Recruitment of HIF-1α and HIF-2α to common target genes is differentially regulated in neuroblastoma: HIF-2α promotes an aggressive phenotype

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Summary

In neuroblastoma specimens, HIF-2α but not HIF-1α is strongly expressed in well-vascularized areas. In vitro, HIF-2α protein was stabilized at 5% O2 (resembling end capillary oxygen conditions) and, in contrast to the low HIF-1α activity at this oxygen level, actively transcribed genes like VEGF. Under hypoxia (1% O2), HIF-1α was transiently stabilized and primarily mediated acute responses, whereas HIF-2α protein gradually accumulated and governed prolonged hypoxic gene activation. Knock-down of HIF-2α reduced growth of neuroblastoma tumors in athymic mice. Furthermore, high HIF-2α protein levels were correlated with advanced clinical stage and high VEGF expression and predicted poor prognosis in a clinical neuroblastoma material. Our results demonstrate the relevance of HIF-2α in neuroblastoma progression and have general tumor biological implications.

Introduction

The oxygen pressure within solid tumors is heterogeneous, ranging from approximately 5% O2 in well-vascularized regions to anoxia near necrotic regions, but is on average in the hypoxic range (about 1% O2) (Brown and Wilson, 2004; Goda et al., 1997; Höckel and Vaupel, 2001). In response to hypoxia, tumor cells adapt by changing the transcription of genes involved in angiogenesis, cell survival, and metabolism. The hypoxia-inducible transcription factors HIF-1α and HIF-2α are critical for this adaptive response (Harris, 2002; Semenza, 2003). At hypoxia the α subunits are stabilized, heterodimerize with the constitutively present partner HIF-1β/ARNT, and together with coactivators such as CBP/p300 regulate genes via specific hypoxia response elements (HREs) (Semenza, 2003). In the presence of oxygen, prolyl hydroxylases (PHDs) modify the HIF-α proteins at two conserved prolines, resulting in HIF interaction with the von Hippel-Lindau (VHL)-E3 ligase protein complex, targeting HIFs for ubiquitylation and subsequent proteasomal degradation (Epstein et al., 2001; Huang et al., 1998; Kallio et al., 1999). HIF transcriptional activity is further regulated by an oxygen-dependent asparagyl hydroxylase, FIH-1 (factor inhibiting HIF) leading to reduced interaction with CBP/p300 coactivators (Lando et al., 2002; Mahon et al., 2001). In addition to hypoxia, HIF-1α protein synthesis, stability, and activity can also be regulated by other mechanisms, for instance, in response to growth factor-induced signaling (Semenza, 2003).

There are several known and well-characterized HIF-1α target genes (Semenza, 2003; Wenger et al., 2005), whereas no exclusive HIF-2α target gene has yet been identified, with the possible exception of the recently reported Oct-4 (Covello et al., 2006). There is a redundancy regarding HIF targets (Sowter et al.,...
a role in normal SNS development has been proposed (Tian et al., 1998), whereas HIF-2α protein is preferentially targeted by HIF-1α and HIF-2α, as opposed to HIF-1α and HIF-2α protein in well-vascularized tumor areas. The childhood tumor neuroblastoma is derived from SNS precursor cells or immature SNS neuroblasts (Hoehner et al., 1996). We have previously shown that neuroblastoma cells respond to low oxygen by induction of a hypoxic phenotype and that hypoxic neuroblastoma cells become differentiated and gain SNS stem cell characteristics (Jögi et al., 2002, 2004), features that in the clinical setting are associated with increased aggressiveness and adverse patient outcome (Wei et al., 2004). Here we report that neuroblastoma specimens frequently have strong expression of HIF-2α protein in well-vascularized tumor areas. In vitro, HIF-2α, as opposed to HIF-1α protein, is strongly induced at physiological oxygen concentrations (5% O2). Furthermore, under chronic hypoxia (1% O2), HIF-1α protein is degraded over time, whereas HIF-2α is continuously accumulated. Global gene expression analysis revealed genes closely following the HIF stabilization patterns at 1% and 5% O2. HIF subcellular localization and genomic DNA binding, together with specific HIF knockdown, indicated a temporally regulated mode of HIF utilization during cellular adaptation to hypoxia, where HIF-1α primarily mediates fast (acute) and HIF-2α mediates late (chronic) responses to hypoxia. In addition, expression of known hypoxia-driven genes were induced in a HIF-2α-dependent manner at 5% O2, indicating a role of HIF-2α in promotion of an aggressive neuroblastoma phenotype at physiological as well as chronic hypoxic oxygen levels. Accordingly, transient knockdown of HIF-2α reduced growth of xenografted neuroblastoma tumors in athymic mice. Immunohistochemical evaluation of a clinical neuroblastoma tumor material further demonstrated a significant correlation between HIF-2α and VEGF protein levels, and a strong and significant correlation between high HIF-2α protein content and unfavorable patient outcome was found. Taken together, these data clearly implicate HIF-2α as an important factor determining neuroblastoma aggressiveness, and HIF-2α could therefore represent an important target in neuroblastoma therapy.

