The HIF-2alpha-Driven Pseudo-Hypoxic Phenotype in Tumor Aggressiveness, Differentiation, and Vascularization.

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The HIF-2α-driven pseudo-hypoxic phenotype in tumor aggressiveness, differentiation and vascularization

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Abstract

Cellular adaptation to diminished tissue oxygen tensions, known as hypoxia, is largely governed by the hypoxia inducible transcription factors, HIF-1 and HIF-2. Tumor hypoxia and high HIF protein levels are frequently associated with aggressive disease. In recent years, high tumor cell levels of HIF-2 and the oxygen sensitive subunit HIF-2α have been associated with unfavorable disease and shown to be highly expressed in tumor stem/initiating cells originating from neuroblastoma and glioma, respectively. In these cells, HIF-2 is active under non-hypoxic conditions as well, creating a pseudo-hypoxic phenotype with clear influence on tumor behavior. Neuroblastoma tumor initiating cells are immature with a neural crest-like phenotype, and downregulation of HIF-2α in these cells results in neuronal sympathetic differentiation, and the cells become phenotypically similar to the bulk of neuroblastoma cells found in clinical specimens. Knockdown of HIF-2α in neuroblastoma and glioma tumor stem/initiating cells leads to reduced levels of VEGF and poorly vascularized, highly necrotic tumors. As high HIF-2α expression further correlates with disseminated disease as demonstrated in neuroblastoma, glioma and breast carcinoma, we propose that targeting HIF-

2α and/or the pseudo-hypoxic phenotype induced by HIF-2 under normoxic conditions has great clinical potential.

Introduction

Mammalian cells, including tumor cells, require oxygen for maintenance of an efficient energy supply, and lack of oxygen eventually leads to cell death due to impaired energy requiring processes. Cells can withstand fluctuations in oxygen levels by adapting to a decrease in oxygen involving reduction of energy consumption and increase in anaerobic metabolism. During the adaptation process, there is a dramatic shift in the expression of genes regulating a number of cellular functions including glucose transport and metabolism, angiogenesis and cell survival. Central for this phenotypic shift are the hypoxia inducible factors (HIFs), HIF-1 and HIF-2. These factors are heterodimeric transcription factors composed of a unique alpha subunit and a beta subunit (ARNT/HIF-1β) shared by all three HIFs. Classically, HIFs are regulated by degradation of the alpha subunit at high oxygen levels and by stabilization at hypoxia (reviewed in Kaelin and Ratcliffe 2008). In the dimeric, active state, HIF-1 and HIF-2 bind to hypoxia responsive elements (HREs) located in genes regulated by hypoxia and HIFs. Although HIF-1 and HIF-2 seem to activate hypoxia-responsive genes by similar means (Tian et al. 1997; Wiesener et al. 1998), the HIF-α subunits work in a non-redundant manner and several differences in gene regulation have been proposed, many of which emphasize the predominant role of HIF-1 in regulating the transcriptional response to hypoxia (Iyer et al. 1998; Ryan et al. 1998; Hu et al. 2003; Park et al. 2003; Sowter et al. 2003). As detailed below, HIF-2 is also crucial for the hypoxic response, and, as opposed to HIF-1, it is active at prolonged hypoxia as well (Holmquist-Mengelbier et al. 2006). The less studied HIF-3 is present in several splice variants lacking the C-terminal transactivation domain and is thought to negatively regulate HIF-1 and HIF-2

by sequestering the HIF-1 and HIF-2 alpha subunits, thereby blocking their binding to HREs (Makino et al. 2001; Maynard et al. 2007). Although the main mode of HIF activation is via stabilization of the alpha subunits, HIF-2, but also HIF-1, can be stable and transcriptionally active at physiological or even higher oxygen tensions, as will be a central theme in this review. We propose that this phenomenon, at least regarding HIF-2, is largely linked to its role and regulation during normal development.

The phenotypes obtained by elimination of either *Hif1a* or *Epas1/Hif2a* clearly show that both genes are needed for proper development and that they are non-redundant. Importantly, and the reason for the focus on HIF-2α in this review, HIF-1α and HIF-2α are differently regulated in tumors such as neuroblastoma, breast cancer, non-small cell lung carcinoma (NSCLC) and glioma and seem to have different impact on tumor behavior and patient outcome in these tumors (Holmquist-Mengelbier et al. 2006; Helczynska et al. 2008; Heddleston et al. 2009; Kim et al. 2009b; Li et al. 2009; Noguera et al. 2009).

Phenotypic effects of HIF-2α elimination

While elimination of *Hif1a* has profound and repeatable effects on embryonal development, the effects of knocking out *Hif2a* have turned out to be much more complex and dependent on the genetic background, as summarized below. Despite displaying incompletely overlapping phenotypes, four different *Hif2a* knockout mice have been instrumental in identifying putative roles for *HIF2A* during normal development (Tian et al. 1998; Peng et al. 2000; Compernolle et al. 2002; Scortegagna et al. 2003). While $Hif1a^{-/-}$ animals are dead by embryonic day E11 with severe disorganization of vascular networks and gross neural tube defects, the effects of eliminating *Hif2a* - at least during early development - appears less general.

