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# Hypoxia-Induced Erythropoietin Expression in Human Neuroblastoma Requires a Methylation Free HIF-1 Binding Site

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**Abstract** The glycoprotein hormone Erythropoietin (EPO) stimulates red cell production and maturation. EPO is produced by the kidneys and the fetal liver in response to hypoxia (HOX). Recently, EPO expression has also been observed in the central nervous system where it may be neuroprotective. It remained unclear, however, whether EPO is expressed in the peripheral nervous system and, if so, whether a neuronal phenotype is required for its regulation. Herein, we report that EPO expression was induced by HOX and a HOX mimetic in two cell lines derived from neuroblastoma (NB), a tumor of the peripheral nervous system. Both cell lines with inducible EPO expression, SH-SY5Y and Kelly cells, expressed typical neuronal markers like neuropeptide Y (NPY), growth-associated protein-43 (GAP-43), and neuron-specific enolase (ENO). NB cells with a more epithelial phenotype like SH-SHEP and LAN-5 did not show HOX inducible *EPO* gene regulation. Still, oxygen sensing and up-regulation of hypoxia-inducible factor-1 (HIF-1) were intact in all cell lines. We found that CpG methylation of the HIF binding site (HBS) in the *EPO* gene 3′ enhancer was only present in the SH-SHEP and LAN-5 cells but not in SH-SY5Y and Kelly cells with regulated EPO expression. The addition of recombinant EPO to all NB cells, both under normoxic and hypoxic conditions, had no effect on cell proliferation. We conclude that the ability to respond to HOX with an increase in EPO expression in human NB may depend on CpG methylation and the differentiation status of these embryonic tumor cells but does not affect the proliferative characteristics of the cells. J. Cell. Biochem. 93: 153–161, 2004. 

**Key words:** erythropoietin; neuroblastoma; Hypoxia-inducible factor-1; oxygen sensing; tissue specific gene expression

The glycoprotein hormone Erythropoietin (EPO) is the main stimulator of erythropoiesis and is produced in the kidneys and the fetal liver in response to hypoxia (HOX) [Jelkmann, 1992; Dame et al., 1998; Ebert and Bunn, 1999]. The effects of EPO are mediated through its receptor, EPOR [Youssoufian et al., 1993]. Expression of EPO and EPOR is not restricted to the erythroid lineage, but is also found in hematopoietic stem cells, endothelial cells, smooth and skeletal muscle cells, and cells of neuronal origin [Komatsu et al., 1989; Anagnostou et al., 1990; Masuda et al., 1993; Carlini et al., 1999]. In neuronal cells, EPO provides protection from oxidative stress in culture and protects neurons from ischemic damage in vivo [Morishita et al., 1997]. EPO is expressed in different regions of the developing fetal brain and can be measured in the liquor of newborns [Juul et al., 1998; Dame et al., 2000].

HOX induced EPO expression is controlled by the hypoxia-inducible factor-1 (HIF-1), which is a heterodimer of the oxygen-regulated subunit HIF-1α and the constitutive HIF-1β subunit [Wenger, 2002]. Under normoxic conditions HIF-1α is targeted for ubiquitin-dependent

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protein degradation by hydroxylation of proline residues 402 and 564 by prolyl-hydroxylases in an oxygen-dependent manner [Epstein et al., 2001]. Hydroxy-proline-HIF-1α binds to the product of the von Hippel–Lindau tumor suppressor gene (*pVHL*), which recruits an E3 ubiquitin-protein ligase complex [Maxwell et al., 1999]. Under HOX, proteasomal protein degradation ceases due to the lack of proline hydroxylation, HIF-1α accumulates and is translocated into the nucleus [Wenger, 2002]. A HIF-1 complex is formed by dimerization of stabilized HIF-1α and HIF-1β that binds to the HIF-1 DNA-binding site (HBS) within the HOX response element (HRE) in the 3′-flanking EPO enhancer [Ebert and Bunn, 1999]. In addition, CpG methylation has been found to determine whether cells express the *EPO* gene or not [Wenger et al., 1998]. For different hepatoma cells lines a clear correlation between EPO protein expression and the degree of EPO 3′ HBS methylation was found which indicated that methylation of the HBS would prevent activation of the EPO enhancer and thus expression of the *EPO* gene [Wenger et al., 1998].