**Results**

**HIF-1α and HIF-2α induction patterns differ in neuroblastoma cells**

We immunostained consecutive sections of neuroblastoma specimens for HIF proteins and the blood vessel endothelial marker CD31. We consistently (18/20 tumors) found weak to strong HIF-2α immunoreactivity in tumor cells adjacent to blood vessels (Figures 1A–1B), while HIF-1α staining in vascularized areas was negative (data not shown). Importantly, the HIF-2α protein was frequently localized to nuclei, consistent with functional activity also in well-vascularized lesions. These observations prompted us to analyze in closer detail the kinetics by which HIF protein levels and functions change in cultured neuroblastoma cells at different oxygen levels. At 1% O2, HIF-1α levels were rapidly induced to peak within hours and thereafter gradually declined to low levels at 72 hr in both SK-N-BE(2)c and KCN-69n neuroblastoma cell lines (Figures 1C and 1D). Analysis of HIF-2α protein levels at 1% O2 revealed an almost opposite pattern. After an initial induction at 2 hr of hypoxia, HIF-2α protein levels increased over time and were upregulated after 72 hr in both analyzed neuroblastoma cell lines (Figures 1C and 1D). To mimic more physiological growth conditions with an oxygen pressure closer to that present in end capillaries, i.e., around 5% O2 (Goda et al., 1997), we exposed neuroblastoma cells to 5% O2 and analyzed HIF protein levels (Figures 1E and 1F). Whereas basal or induced HIF-1α protein was hardly detectable, a gradual and prominent increase over time in HIF-2α protein levels was seen in both cell lines, in agreement with the in vivo situation with high HIF-2α levels in well-oxygenated tumor areas. The steady-state levels of HIF-1α mRNA remained virtually unaffected (Figures 1G and 1H), while HIF-2α mRNA levels were upregulated at 1% and 5% O2 in both cell lines (Figures 1I and 1J).

The differential changes over time of the HIF proteins at 1% O2 and the seemingly efficient degradation of HIF-1α at 5% O2 might reflect the expression levels of HIF-regulating PHDs under these conditions. In SK-N-BE(2)c (Figures 2A–2C) and KCN-69n (data not shown) cells, no consistent induction or reduction of PHD1 mRNA levels was seen at either 21%, 5%, or 1% O2. In contrast, as expected (Marxsen et al., 2004), a robust increase in PHD2 and PHD3 mRNA at both 5% and 1% O2 was observed in both tested cell lines. PHD2 protein levels were induced at 1% O2 in both tested cell lines, whereas no or limited induction was seen at 5% O2 (Figures 2D–2G). Thus, despite the raise in PHD2 and PHD3 expression with time, HIF-2α protein increased at both 5% and 1% O2, suggesting that HIF-2α is less sensitive to PHD2- and PHD3-induced degradation than HIF-1α is in neuroblastoma cells. Alternatively, as HIF-2α mRNA levels increase at 5% and 1% O2, high de novo HIF-2α protein synthesis might counteract an increased PHD-induced degradation. Both HIF proteins were rapidly stabilized by the PHD inhibitors CoCl2 and DIP (Figure S1). After 24 hr, HIF-1α protein was still present in cultures containing inhibitors, while HIF-2α protein in CoCl2-treated cells decreased after 24 hr, in contrast to the sustained HIF-2α levels observed in DIP-treated and hypoxic cells (Figures S1A and S1B). As the HIF-2α mRNA levels increased in DIP- and CoCl2-treated cells, but to a lesser extent in CoCl2-treated cells (Figures S1C–S1F), it is possible that the high and sustained HIF-2α protein levels at hypoxia reflect the combined result of increased protein synthesis and less efficient protein degradation.
Subcellular localization and function of HIFs in neuroblastoma cells at 1% and 5% O2

To evaluate the functional activity of HIF proteins at hypoxia and physiological oxygen tensions, we initially assessed their subcellular localization at 21%, 5%, or 1% O2 in SK-N-BE(2)c cells. HIF-2α protein present at 21% O2 was mainly detected in cytosolic extracts. At 5% O2, induced HIF-2α was initially mainly cytoplasmic, but after 72 hr most protein was accumulated in the nucleus (Figures 3A and 3B). As expected, no or small amounts of HIF-1α protein was detected at 21% or 5% O2. In contrast, massive stabilization and nuclear localization of both HIF-1α and HIF-2α proteins were seen at 1% O2 (Figures 3A and 3B). After 72 hr of growth, the small amount of HIF-1α protein detectable at 1% O2 appeared to be nuclear.