Hif2a expression during development is most abundant in vascular endothelial cells and disrupted vascular development of specific (although distinct) organs has been observed in various *Hif2a*$^{-/-}$ mice. Furthermore, whether or not attributable to vascular system defects, some *Hif2a*$^{-/-}$ mice have succumbed to embryonic death displaying hemorrhage. In particular, the *Hif2a*$^{-/-}$ mice created by Peng et al. showed varying degrees of vascular disorganization despite apparently normal blood vessel formation, suggesting that HIF-2α is required for normal remodeling/maturation post-vasculogenesis (Peng et al. 2000). *Hif2a*$^{-/-}$ mice in other studies, however, appeared normal in vascular development (despite hemorrhage) or displayed only subtle changes during late stages of pulmonary vascularization. In the Compernolle et al. study, *Hif2a* knockout mice died neonatally due to respiratory distress syndrome, apparently caused by impaired fetal lung maturation because of reduced VEGF levels and insufficient surfactant production (Compernolle et al. 2002).

Creating *Hif2a*$^{-/-}$ animals by hybrid mating allowed Scortegagna et al. (Scortegagna et al. 2003) to study effects of *Hif2a* loss in the post-natal mouse. These mice suffered from biochemical/metabolic abnormalities and multiple-organ pathology, specifically in sites of high-energy demand including the heart, liver, testis and bone marrow, indicating a syndrome related or similar to mitochondrial disease. Overall, adult *Hif2a*$^{-/-}$ mice showed greater oxidative stress as well as a reduced response to oxidative stress, suggesting an important role for HIF-2α in ROS homeostasis. The fact that *Hif2a* itself is regulated by ROS may indicate a role as a primary sensor of oxidative stress. In support, ROS accumulation and improved response to radiation therapy by *HIF2A* inhibition was recently described in human tumor cells (Bertout et al. 2009). These findings may implicate a role for *HIF2A* in radiation and chemotherapy resistance in tumor and possibly normal stem cells.

In addition, *Hif2a*$^{-/-}$ mice display defects in hematopoietic development due to greatly reduced EPO levels in the kidney (Scortegagna et al. 2003; Scortegagna et al. 2005; Rankin et al. 2007). Administration of exogenous EPO reverts this phenotype as well as some of the other defects associated with *Hif2a* elimination (Scortegagna et al. 2005). Further supporting a role for *HIF2A* in EPO production, a gain-of-function mutation in the *HIF2A* gene has been found associated with familial erythrocytosis (Percy et al. 2008a; Percy et al. 2008b).

HIF-2 during normal sympathetic nervous system (SNS) development

In 1998, Steven McKnight and colleagues showed that *Hif2a* expression was transient but prominent in developing sympathetic ganglia and paraganglia (Organ of Zuckerkandl) (Tian et al. 1998), the latter organ is the main site of catecholamine synthesis during development and is thus tyrosine hydroxylase (TH) positive, and it was later confirmed that HIF-2α is expressed also in developing human fetal SNS (Nilsson et al. 2005) (Fig. 1). Supporting a direct role for *Hif2a* in catecholamine production, the *Hif2a* deficient 129/SvJ mice contained substantially reduced levels of catecholamines, displayed bradycardia, and died at mid-gestation at a developmental stage corresponding to when *Hif2a* levels in the Organ of Zuckerkandl were the highest in heterozygous animals (Tian et al. 1998). Strikingly, the mid-gestational death was rescued by feeding the mothers DOPS, a substance that can directly convert into norepinephrine. Although a sympathetic phenotype was less pronounced in other *Hif2a*$^{-/-}$ mice – particularly as a cause of death - altered catecholamine content or DOPS-mediated rescue was recorded at least to some degree in all other *Hif2a* knockout animals (Peng et al. 2000; Compernolle et al. 2002; Scortegagna et al. 2003). These findings are consistent with the reported role of *Hif2a* in activating transcription of the DDC and DBH enzymes and thereby regulating catecholamine synthesis in fetal rat sympathoadrenal progenitor cells regardless of oxygen tension (Brown et al. 2009). In further support of a role

for *Hif2a* in sympathetic development, mice lacking the HIF prolyl hydroxylase PHD3 displayed an increased number (but reduced functionality) of sympathetic cells in the adrenal medulla, carotid body and the superior cervical ganglia due to reduced apoptosis (Bishop et al. 2008). A reasonable assumption based on the role of PHD3 in targeting HIFs for degradation is that HIF protein levels in general would be higher in *PHD3*$^{-/-}$ animals, and *in vitro* studies have suggested that PHD3 is more important in regulation of HIF-2α than HIF-1α (Appelhoff et al. 2004; Henze et al. 2009). Indeed, the sympathetic phenotype of *Phd3*$^{-/-}$ mice was intriguingly reversed by crossing animals with heterozygous *Hif2a*$^{+/-}$ (but not *Hif1a*$^{+/-}$) mice, again indicating that proper control of *Hif2a* expression is crucial for normal SNS development (Bishop et al. 2008). This notion is perhaps embodied by the link between high HIF-2α expression and immature, aggressive phenotypes of the sympathetic nervous system malignancy neuroblastoma, as discussed below.

