucleotide was used in EMSAs with the same nuclear extracts as before, the slower migrating complex was mainly observed and an anti-COUP-TF antibody was able to induce a supershift in this reaction, whereas an anti-HNF-4α antibody failed to do so (Fig. 4, lanes 4 and 5).

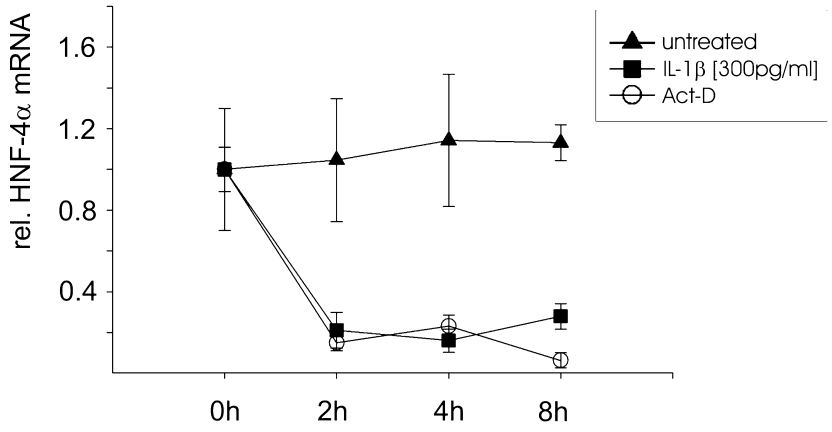

Figure 3. IL-1β reduces HNF-4α mRNA expression in HepG2 cells. Real-time RT-PCR quantification of HNF-4α mRNA in response to IL-1β. Cells were incubated in the absence or presence of IL-1β or actinomycin D for the indicated periods. HNF-4α mRNA was normalized to L28 mRNA and related to the respective 0 h group (means +SDs of five separate experiments).