Cytoplasmic and nuclear localization of HIF-1α and HIF-2α proteins in neuroblastoma cells at 1% O2 was assessed by immunohistochemistry and Western blotting. HIF-1α and HIF-2α proteins were detected in both cytoplasmic and nuclear fractions, with HIF-2α being predominantly cytoplasmic and HIF-1α being predominantly nuclear. These findings suggest that HIF-2α may play a more active role in regulating gene expression under hypoxic conditions, while HIF-1α may be more involved in anaerobic metabolism.

Subcellular localization and function of HIFs in neuroblastoma cells at 1% and 5% O2

To evaluate the functional activity of HIF proteins at hypoxia and physiological oxygen tensions, we initially assessed their subcellular localization at 21%, 5%, or 1% O2 in SK-N-BE(2)c cells. HIF-2α protein present at 21% O2 was mainly detected in cytosolic extracts. At 5% O2, induced HIF-2α was initially mainly cytoplasmic, but after 72 hr most protein was accumulated in the nucleus (Figures 3A and 3B). As expected, no or small amounts of HIF-1α protein was detected at 21% or 5% O2. In contrast, massive stabilization and nuclear localization of both HIF-1α and HIF-2α proteins were seen at 1% O2 (Figures 3A and 3B). After 72 hr of growth, the small amount of HIF-1α protein detectable at 1% O2 appeared to be nuclear.

HIF protein data at prolonged hypoxia and at 5% O2 (Figures 1 and 3), and the fact that hypoxia-driven genes like VEGF and DEC1/BHLHB2 are still expressed during chronic hypoxia (Jögi et al., 2002; Miyazaki et al., 2002), suggest that HIF-2α rather than HIF-1α is the major transcriptional regulator of prolonged hypoxic responses and that HIF-2α is active at physiological oxygen levels. The capacities of HIF-1α and HIF-2α to bind the HREs of the VEGF and DEC1/BHLHB2 promoters at these conditions were tested by chromatin immunoprecipitation (ChIP) assays. The most robust binding of HIF-1α to the VEGF-HRE was detected after 4 hr at 1% O2, whereas interaction with HIF-2α was most pronounced after 24 hr at 5% O2 (Figure 3C).
with a similar microarray expression pattern were identified by using as a template for further microarray data analyses. Genes ining data structure regardless of factual HIF dependence, was tion at 1% and the delayed upregulation at 5% O2 (Figure 4A).

Notably, HIF-2 binding was detected primarily at 4 hr at both 1% O2. A similar HIF-binding pattern was seen when analyzing occupancy of the DEC1/BHLHB2-HRE. These observations were independently validated using Q-PCR being hypoxia driven, including SERPINB9 and TRIO, both highly implicated in tumorigenesis and cell survival (van Houdt et al., 2005; Zheng et al., 2004). The early and sustained expression pattern at 1% O2 highly resembling that of HREs, as demonstrated in cells transfected with a vector containing three copies of the EPO-HRE coupled to a luciferase reporter gene. For reference, cells were cotransfected with a CMV Renilla luciferase vector. Cells were cultured at 21, 5%, and 1% O2 for 24 hr followed by measurements of luciferase activity. Ratios between HRE and Renilla luciferase activities (HRE/CMV) were calculated and normalized to the control samples (21% O2) in each experiment. Error bars show standard deviation of triplicate experiments.

Gene induction at 1% and 5% oxygen correlates with differential regulation of HIF-α stability