Neuroblastoma (NB) is the most frequent solid malignancy of childhood and affects primarily infants [Berthold, 1990]. NB develops from immature cells derived from the neural crest. Some tumors show signs of neuronal differentiation characterized by the histological grading of Hughes et al. [1974]. The biological and clinical behavior of NB is heterogeneous, ranging from spontaneous differentiation or complete regression without prior therapeutic intervention to rapid and fatal progression in spite of aggressive therapy. The determinants for this change in phenotype have remained essentially unknown. Because HOX and necrosis are characteristic findings in aggressive tumors like human NB that can "outgrow" their blood supply [Nordsmark et al., 1994] we were interested if human NB cell lines of different phenotype express EPO in an oxygen dependent manner.

## MATERIALS AND METHODS

### Cell Culture

The human NB cell lines were obtained from Dr. M. Schwab (German Cancer Research Center, Heidelberg, Germany) as previously reported [Rössler et al., 1999]. LAN-5 and Kelly harbor MYCN amplification, SH-SY-5Y and SH-SHEP are neuroblastic- and epithelial-like clones of the parental NB cell line SK-N-SH [Ross et al., 1983]. This parental cell line was established from a bone marrow aspirate of a 4-year-old girl with a highly malignant tumor [Biedler et al., 1973]. SK-N-LO and SK-N-MC cells were obtained from Dr. A. Voigt (Department of Pediatrics, University of Jena, Germany). Cells were grown in RPMI-1640 medium (Bio Whittaker, Cambrex Company, Verviers, Belgium) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% $CO_2$ in air.

HOX was defined as incubation with 3% $O_2$, 5% $CO_2$, and 92% $N_2$ in a humidified atmosphere. Control normoxic cells were placed in an incubator (5% $CO_2$, 21% $O_2$, and 74% $N_2$) for the same period of time. Ciclopirox (Sigma, Buchs, Switzerland) as a chemical inducer of HIF-1 [Wanner et al., 2000] was used at a final concentration of 20 μM (CX).

### Proliferation Assays

For cell proliferation experiments, cells seeded onto 12-well plates (for Coulter Counter measurements) or 96-well plates (for MTT-(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay) were switched to RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) containing serum-free supplement (Sigma, St. Louis, MO) at time of treatment. Recombinant EPO (Boehringer, Mannheim, Germany) in different concentrations was added to the medium. After different time intervals, cells were counted by a Coulter particle counter (Hialeah, FL) or cell viability was determined by measuring reduced MTT absorbance after its extraction.

### Real Time RT-PCR

RNA was isolated using a Quiagen RNA isolation kit (Valencia, CA) and cDNA was synthesized from 1 μg of total RNA as described previously [Stolze et al., 2002]. The 10 pmol of both forward and reverse primers were used for each PCR reaction. The primer sequences were as following: EPO sense: 5′ TCT GGG AGC CCA GAA GGA AGC CAT, EPO reverse: 3′ CTG GAG TGT CCA TGG GAC AG, EPOR sense: 5′ AGT GAG CAT GCC CAG GAT ACC TAT, EPOR reverse: 3′ CTA AGA GCA AGC CAC ATA GCT GGG, NPY sense: 5′ GCG CGC CAG CCA CCA TGC TAG G, NPY reverse: 3′ GGC TGC ATG

CAT TGG TAG G, GAP-43 sense: 5′ GTC AGG TCT CCA TTG AGG, GAP-43 reverse: 3′CTG GCC TGT GCA CAG CCC CAC, ENO sense: 5′ GTC AGG TCT CCA TTG AGG, ENO reverse: 3′CTG GCC TGT GCA CAG CCC CAC, β-actin sense: 5′ CGG GAA ATC GTG CGT GAC AT, β-actin reverse: 3′ GAA CTT TGG GGG ATG CTC GC. The PCR with the temperature profile "94°C for 1 min, 60°C for 1 min, and 72°C for 2 min" was run with the EPO and EPOR primers for 35 cycles, with NPY, GAP-43, and ENO primers for 35 cycles and with β-actin primers for 27 cycles. The PCR products were then run on a 2% agarose gel. β-actin was used as a control to ensure equal amount of cDNA in each PCR. For EPO quantification forward primer 5′ CTC CGA ACA ATC ACT GCT 3′ and reverse primer 5′ GGT CAT CTG TCC CCT GTC T 3′ were used in a two step real-time PCR with a denaturation step at 95°C for 10 min and then 40 cycles of 95°C 15 s and 60°C 1 min (SYBR-Green, GeneAmp 5700 Sequence Detection System, Applied Biosystem, Weiterstadt, Germany).