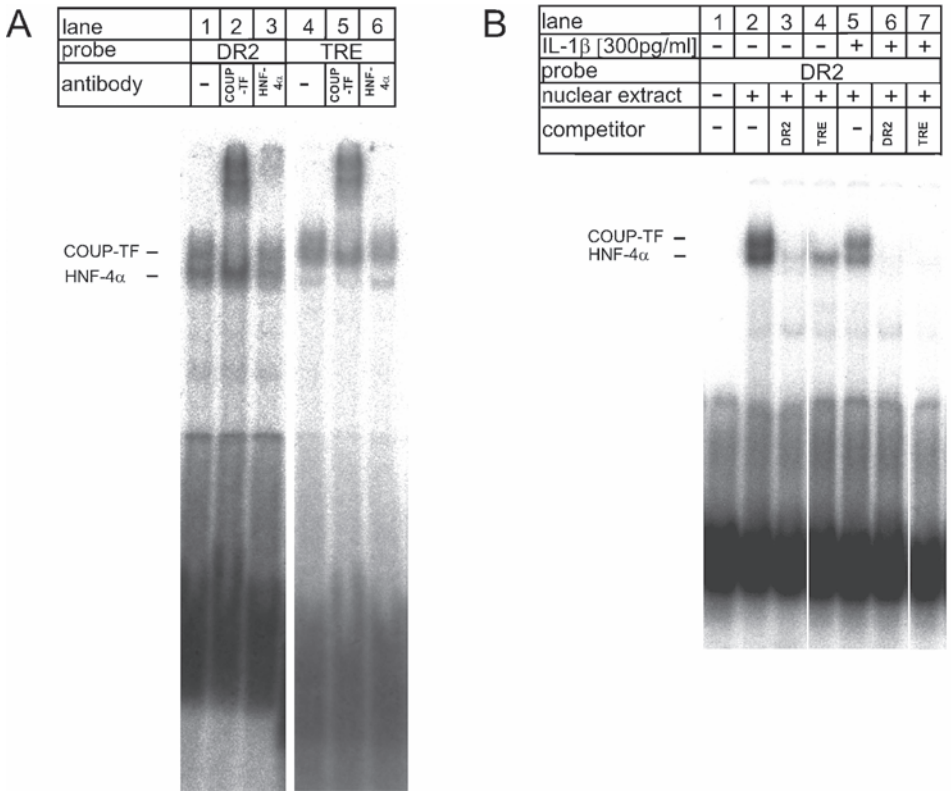

Figure 4. IL-1β suppresses DNA binding of HNF-4α to the DR2 site. (*a*) Electrophoretic mobility shift assays (EMSAs) of nuclear extracts from HepG2 cells with oligonucleotides containing either the DR2 sequence of the erythropoietin (EPO) enhancer or the thyroid hormone response element (TRE) sequence as a probe. Supershift experiments revealed that HNF-4α and chicken ovalbumin upstream promoter transcription factor (COUP-TF) bound to the DR2 probe, forming two complexes of differing electrophoretic mobility (lanes 1–3). Supershift experiments with the labeled TRE oligonucleotide (lanes 4–6) demonstrated specific binding of COUP-TF. All samples were resolved on the same gel. (*b*) EMSAs performed with nuclear extracts of untreated HepG2 cells (lanes 1–4) and cells treated with 300 pg/mL of IL-1β (lanes 5–7). Unlabeled DR2 or TRE oligonucleotides were used as competitor in 250-fold excess as noted. All samples were resolved on the same gel.

Effect of IL-1β on DNA-binding of HNF-4α. When DNA-binding was analyzed in nuclear extracts of HepG2 cells treated with IL-1β for 4 h, the overall DNA-binding activity of the DR2 probe was clearly decreased when compared to control cell extracts (Fig. 4b, compare lanes 2 and 5). Competition experiments were performed to further address the question of specificity of the influence of IL-1β on DNA binding of HNF-4α. To study HNF-4α DNA binding by EMSA without interference by COUP-TF, we added the unlabeled TRE oligonucleotide in 250-fold excess as a competitor to the binding reaction. As a

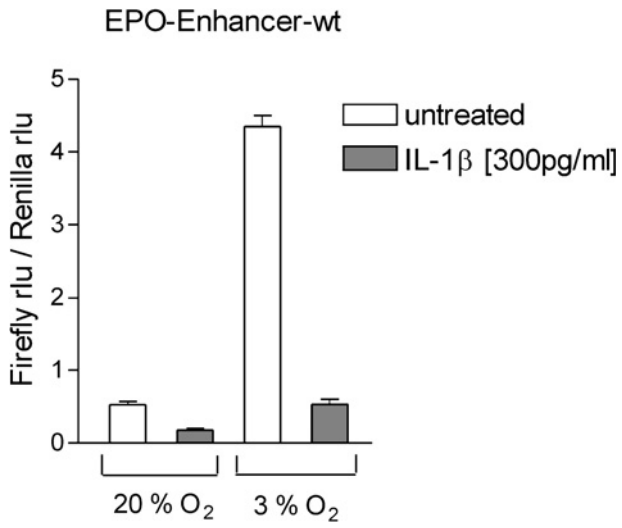

Figure 5. IL-1β suppresses the hypoxic inducibility of the EPO enhancer in HepG2 cells. HepG2 cells were transiently transfected with the pEPO-Enhancer-wt firefly luciferase reporter plasmid and cotransfected with a SV40-Renilla luciferase plasmid. Cells were treated with IL-1β (300 pg/mL) and cultivated under normoxic (20 % O_2) or hypoxic (3 % O_2) conditions for 24 h as noted. Firefly luciferase relative light units (rlu) were normalized to SV40-Renilla luciferase rlu (means + SDs of four separate experiments).

result, the formation of the protein-DNA complex representing COUP-TF was prevented, but the HNF-4α-DNA complex was still detectable (Fig. 4b, lane 4). In nuclear extracts from IL-1β-treated HepG2 cells, the addition of unlabeled DR2 competitor strongly reduced complex formation, whereas the TRE oligonucleotide as a competitor led to a complete loss of specific DNA binding to the labeled DR2 probe (Fig. 4b, lane 7). Thus, treatment with IL-1β suppressed DNA binding of HNF-4α in these samples.