As demonstrated previously (Jögi et al., 2002), and supported by data presented here, the overall response and adaptation to hypoxia are similar among neuroblastoma cell lines. A representative cell line for this response is SK-N-BE(2)c, and mRNA from these cells grown at 1%, 5%, and 21% O2 for 0–72 hr was analyzed using microarray assays. Microarray expression data of tyrosine hydroxylase (TH) showed correlation to the HIF-1α and HIF-2α protein level patterns with regard to the rapid induction at 1% and the delayed upregulation at 5% O2 (Figure 4A). These observations were independently validated using Q-PCR (Figure 4B). The TH expression pattern, representing an interesting data structure regardless of factual HIF dependence, was used as a template for further microarray data analyses. Genes with a similar microarray expression pattern were identified by calculating the projection length for each gene vector in the 1% O2 series (n = 12,407; genes with valid expression data for all seven time points) onto the TH log2 expression vector (TH 1% O2) (Figure S2A). Low absolute-valued projection lengths, centering on zero, were found to show no specific correlation to the TH vector. High projection lengths correlated well to the expression pattern of TH 1% O2, whereas the most extreme negative projection lengths were anticorrelated to TH 1% O2. By permuting the sample labels 1000 times and assaying projection length distribution in comparison to that of the observed data, a statistically significant projection length cut-off level was defined (Figure S2B). Genes having a projection length larger than 4.1 (p = 0.040) were considered significantly induced. These calculations gave a data set of 75 array reporters representing 64 known genes and five ESTs (Figure 5) with an expression pattern at 1% O2 highly resembling that of TH (cf. Figures 4 and 5). Several of the identified genes have been demonstrated to be hypoxia responsive (Hu et al., 2003; Jögi et al., 2004; Semenza, 2003; Wenger et al., 2005). Using the TFASTA sequence comparison program, two ESTs were identified as the human endogenous retroviral genes ERV3 (EN1_HUMAN, E = 1.1 × 10^-25) and ENT1 (ENT1_HUMAN, E = 3.2 × 10^-74). Some of the 64 genes have previously not been highlighted as being hypoxia driven, including TRIO and SERPINB9, both highly implicated in tumorigenesis and cell survival (van Houdt et al., 2005; Zheng et al., 2004). The early and sustained TH pattern seen at 1% O2 was not significant for any group of genes at 5% or 21% O2 (data not shown), but instead several of the 75
identified array reporters showed over time a slow increase in expression at 5% $O_2$ (Figure 5). Principal component analysis on the log2 ratio values followed by one-way ANOVA ($p < 0.001$) and Bonferroni-corrected pairwise Student’s t tests ($p < 0.001$) between the oxygen treatment groups revealed that this was a general pattern among the 75 array reporters (Figure 4C). In summary, we demonstrate a strong, rapid, and significant induction of the identified genes at 1% $O_2$. In contrast, no significant induction was observed at 21% $O_2$. However, a slow induction was detected at 5% $O_2$, very similar to that documented for $TH$ (cf. Figures 4 and 5). We confirmed this general expression pattern for a set of the 64 genes in two neuroblastoma cell lines using Q-PCR as exemplified by DEC1/BHLHB2 and NDRG1 (Figures 6A–6D).

**HIF target genes differentially utilize HIF proteins at acute and prolonged hypoxia and at 5% $O_2$**

To distinguish between HIF-1$α$- and HIF-2$α$-driven transcription, small inhibitory RNAs (siRNAs) against HIF-1$α$ and HIF-2$α$ were employed, focusing on the transcriptional activity of genes identified in our microarray analysis (Figure 5). We first confirmed a specific reduction of HIF mRNA and protein by cognate siRNA treatment at 4 and 24 hr at 1% $O_2$, and at 5% $O_2$ for 24 hr (Figures 7A, 7B, 7G, and 7H and Figure S3A). Upon acute (4 hr) hypoxic treatment, induced expression of VEGF and DEC1/BHLHB2 was downregulated by siRNA against HIF-1$α$ but not HIF-2$α$ (Figures 7C and 7E). However, at prolonged hypoxia (1% $O_2$ for 24 hr) HIF-2$α$ siRNA, in addition to HIF-1$α$ siRNA, substantially reduced VEGF and DEC1/BHLHB2 mRNA levels (Figures 7D and 7F). Taken together, our results strongly suggest that HIF-1$α$ and HIF-2$α$ act on the same genes but during different temporal windows, with HIF-1$α$ primarily activated at the acute hypoxic phase and HIF-2$α$ at later, prolonged stages of cellular adaptation to hypoxia.