### DNA Preparation, Digestion, and PCR for CpG Status Analysis

Genomic DNA was isolated form the NB cell lines with the genomic DNA isolation Kit (MBI Fermentas) and digested with the restriction enzymes PstI and TaiI according to the manufacturer's instructions (MBI Fermentas). TaiI does not cut DNA if the cytosine residue at the restriction site is methylated. Thereafter, PCR was performed of the digestion products (94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) with primers spanning the PstI-PstI and the TaiI-PstI fragment of the enhancer with primers covering the respective restriction sites of the human EPO enhancer [Wenger et al., 1998]. Amplified DNA was separated on a 2% agarose gel. Two products of 849 bp (PstI-PstI; only found if TaiI had not cut at the internal and thus methylated TaiI site) or of 707 bp (TaiI-PstI; found in all digested samples, serves as a control that intact DNA was in the sample) were detected.

### Western Blotting

Cellular proteins were prepared from 60 mm dishes of sub confluent cells. Cells were washed with ice cold PBS, drained, then lysed on the plates with 100 μl extract buffer (300 mM NaCl, 10 mM Tris pH 7.9, 1 mM EDTA, 0.1% NP-40, 1× Protease Inhibitor Cocktail, Roche, Basel, Switzerland) for 20 min on ice. The extract was spun down in a microfuge (5,000 rpm, 4°C, 5 min) and the protein concentration was measured using the Bio-RAD Laboratories protein assay reagent. Western analysis were performed according to standard procedures as recently described [Stolze et al., 2002] using the primary monoclonal anti-HIF-1α antibody (Transduction Laboratories, San Diego, CA, diluted 1:250) and anti-α-tubulin antibody (diluted 1:500, Santa Cruz Biotechnology, Heidelberg, Germany) to detect the respective protein as loading control. Immunoreactive proteins were visualized using ECL detection and X-ray films.

### ELISA

Serum free medium was added to NB cell lines of equal cell number for 24 h. The supernatant was centrifuged and EPO was quantified by a commercial ELISA kit (R&D Systems, Minneapolis, MN).

### Statistics

For the data in Figure 2 a paired t-test with a two-tail *P*-value was calculated. For the data in Figure 3 a one-way analysis of variance (ANOVA) was performed followed by Dunnett's post test. A two-tail *P*-value was calculated.

## RESULTS

Qualitative PCR for EPO and EPOR mRNA revealed that all four NB cell lines examined expressed EPOR mRNA while significant EPO expression was only found in SH-SY5Y and Kelly cells (Fig. 1). Expression of the EPO gene was almost undetectable in SH-SHEP and LAN-5 cells. In an attempt to further characterize the EPO mRNA expressing cells we determined the expression of typical neuronal markers like neuropeptide Y (NPY), growth-associated protein-43 (GAP-43), and neuron-specific enolase (ENO). It was evident that EPO was preferentially expressed in cells of the neuronal phenotype, particularly those with NPY and higher GAP-43 and ENO expression (Fig. 1).

EPO mRNA is strongly up-regulated 5–15 fold as quantified by real-time RT-PCR in SH-SY5Y and Kelly cells but not in the NB cells with a more epithelial phenotype like SH-SHEP and LAN-5 (Fig. 2A). Measurement of EPO protein secreted into the culture supernatant confirmed

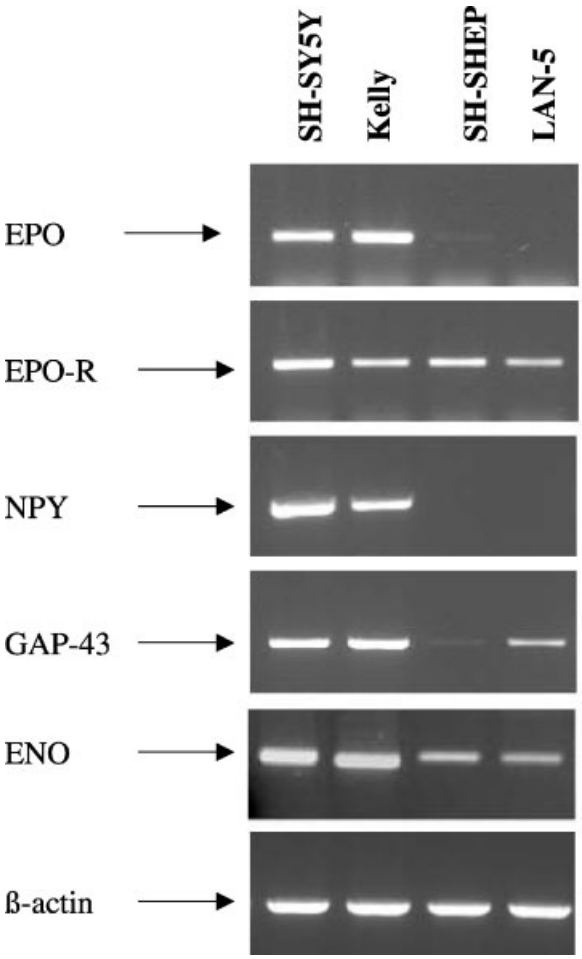


**Fig. 1.** RT-PCR of mRNA for Erythropoietin (EPO), EPOR, neuropeptide Y (NPY), growth-associated protein-43 (GAP-43), neuron-specific enolase (ENO), and β-actin under hypoxic conditions for 24 h in neuroblastoma (NB) cell lines with neuronal (SH-SY5Y, Kelly) and more epithelial (SH-SHEP, LAN-5) phenotype.