Effect of IL-1β on the hypoxic inducibility of the EPO enhancer. To investigate the effect of IL-1β on the EPO enhancer, reporter gene assays were performed. The pEPO-Enhancer-wt plasmid contained the hypoxia responsive 3′-enhancer of the EPO gene. To mimic the situation *in vivo*, this sequence was inserted downstream of the luciferase gene. HepG2 cells transfected with the pEPO-Enhancer-wt plasmid showed an 8-fold hypoxic induction of the reporter activity compared to normoxic controls. This hypoxic induction was completely suppressed by treatment with IL-1β ($p< 0.001$, Student's *t*-test) (Fig. 5).
Furthermore, U2OS cells were used as model. Because these cells do not express HNF-4α protein intrinsically, a pHNF-4α-dsRed2 expression plasmid or an empty pcDNA3 control vector was co-transfected. U2OS cells transfected with both the pEPO-Enhancer-wt plasmid and the pHNF-4α-dsRed2 expression vector showed a 15-fold hypoxic induction of the reporter activity compared to normoxic controls. Similar to the results obtained in HepG2 cells, this hypoxic induction was completely suppressed by treatment with IL-1β ($p<0.001$, Student's *t*-test) (Fig. 6a). When the pcDNA3 control vector was co-transfected instead of the pHNF-4α-dsRed2expression plasmid, neither a hypoxic induction nor a significant effect of IL-1β on reporter gene activity was detected (Fig. 6a).

Role of the HIF-1 binding site in the suppression of EPO enhancer activity by IL-1β. No hypoxia-inducible luciferase activity was observed in reporter gene experiments with the pEPO-Enhancer-dHIF plasmid, containing the same sequence as the pEPO-Enhancer-wt plasmid but without HIF-1-binding site, and cotransfection of the pHNF-4α-dsRed2 expression plasmid. Treatment with IL-1β had only a minor effect on luciferase activity (Fig. 6b). Furthermore, cells cotransfected with pcDNA3 showed no hypoxic inducibility but decreased luciferase activity after IL-1β stimulation. Despite being statistically significant, the biological meaning of this finding demands further clarification. It is of note that the absolute relative light unit (rlu) count was dramatically reduced compared to the experiments with the pEPO-Enhancer-wt plasmid.

Effect of IL-1β on HNF-4α-dsRed2 fusion proteins. To further strengthen the results from the reporter gene experiments, the effect of IL-1β on HNF-4α-dsRed2 fusion proteins was investigated by fluorescence microscopy and Western blotting. In untransfected control cells, only background fluorescence was observed in contrast to pHNF-4α-dsRed2/pEPO-Enhancer-wt cotransfected cells where strong red fluorescent signals were obtained (Fig. 7a). The fluorescent signals were mainly located in the nuclei of cells, and were not affected by the oxygen concentration. However, IL-1β clearly reduced HNF-4α-dsRed2 fluorescence intensities irrespectively of the oxygen concentration (Fig. 7a). Moreover, Western blot analysis with whole cell extracts of similarly transfected and stimulated U2OS cells revealed a strong decrease of HNF-4α-dsRed2 fusion proteins after IL-1β stimulation (Fig. 7b).

Discussion

Blunted EPO production in response to inflammatory cytokines is considered as one of the main reasons for the development of ACD [4, 28, 29]. The present study indicates that IL-1β, at least partially, suppresses hypoxic inducibility of the EPO enhancer via inhibition of the action of HNF-4α.