The protein stabilization and subcellular localization data shown in Figures 1 and 3 in combination with the microarray analysis suggested that HIF-2$α$ could be the dominant active HIF at 5% $O_2$. To directly test this notion, the expression levels of five selected genes identified in the microarray, $TH$, VEGF, DEC1/BHLHB2, NDRG1, and SERPINB9, were investigated in cells grown at 5% $O_2$ in the absence or presence of siRNA against either HIF-1$α$ or HIF-2$α$. As shown in Figures 7I–7M, the expression of these genes was substantially reduced by HIF-2$α$ siRNA. HIF-1$α$ siRNA also reduced $TH$, DEC1/BHLHB2, and NDRG1 expression, albeit with lower efficiency than siRNA against HIF-2$α$. Interestingly, VEGF and SERPINB9 mRNA levels were virtually unaffected by HIF-1$α$ siRNA at 5% $O_2$ but were clearly reduced by HIF-2$α$ knockdown (Figures 7J and 7M). These results demonstrate that HIF-2$α$ is actively regulating hypoxia-driven genes at physiological oxygen tensions. We also found that knockdown of either HIF-1$α$ or HIF-2$α$ reduced the mRNA levels of the glycolytic enzyme PGK1, indicating that HIF-2$α$ can affect putative HIF-1$α$-specific target genes at physiological oxygen levels, at least in neuroblastoma cells (Figure 7N). However, not all genes induced at 5% $O_2$ appeared to be primarily regulated by HIF-2$α$, as exemplified by BNIP3 (Figure 7O).

**HIF-2$α$ promotes an aggressive neuroblastoma phenotype**

The specific effect of HIF-2$α$ siRNA treatment on VEGF expression at 5% $O_2$, the known proangiogenic activity of VEGF, and the demonstrated correlation between blood vessel density and aggressiveness in many tumor forms (Carmeliet, 2005) prompted us to investigate if HIF-2$α$ and VEGF were coexpressed in neuroblastoma specimens and whether HIF-2$α$ protein is associated with an unfavorable outcome. Neuroblastoma specimens were immunohistochemically stained with anti-VEGF antibodies and screened for colocalization of VEGF and HIF-2$α$ immunoreactivity in well-vascularized tumor areas, as defined by the presence of CD31-positive vascular endothelial cells. As illustrated in Figures 8A–8C, a strikingly concordant pattern of localized HIF-2$α$, VEGF, and CD31 immunoreactivity...
was frequently observed. These data suggest that HIF-2α may drive the expression of VEGF and, hence, angiogenesis and tumor growth. To assess the importance of HIF-2α on tumor growth in vivo, neuroblastoma cells transiently transfected with siRNA against HIF-2α were injected into athymic mice. The time for tumor take did not significantly differ between the HIF-2α siRNA (4.3 ± 1.4 days) and control siRNA (4.6 ± 1.2 days) groups. However, strikingly, tumor growth was significantly impaired by targeting HIF-2α (Figure 8D). In contrast, transient knockdown of HIF-1α did not significantly affect xenograft tumor growth (Figure S3B).

To further investigate the in vivo effects of HIF-2α protein expression, we analyzed 93 primary neuroblastoma tumors arranged in a tissue microarray, addressing the hypothesis of local coexpression of HIF-2α and VEGF (cf. Figures 8A and 8B). We also analyzed whether HIF-2α levels could provide prognostic information regarding disease outcome. Tissue microarray sections, immunohistochemically stained for HIF-2α and VEGF, were scored as fractions of positive cells (range) as well as according to general intensity of positive cells. HIF-2α and VEGF correlated positively irrespective of whether range (p < 0.001) or intensity (p = 0.009) was used as the immunoreactivity.
Discussion

HIF-1α has been directly or indirectly linked to the regulation of most investigated hypoxia-induced genes. The corresponding role of HIF-2α, on the other hand, is less clear (Park et al., 2003; Poellinger and Johnson, 2004; Takahashi et al., 2004). Gene elimination data indicate that HIF-2α function is required during development (Peng et al., 2000; Tian et al., 1998). In cancer, the HIF proteins are frequently coexpressed, thereby raising the questions of what their specific roles in growth and development of tumors are, and which genes are regulated by HIF-1α and by HIF-2α, respectively. Based on our data and the reported substantial redundancies in the utilization of the HIFs (Raval et al., 2005; Sowter et al., 2003; Warnecke et al., 2004), we argue that the answer lies not in which genes are transcribed by HIF-1α or HIF-2α, but rather in the conditions under which HIF-1α and HIF-2α are stabilized, transcriptionally active, and subsequently utilized by hypoxia-regulated genes. We suggest that there is a temporal shift in HIF utilization, where HIF-1α is primarily active during the acute phase of hypoxic adaptation, and HIF-2α dominates during later, more chronic phases of hypoxia (Figure 8H).