that only the NB cells of neuronal phenotype produce detectable amounts of EPO after hypoxic incubation. EPO protein concentration was $2 \pm 2$ mU/ml in supernatant from SH-SY5Y cells and $10 \pm 3$ mU/ml in Kelly cells, respectively but no EPO protein was detected in the supernatant of the more epithelial-like NB cells SH-SHEP and LAN-5 after hypoxic incubation (Fig. 2B). The hepatoma cell line Hep-G2 was used as a control for HOX induced up-regulation of EPO.

For further comparison of EPO production in NB cells of the two phenotypes we used the more neuronal (SH-SY5Y) and the more epithelial (SH-SHEP) differentiated cell line that both originate from the same tumor [Ross et al., 1983]. In addition to HOX we used the chemical HOX mimetic and potential iron chelator Ciclopirox to induce EPO expression [Wanner et al., 2000]. EPO mRNA expression was inducible only in SH-SY5Y cells after 16 h of HOX or the addition of Ciclopirox (Fig. 3A). Likewise, EPO mRNA expression was induced by HOX or Ciclopirox in Kelly cells but not in LAN-5 cells and EPO protein was not found in the supernatant of SH-SHEP and LAN-5 after hypoxic incubation or the addition of Ciclopirox (data not shown).

To test whether the NB cell lines not capable of increasing EPO expression had a general defect in their oxygen sensing capability we analyzed accumulation of HIF-1α after HOX or Ciclopirox treatment. All four NB cell lines accumulated HIF-1α protein although to different degrees (Fig. 3B). In fact, if related to tubulin levels SH-SHEP cells appeared to have a stronger inducibility of HIF-1α compared to SH-SY5Y indicating that the lack of EPO mRNA induction is not due to a general defect in oxygen sensing in SH-SHEP cells.

In previous studies it had been shown that oxygen-regulated *EPO* gene expression is dependent on a methylation free CpG island in the HBS of the 3′ EPO enhancer [Wenger et al., 1998]. If HBS is methylated, HIF-1 cannot bind to the enhancer and as a consequence is not able to up-regulate EPO expression. To test if HBS methylation is responsible for defective EPO regulation in epithelial-like SH-SHEP and LAN-5 cells, DNA from different NB cells was digested with PstI and TaiI and resulting fragments were detected by PCR. Two products of 849 bp (PstI-PstI; only found if TaiI had not cut at the internal and thus methylated TaiI site) or of 707 bp (TaiI-PstI; found in all digested samples, serves as a control how much intact DNA was in the sample) were detected. In Figure 4 only SH-SY5Y and Kelly cells show almost no PstI-PstI amplified fragments despite strong signals for PstI-TaiI indicating that the TaiI site was not methylated and thus DNA cut at the TaiI site. In contrast, all other non-EPO producing cells showed equal intensity for the PstI-PstI and the PstI-TaiI fragments. Thus, CpG methylation within the HBS in the more epithelial-like NB cell lines could account for the lack of EPO up-regulation by HOX. The more epithelial-like NB cell line, SK-N-LO and the cell line SK-N-MC that both do not produce