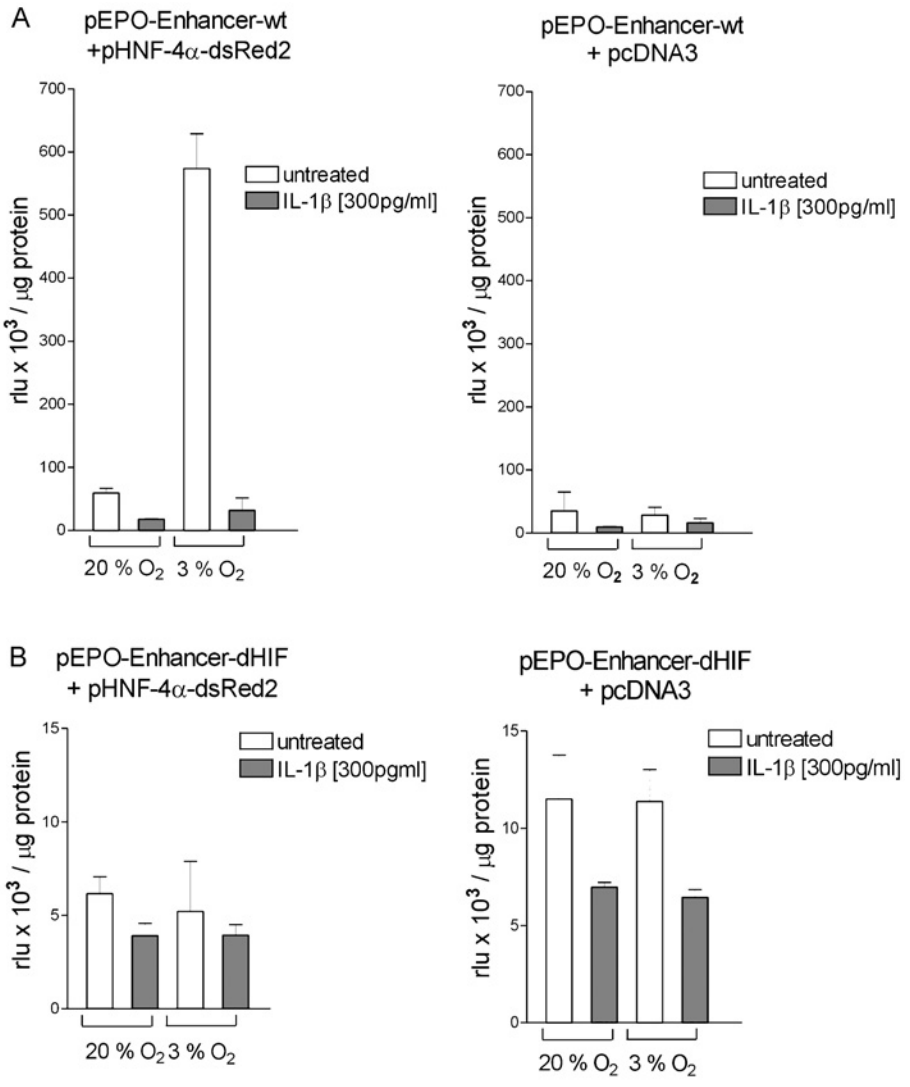

Figure 6. IL-1β blocks the hypoxic inducibility of the EPO enhancer via HNF-4α. (*a*) Reporter gene assay of U2OS cells transiently cotransfected with the pEPO-Enhancer-wt plasmid and the pHNF-4α-dsRed2 expression plasmid or the empty pcDNA3 vector as control. Cells were treated with IL-1β (300 pg/mL) and cultivated under normoxic (20 % O_2) or hypoxic (3 % O_2) conditions for 24 h as noted. Luciferase activity was normalized to total cellular protein (means + SDs of four separate experiments). (*b*) U2OS cells transiently transfected with the pEPO-Enhancer-dHIF plasmid together with the pHNF-4α-dsRed2 expression plasmid or empty pcDNA3 as control. Treatment with IL-1β, cultivation and luciferase determination was performed under the same conditions as described above. Luciferase activity was normalized to total cellular protein (means + SDs of four separate experiments).

HNF-4α plays an important role in the tissue-specific and hypoxia-inducible expression of the EPO gene in kidney and livers [18]. HNF-4α is required for EPO production in hepatocytes [19, 30]. With respect to EPO expression by neuronal cells [31, 32], it is of interest that human neuroblastoma cells express EPO mRNA in an HNF-4α-independent manner [33]. Probably a different, yet unknown transcription factor substitutes for HNF-4α in these cells. In addition, CpG methylation of the HIF binding site in the EPO gene enhancer is missing in some neuroblastoma cell lines [34]. Usually, EPO expression increases maximally only on interaction of the EPO promoter with the enhancer complexed with HIF, HNF-4α, Sp1, and the co-activators CBP/P300 and SRC-1 [21, 22, 24]. Mutations of either the HIF or the HNF-4α response elements result in the loss of hypoxia-inducible EPO expression [35]. Mazure et al. [25] reported that severe hypoxia with an oxygen concentration of < 0.1 % O_2 in the surrounding atmosphere down-regulates expression of HNF-4. Taking into account that all of their experiments were carried out on regular culture dishes in which oxygen supply is diffusion-limited, one can assume that the cellular oxygen pressure was close to zero [36]. We applied less severe hypoxia (3 % O_2) and HNF-4α protein and RNA levels remained unaffected under this condition. Thus, our results are not in contrast to those reported by Mazure et al. [25].