In conclusion, high levels of HIF-2α intensity correlated significantly to high clinical stage (p = 0.028). To evaluate the prognostic significance of HIF-2α protein levels, Kaplan-Meier survival analysis was performed. A clear and significant difference in survival (p = 0.004) was seen, with high HIF-2α intensity predicting low overall survival (Figure 8F). A similar analysis for VEGF did not demonstrate a significant correlation to survival (data not shown), suggesting that HIF-2α affects tumor aggressiveness beyond the induction of VEGF expression. A further analysis of only the high-stage tumors (stages 3 and 4) revealed that HIF-2α intensity predicted survival also in this material (p = 0.043) (Figure 8G), making high HIF-2α intensity one of the downstream effectors contributing to this aggressive phenotype.
As presented here, HIF-2α, in contrast to HIF-1α protein, is highly expressed in well-vascularized and apparently nonhypoxic lesions of the SNS-derived tumor neuroblastoma. These observations were corroborated by in vitro data showing that HIF-2α is stabilized and localized in the nucleus at physiological growth conditions (5% O2) as well as at prolonged hypoxia (1% O2). HIF-1α levels, on the other hand, were low at 5% O2, especially in comparison to the levels at 1% O2. Microarray data, together with the demonstrated binding of HIF-2α to the VEGF and DEC1/BHLHB2 HREs in vivo and the selective downregulation of VEGF expression and other genes at 5% O2 by HIF-2α siRNA, directly and unequivocally showed that HIF-2α is highly involved in regulation of classic hypoxia-driven genes at physiological oxygen tensions. One of these genes, NDRG1, is a known HIF-1α target but is induced at prolonged hypoxia in HIF-1α-negative cells (Cangul, 2004), supporting our hypothesis that HIF-2α governs gene regulation at chronic hypoxia. The expression of some of the investigated genes was also reduced by HIF-1α siRNA, showing that also HIF-1α can be active at physiological oxygen tensions, but interestingly, the induction of VEGF was seemingly unaffected by siRNA against HIF-1α at 5% O2. The finding that PGK1 expression is slightly regulated by HIF-2α at 5% oxygen is interesting, as PGK1 has been shown to be a HIF-1α-driven gene in many cell systems (Covello et al., 2005; Dayan et al., 2006; Hu et al., 2003; Wang et al., 2005). Our results suggest that there are important differences in HIF usage between different cell types, although there are few studies that address the HIFs at near-physiological oxygen tensions. In support of our methodological approach, several of the genes identified by our time course microarray analysis have functional HREs (Semenza, 2003; Wenger et al., 2005) and showed slow upregulation at 5% O2, establishing a general pattern as compared to the 1% and 21% O2 series (Figure 4C). Among the genes showing this mode of regulation were DEC1/BHLHB2, NDRG1, STC1, and VEGF, all known to be induced by hypoxia and implicated in tumorigenic processes (Chakrabarti et al., 2004; Ryan et al., 1998; Wang et al., 2004; Yeung et al., 2005). In this group, we also identified several genes that have previously not been described as hypoxia responsive. Of significant interest were TRIO and SERPINB9. Amplification of TRIO in bladder neoplasms is associated with invasive and rapid growth, and when overexpressed in fetal kidney cells, TRIO increases tumorigenicity and invasiveness (Yoshizuka et al., 2004; Zheng et al., 2004). Overexpression of SERPINB9 is associated with metastatic melanoma, and expression of this gene predicts poor prognosis in anaplastic large cell lymphoma (ten Berge et al., 2002; van Houdt et al., 2005). SERPINB9 was also validated in our study as a HIF-2α-regulated gene at 5% oxygen. The human endogenous retroviral ERV3 protein has unknown functions but is normally expressed in the developing SNS (Andersson et al., 2002), adding to the list of hypoxia-regulated genes that support our previous finding that hypoxia drives neuroblastoma cells toward a neural crest-like stem cell phenotype (Jögi et al., 2002).

Hypoxia and activation of HIF-1α correlate with tumor progression and poor prognosis in several different cancers (Semenza, 2003). We show here that HIF-1α-driven genes can also be transcribed by HIF-2α at 5% O2, suggesting that HIF-2α in neuroblastoma has the potential to act oncogenically at physiological oxygen levels as well as at prolonged hypoxia. Published data indicate that HIF-2α could exert the effects of explain some diverging published data, as the differential temporal usage and oxygen sensitivity of the HIFs, so far, appear to have been largely overlooked.
an oncogene (Covello et al., 2005; Kondo et al., 2003; Raval et al., 2005). However, HIF-2α, and HIF-1α as well, has been claimed to function as a tumor suppressor protein in glioblastoma and teratoma models (Acker et al., 2005). In that report and in agreement with our findings, overexpression of HIF-2α leads to increased VEGF expression and vascularization. In disagreement with our findings and the above-mentioned published data, the net effect of HIF-2α (and HIF-1α) overexpression on xenograft tumor growth was negative, presumably due to HIF-induced apoptosis. Apparently there are tissue-specific differences in response to high HIF protein levels (Blancher et al., 2000), although the Acker et al. data await confirmation in, for example, a larger and clinically characterized glioblastoma material, especially since VEGF and HIF-1α expression have been positively correlated to glioma progression (Jensen, 2006). In summary, HIF-2α activation is undoubtedly correlated with high tumor vascularization, and a role of HIF-2α in embryonal vascularization has been suggested (Peng et al., 2000). Here we show that preferentially HIF-2α and not HIF-1α mediates transcriptional activation of VEGF at physiological oxygen tensions. Accordingly, in a clinical neuroblastoma material there was a significant correlation between high VEGF and HIF-2α protein levels. Thus, there are strong implications for a direct involvement of HIF-2α in neuroblastoma angiogenesis, and our data suggest that this process could occur independently of pronounced hypoxia.