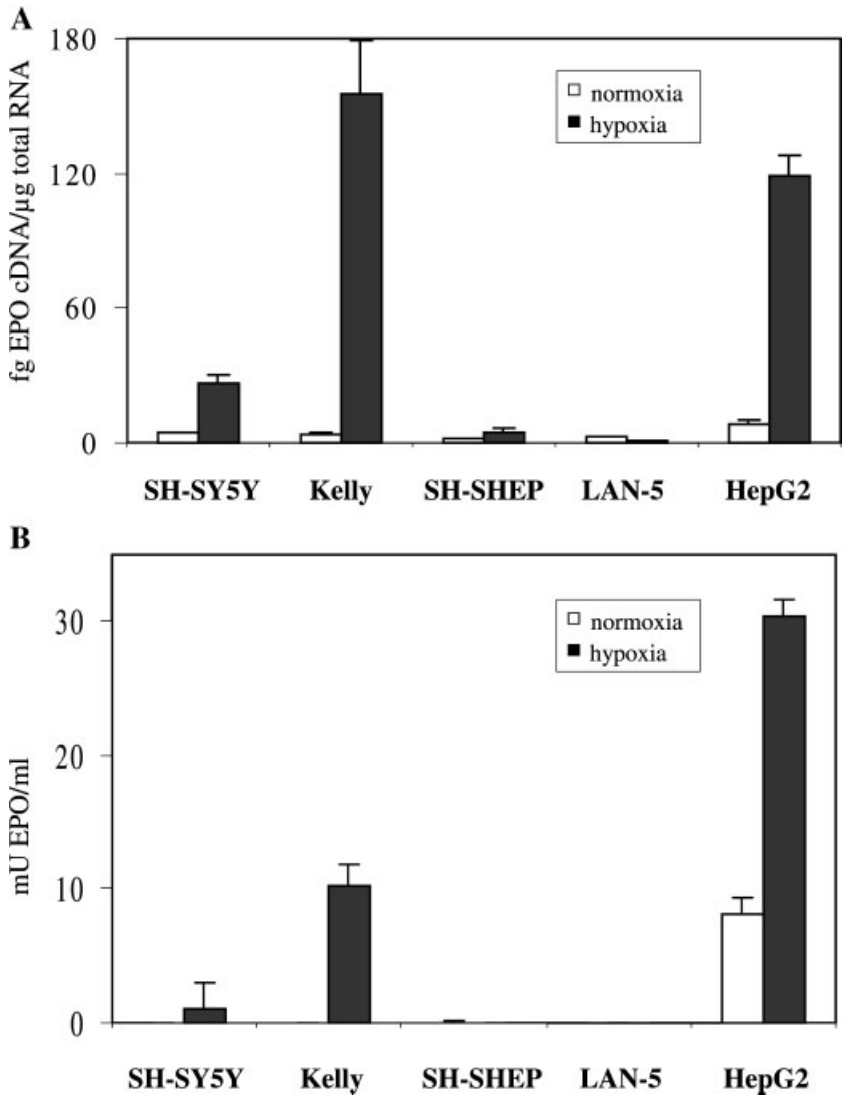


**Fig. 2.** **A**: Quantitation of EPO mRNA cultured 24 h under normoxia □ and hypoxia (HOX) ■ by real-time RT-PCR analysis. Results are expressed in femtogram (fg = $10^{-15}$ g) EPO cDNA per µg total RNA. Data are the means ± SD of four separate experiments in which each cDNA level was measured in duplicate. **B**: Measurement of EPO in the supernatant of NB cell lines cultured 24 h under normoxia □ and HOX ■ by ELISA.

HOX inducible EPO (data not shown) were included since they have been demonstrated to show CpG methylation in the EPO enhancer [Wenger et al., 1998].

Finally, we tested whether EPO might have any influence on the proliferation of NB cells. When recombinant EPO (10 U/ml) was added to SH-SY5Y and SH-SHEP cells for 0, 24, 48, and 72 h no effect on proliferation was observed (Fig. 5). Compared to the untreated control cells with respect to the cell number as well as cell viability determined by the MTT assay, EPO did neither promote nor inhibit proliferation. The same results were obtained with all other NB cell lines available with different concentrations of EPO (10, 5, 2.5 and 1.25 U/ml and mU/ml). MTT assay results showed no difference in the number of viable cells when treated with or without EPO, respectively (data not shown).

## DISCUSSION

The importance of EPO and EPOR expression in the nervous system has not been yet sufficiently clarified but EPO seems to play a role as a factor for differentiation and neuroprotection [Morishita et al., 1997]. EPO has been reported to be without influence on proliferation [Berdel