Our assumption that IL-1β lowers the level of HNF-4α by increasing its degradation through the proteasome system in HepG2 cells is consistent with studies by Wang et al. [37]. However, in contrast to these investigators, we applied low concentrations of IL-1β (300 pg/mL) that reduce EPO secretion but do not exert cytotoxic effects on the cells [5]. Our concept is further supported by two facts. First, indirect evidence is provided with the reporter gene experiments shown

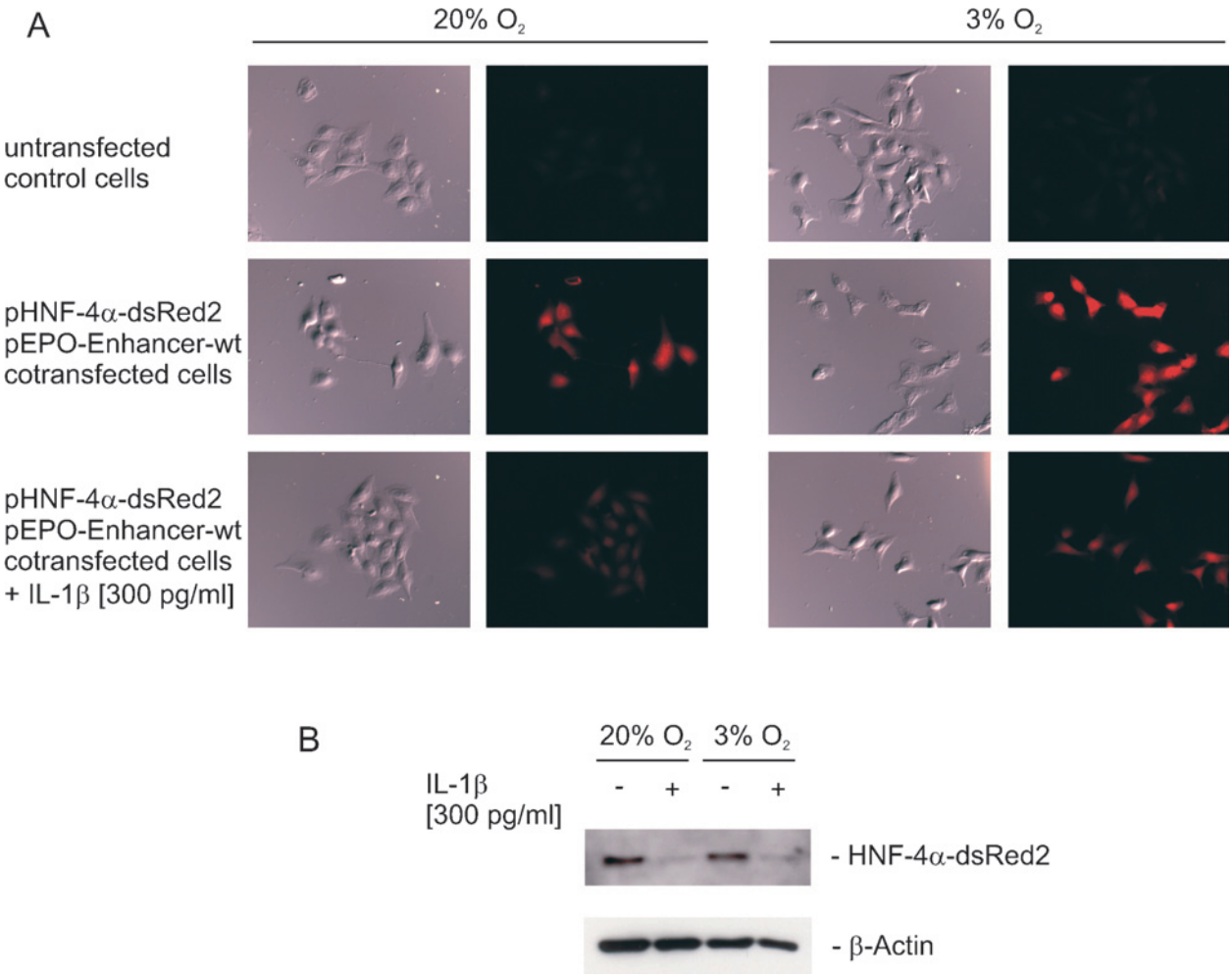

Figure 7. IL-1β decreases HNF-4α-dsRed2 fusion protein. (*a*) Fluorescence microscopy of U2OS cells left untransfected or cotransfected with pEPO-Enhancer-wt and pHNF-4α-dsRed2. Cells were either stimulated with IL-1β (300 pg/mL) or left untreated and exposed to normoxia (20 % O_2) or hypoxia (3 % O_2) for 4 h. (*b*) Western blot analysis of HNF-4α-dsRed2 fusion proteins in cotransfected U2OS cells. Cells were treated as described in (*a*). Reprobing with the β-actin antibody served as loading control.

in Figure 6. These suggest that proteasomal degradation of HNF-4α is the primary mechanism by which IL-1β reduces HNF-4α activity since it is unlikely that IL-1β will affect the transcriptional control of the expression plasmid. Second, more direct support is provided with the results of the fluorescence microscopy and Western blotting experiments shown in Figure 7, where a decrease of HNF-4a-dsRed2 fusion protein upon IL-1β stimulation was observed.