By knocking down HIF-2α in cultured neuroblastoma cells and subsequently injecting them subcutaneously in athymic mice, early growth of the resulting tumors was reduced, demonstrating an important role of HIF-2α in neuroblastoma growth. This result and the finding that high HIF-2α protein levels strongly predict adverse outcome in clinical neuroblastoma material provide compelling support for the involvement of HIF-2α in determining an aggressive neuroblastoma behavior. Importantly, our data also suggest that scoring HIF-2α protein levels in high-stage neuroblastomas will give direct prognostic information beyond that obtained by clinical staging. In addition, we show that HIF-2α can drive expression of genes involved in tumor migration and invasion, such as SERPINE1. In the clinical setting, this could lead to HIF-2α-dependent development of highly aggressive tumor cells also in less hypoxic tumor areas, contributing to the phenotypical heterogeneity frequently seen in neuroblastoma and in other solid tumors. Differences in the kinetics by which HIF-1α and HIF-2α proteins become accumulated at hypoxia have also been observed in HeLa, lung epithelial, and breast carcinoma cells (Holmquist et al., 2005; Uchida et al., 2004; Wiesener et al., 1998), suggesting that the HIF accumulation patterns seen here and a putative oncogenic role of HIF-2α are not unique to neuroblastomas. Taken together, we present evidence in support of a role of HIF-2α in neuroblastoma angiogenesis and growth, also under nonhypoxic conditions. The demonstration of differential HIF utilization under acute versus prolonged hypoxia further suggests that any HIF-related therapeutic strategies may benefit from selectively targeting either HIF-1α or HIF-2α.

**Experimental procedures**

**Cell culture, western blot analysis, and quantitative real-time PCR**

The SK-N-BE(2)c and KON-69n human neuroblastoma cells were grown at reduced oxygen levels as described (Holmquist et al., 2005). For inhibition of PHD activity, cells were treated with 100 μM cobalt chloride (CoCl₂) or 200 μM 2,2′-dipyridyl (DIP). Western blotting and cellular fractionation were performed as described (Nilsson et al., 2005). Primary antibodies were as follows: anti-actin mAb (ICN Biomedicals), anti-HIF-1α and HIF-2α mAb (Novus Biologicals), anti-GAPDH mAb (Chemicon), anti-lamin B goat antisera (Santa Cruz), and anti-PHD2 Ab (Novus). Horseradish peroxidase-conjugated secondary antibodies and Super Signal substrate (Pierce) were used for chemiluminescence detection.

RNA extraction, cDNA synthesis, and Q-PCR reactions with SYBR Green PCR master mix (Applied Biosystems) were performed as described (Löfstedt et al., 2004). Expression levels of genes of interest were normalized to the expression of three housekeeping genes (SDHA, YWHAZ, and UBC) not affected by reduced oxygen. Primers were designed using Primer Express (Applied), and sequences are given in Table S1.