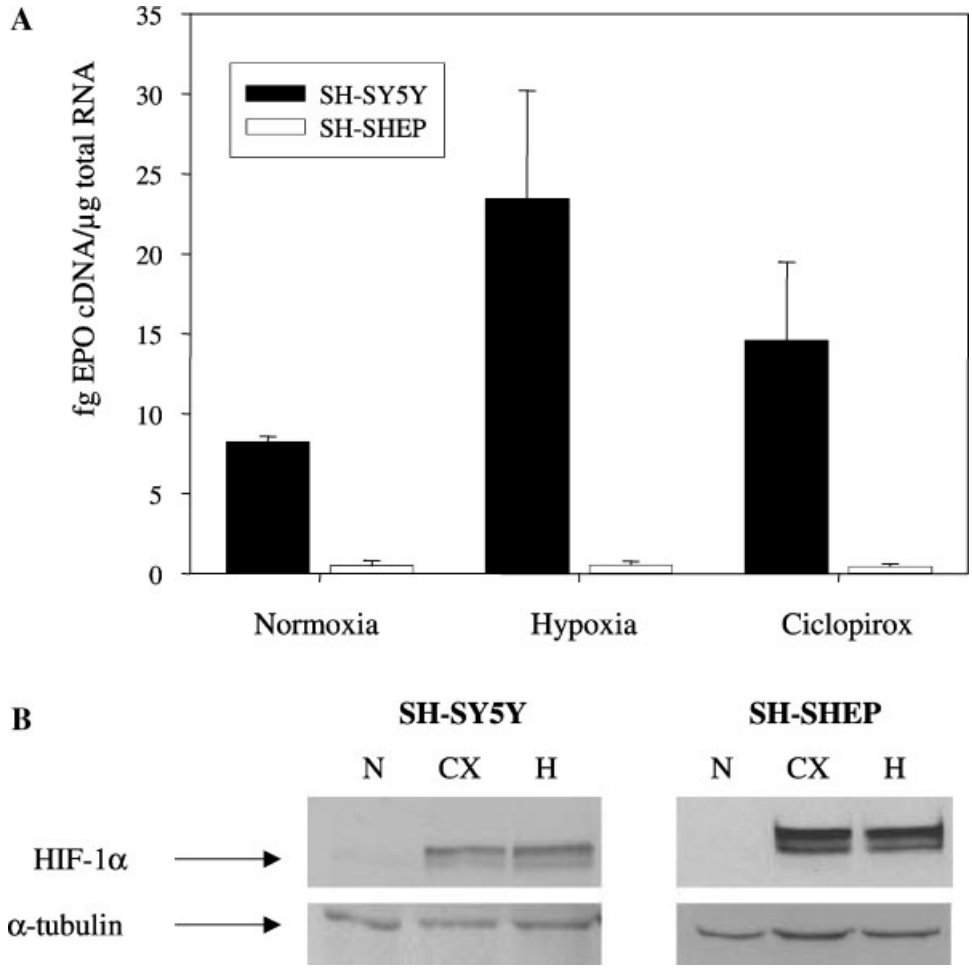


**Fig. 3.** **A**: EPO mRNA expression in SH-SY5Y cells (neuronal phenotype) ■ and SH-SHEP cells (more epithelial phenotype) □ after exposure to normoxia, HOX, or the HOX mimetic Ciclopirox for 6 h. EPO mRNA was quantitated by real time RT-PCR. Data are the means ± SD of four separate experiments in which each cDNA level was measured in duplicate. **B**: Westernblot for hypoxia-inducible factor-1α (HIF-1α) from extracts of SH-SY5Y and SH-SHEP cells exposed to normoxia (N), HOX (H), or the HOX mimetic Ciclopirox (CX) for 6 h. α-tubulin was used as a loading control. Blots are representative for at least three separate experiments.

et al., 1991] but has also been claimed to be mitogenic for tumor cells [Westenfelder and Baranowski, 2000; Acs et al., 2001]. To resolve the question whether EPO stimulates tumor growth is of considerable interest since EPO treatment is about to become a standard therapeutic to fight the anemia in cancer patients undergoing chemotherapy.

NB is an embryonic tumor where the malignant cells develop from immature neuronal cells [Berthold, 1990]. Here, we show that preferentially neuronal NB cell lines express EPO and EPOR whereas a more epithelial phenotype predisposes to EPOR expression but not HOX inducible EPO synthesis. Therefore, both types of cell lines provide cellular models for in vitro studies and two aspects of non-hematological expression of EPO and EPOR can be studied: the expression of EPO in primitive neuronal cells and the potential effect of HOX-induced production of this growth factor on neuronal as well as malignant cells.

EPO is the paradigm of a HOX inducible gene [Jelkmann, 1992; Ebert and Bunn, 1999]. HOX is a critical event in aggressive human cancers and tumor cells, which change their repertoire of expressed genes when challenged with low levels of oxygen [Wenger, 2002]. It was, therefore, of considerable interest to study EPO expression in detail. Interestingly, significant expression and up-regulation of EPO mRNA by HOX was restricted to those cell lines that had neuronal characteristics, namely expressed the mRNAs for NPY, GAP-43, and ENO (Fig. 1B). In