Furthermore, we detected that IL-1β also reduces HNF-4α mRNA expression. In time course experiments with IL-1β-stimulated cells, it became obvious that HNF-4α mRNA decreased within 2 h to about 20 % of the one in the control cultures and remained reduced for at least up to 8 h. It remains to be investigated whether the dramatic decrease of HNF-4α mRNA is due to reduced mRNA synthesis or to enhanced degradation of newly synthesized mRNA. Still, a computer search in the 3'UTR-database (UTResource at http://bighost.area.ba.cnr.it/BIG/UTRHome/) for sequence motifs for mRNA-binding proteins resulted in no matches for A+U rich elements (AREs) in the HNF-4α 3'-UTR [38]. The presence of AREs in the 3'UTRs of many mRNAs accelerates their decay by binding of several RNA-binding proteins (reviewed in [39, 40]). On the other hand, two isolated monomeric cytidine-rich 15-lipoxygenase differentiation control elements (15-LOX-DICE elements), located at positions 35–49 and 526–542 downstream of the stop codon, were identified. 15-LOX-DICE elements are multifunctional *cis*-acting elements in 3'UTRs of numerous eukaryotic mRNAs. In general, they bind several specific RNA-binding proteins, thus conferring mRNA stabilization and translational silencing [41–43]. Whether the two identified monomeric 15-LOX-DICE elements are functional is in question, since at least a dimeric organization is required for *in vitro* activity

[41]. However, the observation that the IL-1β-induced reduction resembled the one caused by the addition of actinomycin D may support the concept of a reduced synthesis. The L28 C_T values were not affected by IL-1β or actinomycin D treatment at the investigated time points (±0.5 C_T values; data not shown). This finding is probably due to the great stability of L28 mRNA, which thereby provides a suitable reference mRNA in such experiments. In conclusion, treatment with IL-1β appears to lower HNF-4α levels both by transcriptional and posttranscriptional mechanisms.

EMSA studies revealed that IL-1β treatment reduced overall DNA binding to the DR2 element of the EPO enhancer, and by supershift and competition experiments it became evident that reduced HNF-4α DNA binding contributes to this effect. Our reporter gene experiments with a plasmid containing the regulatory elements of the EPO enhancer 3' of the luciferase gene confirmed the EMSA results in HepG2 cells. The final proof that HNF-4α is responsible for decreased DNA binding to the EPO enhancer and subsequent reduced transcription comes from cotransfection studies with U2OS cells, which do not express HNF-4α. If an HNF-4α expression plasmid is cotransfected into U2OS cells, these cells gain full hypoxic inducibility of the reporter luciferase and additionally IL-1β susceptibility. When a reporter plasmid without the HIF-1 binding site was tested, HNF-4α expression alone was not able to confer hypoxic inducibility. Taken together, these results support the concept that both the DR2 element and the HIF binding site are necessary for the assembly of the transcriptionally active complex of HNF-4α, HIF-1 and the transcriptional co-activators CBP/P300 and SRC-1 [21, 23, 24]. We conclude that IL-1β down-regulates HNF-4α, most probably by promoting its proteasomal degradation and to a lesser extend by reducing the steady-state HNF-4α mRNA levels. As a consequence, HNF-4α becomes the limiting factor in the transcriptionally active complex of HNF-4α, HIF-1, cBP/P300 and SRC-1, which results in reduced EPO expression. Increasing HNF-4α expression and function, or prevention of its degradation, may be an alternative approach to fight ACD.

Acknowledgements. We are indebted to Dr. Peter Ratcliffe for providing U2OS cells and Dr. Ming-Jer Tsai for the gift of the COUP-TF antiserum. We gratefully acknowledge the excellent technical assistance by Ms. Gabriele Huck. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 367-C8 and GRK 288) and the Mongolian Academy of Sciences.

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