**Patient material and immunohistochemistry**

Routinely fixed paraffin-embedded human neuroblastoma specimens (ethical approval LU 389-98, Lund University, Sweden) were analyzed by HIF-1α and HIF-2α immunohistochemistry. After antigen retrieval, HIF-2α (Novus), VEGF (Santa Cruz), and CD31 (Dako) immunoreactivities were detected using the Envision system and DAKO Techmate 500. A second neuroblastoma material consisting of 93 individual cases, selected based on tissue quality and availability, from patients diagnosed in Spain between 1998 and 2004, was arranged in a tissue microarray (ethical approval no. 59CI8ABR2002) and analyzed by HIF-2α and VEGF immunohistochemistry. Median age at diagnosis was 19 months, follow-up data were available for 79 patients, and follow-up time ranged between 1 and 88 months (S.N., R.N., E.F., and S.P., unpublished data). Forty samples were MYCN amplified, 53 were 1p36 deleted, and distribution in clinical stages (INSS) was as follows: stage 1 (19), stage 2 (5), stage 3 (13), stage 4 (39), stage 4s (7), and unclassified (10). High-stage, MYCN-amplified, and 1p36-deleted tumors were slightly overrepresented compared to a population-based distribution. Established prognostic markers, e.g., clinical stage, MYCN amplification, and 1p36 deletion were all prognostically highly significant in this material (data not shown). Immunoreactivity was independently scored by two pathologists and classified according to range, i.e., fractions of positive cells (0, 0%–10%; 1, 10%–25%; 2, 26%–75%; 3, 76%–100%) and general intensity of positive cells (0, none; 1, mild; 2, moderate; 3, intense). For survival analysis and correlation to stage, groups 0 and 1 (low) and 2 and 3 (high) were pooled, respectively. All statistical calculations were performed using SPSS 12.0.1 (SPSS Inc.).

**Microarray analysis**

mRNA from SK-N-BE(2)c cells grown at 1%, 5%, or 21% O₂ were hybridized and analyzed (and HIF-1α and HIF-2α immunohistochemistry. After antigen retrieval, HIF-2α (Novus), VEGF (Santa Cruz), and CD31 (Dako) immunoreactivities were detected using the Envision system and DAKO Techmate 500. A second neuroblastoma material consisting of 93 individual cases, selected based on tissue quality and availability, from patients diagnosed in Spain between 1998 and 2004, was arranged in a tissue microarray (ethical approval no. 59CI8ABR2002) and analyzed by HIF-2α and VEGF immunohistochemistry. Median age at diagnosis was 19 months, follow-up data were available for 79 patients, and follow-up time ranged between 1 and 88 months (S.N., R.N., E.F., and S.P., unpublished data). Forty samples were MYCN amplified, 53 were 1p36 deleted, and distribution in clinical stages (INSS) was as follows: stage 1 (19), stage 2 (5), stage 3 (13), stage 4 (39), stage 4s (7), and unclassified (10). High-stage, MYCN-amplified, and 1p36-deleted tumors were slightly overrepresented compared to a population-based distribution. Established prognostic markers, e.g., clinical stage, MYCN amplification, and 1p36 deletion were all prognostically highly significant in this material (data not shown). Immunoreactivity was independently scored by two pathologists and classified according to range, i.e., fractions of positive cells (0, 0%–10%; 1, 10%–25%; 2, 26%–75%; 3, 76%–100%) and general intensity of positive cells (0, none; 1, mild; 2, moderate; 3, intense). For survival analysis and correlation to stage, groups 0 and 1 (low) and 2 and 3 (high) were pooled, respectively. All statistical calculations were performed using SPSS 12.0.1 (SPSS Inc.).

**ChIP assay**

Cells were cultured at 21% O₂ or reduced oxygen, and ChIP analyses were performed as previously described (Löfstedt et al., 2004), using antibodies against HIF-1α (Santa Cruz), HIF-2α (Novus), or IgG (Abcam). PCR primers flanking the HREs of the VEGF and DEC1/BHLHB2 promoters are given in Table S1.

**Transfection, luciferase assay, and siRNA**

Triplicates of 8 × 10⁴ SK-N-BE(2)c cells in 24-well culture plates were transfected with 300 ng of a vector containing three copies of the erythropoietin (EPO) HRE in tandem, coupled to a Luciferase reporter gene (Kallio et al., 1999; Löfstedt et al., 2004). As a control of transfection efficiency, 50 ng of a CMV Renilla Luciferase vector (Promega) was included. Transfections were performed with Lipofectamine-2000 for 6 hr in OptiMEM I Reduced Serum Medium (Invitrogen), and cells were harvested after 24 hr of culture at indicated oxygen levels. The luciferase activity was measured and calculated using the Dual-Luciferase Reporter Assay System (Promega). In a set of
Tumor growth was monitored on a daily basis, and volume was calculated by a hypoxia-inducible factor-2α (HIF-2α) or scrambled (control) siRNA. Cells were injected subcutaneously (5 × 10^5 cells/200 μL PBS/mouse) on the back of athymic mice (NMRI strain nu/nu). Six- to eight-week-old female mice weighing 20–25 g at arrival were used and housed in a controlled environment. All procedures were approved by the regional ethical committee for animal research (approval no. M66-05).

Supplemental data

The Supplemental Data Include Supplemental Experimental Procedures, three supplemental figures, and one supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/5/413/DC1/.

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References


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The microarray raw data are available in MIAME compliant format from the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) with accession number E-MEXP-836.