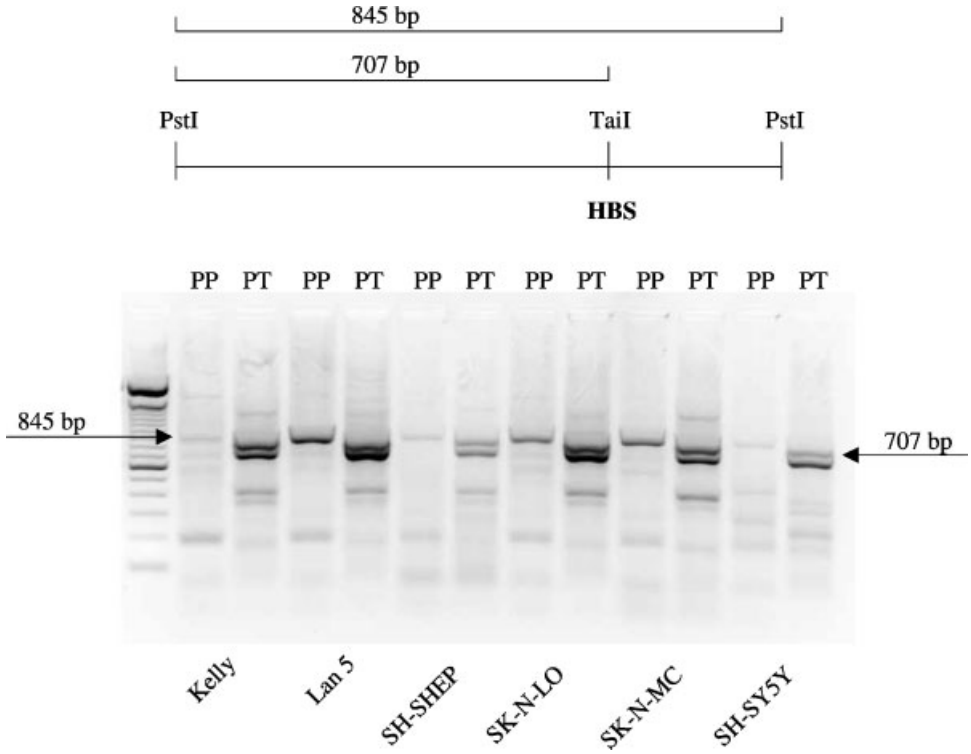


**Fig. 4.** CpG methylation state of the HIF-1 binding site (HBS) in the human EPO 3′ enhancer. After digestion of genomic DNA with Pst I and TaiI DNA fragments were amplified by PCR with primers specific for the PstI-PstI (lanes PP) or the PstI-TaiI (lanes PT) fragment. Methylation of the HBS site prevents cutting by TaiI and thus results in the larger fragment (849 bp). Amplification of the PstI-TaiI fragment (707 bp) is independent from digestion by TaiI and provides information about the amount of DNA available for PCR after the double digest with PstI and TaiI. Equal amounts of DNA from Kelly, LAN-5, SH-SHEP, SK-N-LO, SK-N-MC, and SH-SY5Y were used. PCR products were run on a 2% agarose gel stained with ethidium bromide and photographed under UV light. For better visibility the gel photograph was inverted.

particular, the two cell lines SH-SY5Y and SH-SHEP that originate from the same tumor showed this different character (Fig. 2). Moreover, analysis of the key component in the oxygen sensing pathway, HIF-1α, clearly revealed no defect in oxygen sensing (Fig. 3). Therefore, a more *EPO* gene specific mechanism of regulation should account for the lack of expression of the *EPO* gene. It had been reported before that CpG methylation within the HBS of the *EPO*

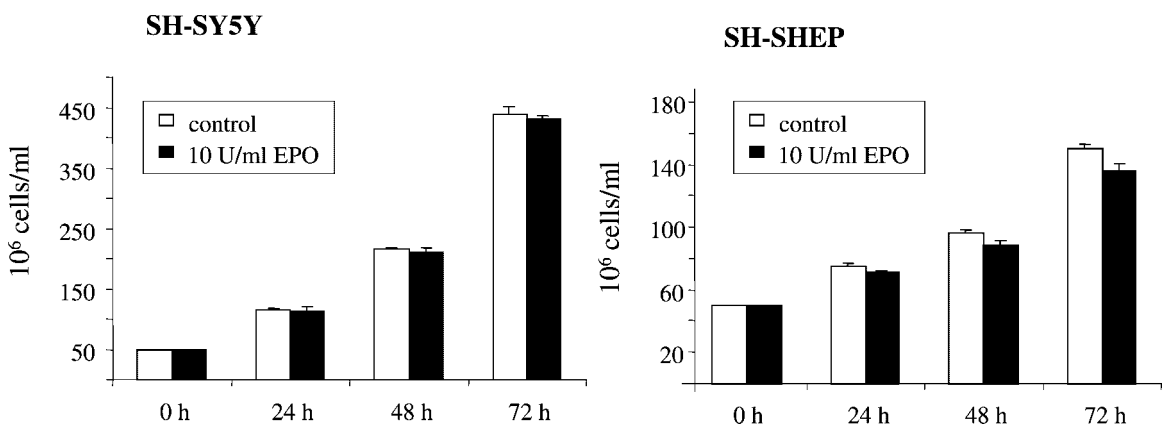


**Fig. 5.** Proliferation of SH-SY5Y and SH-SHEP cells in medium containing serum-free supplement (control) and addition of 10 U/ml EPO. Cell number was determined by a Coulter particle counter after 24, 48, and 72 h. The results are the means ± SD of three separate experiments.

gene is inversely correlated with the degree of EPO expression in different hepatoma cell lines [Wenger et al., 1998]. Our data indicate that the degree of CpG methylation may also play a role in the oxygen-regulated expression on the *EPO* gene. Interestingly, CpG methylation within the EPO enhancer was predominantly found in the epithelial-like NB cells, whereas NB cells of neuronal phenotype did not show methylation. The reason for this difference is not yet clear and may or may not depend on the differentiation of the NB cells. It could however explain why regulated EPO expression is found in the more neuronal like phenotype of NB cells.

HOX inducibility of EPO in neuronal cells is of particular interest. First, these NB cells provide an excellent model for cells that gain hypoxic regulation of a gene by differentiation, which is worth to be studied in more detail in the future. Second, this difference might help to distinguish between neuronal differentiated cells and cells that are more epithelial- or Schwann-cell like or that have not started differentiation yet. The EPO expression status could, therefore, give valuable information with respect to the potential of neuronal differentiation. Since more differentiated tumor cells are not as aggressive as immature cells, knowledge of the EPO expression status could provide additional information regarding the prognosis of the disease. Moreover, EPO expression shows next to the hypoxic regulation strict tissue specificity [Dame et al., 1998]. So far EPO expression has been found in astrocytes in vitro and in vivo but no cell lines are yet available that are of neuronal phenotype and show HOX inducible regulation. NB cell lines may therefore provide an important tool to study tissue specific regulation in comparison with the two available hepatoma cell lines that are so far the only in vitro models for regulated EPO expression [Ebert and Bunn, 1999]. First evidence that considerable differences exist between HOX-induced EPO expression in NB and hepatoma cells is provided by the fact that despite the absence of hepatic nuclear factor-4α (HNF-4α) NB cells express the *EPO* gene [Stolze et al., 2002]. In hepatoma cells HNF-4α had been found to be absolutely required for $O_2$-dependent EPO expression [Ebert and Bunn, 1999].

In previous studies we found that the angiogenesis stimulator VEGF is up-regulated by HOX in a time-dependent matter [Rössler et al., 1999]. Furthermore, the induction of growth arrest or apoptosis was positively correlated with HOX in NB cells transfected with a N-*myc*. Interestingly, in NB tumors overexpression of the oncogene N-*myc* is associated with a poor clinical prognosis in NB although it promotes apoptosis under HOX in NB cells [Rössler et al., 2001]. Herein, we found no correlation, positive or negative, of EPO expression and N-*myc* status of the cells. Since we found EPO and EPOR expression in NB cell lines we considered an—potentially autocrine—effect of EPO on NB cells. So far, EPOR expression was described in two NB cell lines, NMB and SK-N-SH. While the addition of exogenous EPO showed an inhibition of growth and differentiation in NMB cells [Wollman et al., 1996], EPO caused an increase in intracellular $Ca^{2+}$ level in the SK-N-SH cells [Assandri et al., 1999]. In a third report no significant stimulation of clonal growth in one NB cell line was observed [Berdel et al., 1991]. Campana et al. [1998] reported sprouting and signaling by EPO in murine NS20Y and human SK-N-MC cells which was mimicked by a peptide identical to the 17-mer part of the EPO protein. Moreover, EPO has been claimed to stimulate tyrosine phosphorylation and proliferation of breast cancer cells [Acs et al., 2001] and renal carcinoma cells [Westenfelder and Baranowski, 2000]. Our data are in contrast with these reports: exogenously added EPO showed no significant effect on proliferation of the NB cell lines as examined by cell counting and MTT assay.

In summary, we found that EPO and EPOR are expressed by human NB cell lines of different phenotype although we could not yet assign a function of EPO in these cells. Up-regulation of EPO expression under HOX was restricted to NB cells of neuronal phenotype despite an undisturbed oxygen sensing and HIF-1α accumulation in more epithelial NB cells. The reason for the different capability for HOX regulated EPO expression appears to depend on the methylation status of CpGs in the 3′ enhancer of the *EPO* gene. It remains to be studied whether EPO plays a role for neuronal differentiation of these embryonic tumor cells and whether EPO expression could be used as a prognostic factor in NB.

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