Hypoxia in solid tumors and relation to tumor aggressiveness

Direct measurements of the oxygen tension in solid tumors and adjacent non-malignant tissue reveal that tumors, generally, are less well oxygenated and that large parts of solid tumors are hypoxic (Höckel and Vaupel 2001). Although these hypoxic areas often are necrotic, the general histological pattern is rather that tumor cells survive low oxygen tensions and thus can adapt to hypoxic conditions. *In vitro* studies support this conclusion as tumor cells established as cell lines can survive for several days at as low concentrations as 0.1% oxygen. Another interesting aspect of tumor hypoxia is the well-documented association between oxygen shortage and tumor aggressiveness (reviewed in Bertout et al. 2008) . The mechanistic background is probably very complex, but involves cytotoxic resistance, insensitivity to radiation, decreased DNA repair capacity, increased vascularization and increased metastatic potential (reviewed in Semenza 2003; Erler et al. 2006; Löfstedt et al. 2007) and, as will be

discussed in more detail below, dedifferentiation or loss of a differentiated tumor phenotype. As adaptation to hypoxia in tumor cells is largely mediated via stabilization and activation of HIF-1α and HIF-2α, also high levels of HIF proteins have been associated with disseminated disease and poor overall survival. In tumor cell lines, with few exceptions both HIF-1α and HIF-2α are expressed, and we recently postulated that HIF-1α is involved in adaptation to acute, and HIF-2α to prolonged, hypoxia (Holmquist-Mengelbier et al. 2006; Helczynska et al. 2008). For historical reasons, HIF-1α is the isoform that has been most extensively studied in clinical tumor materials and frequently been correlated with aggressive tumor disease, but in recent years high tumor levels of HIF-2α rather than HIF-1α have been shown to associate with negative overall survival and metastatic disease. In, for instance, breast carcinoma, earlier published data link HIF-1α, while later reports link HIF-2α to unfavorable disease (Schindl et al. 2002; Bos et al. 2003; Gruber et al. 2004; Dales et al. 2005; Generali et al. 2006; Giatromanolaki et al. 2006; Kronblad et al. 2006; Helczynska et al. 2008). Whether these contradicting observations reflect real differences in the tumor material analyzed, or can be attributed to methodological shortcomings is presently unknown. Nevertheless, data from tumors of different derivations in which HIF-2 appears to be important for clinical behavior are exemplified in the next paragraph.

Differential tumor HIF expression in relation to patient outcome.

Neuroblastoma. Neuroblastoma is a childhood tumor that arises in precursor cells or immature neuroblasts of the sympathetic nervous system (SNS), which is derived from the neural crest. There is a strong positive correlation between tumor aggressiveness (clinical stage and overall outcome) and immature phenotype (Fredlund et al. 2008). Although several cytogenetic aberrations linked to poor neuroblastoma prognosis have been identified, at the gene level amplification of *MYCN* is the only aberration that strongly associates with

advanced disease, which in turn associates with activated MYC signaling and an immature phenotype (Fredlund et al. 2008). The fully disseminated disease, Stage 4, is highly aggressive and the overall survival of children with this disease stage is less than 40% (Matthay et al. 1999).

As stated above, HIF-2α is expressed during discrete periods of murine and human SNS development, and the fact that neuroblastoma is an SNS-derived tumor appears to be important when the role(s) of HIFs in neuroblastoma is discussed. Both HIF-1α and HIF-2α proteins are expressed and become stabilized at hypoxia in neuroblastoma cell lines (Jögi et al. 2002). There is, however, a distinct difference in stabilization kinetics suggesting that HIF-1 is responsible for the acute and HIF-2 for the prolonged response to hypoxia (Holmquist-Mengelbier et al. 2006). HIF-2α is also less sensitive than HIF-1α to oxygen-dependent degradation, and accumulates already at near-physiological oxygen tensions. Immunohistochemical analysis of HIF expression in neuroblastoma specimens reveals that both HIF-1α and HIF-2α proteins, as expected, can be detected in tumor cell layers adjacent to necrotic areas. While HIF-1α is mainly restricted to perinecrotic zones, HIF-2α protein is also expressed at other locations, most notably in cells adjacent to blood vessels. Presence of tumor cells staining intensely for HIF-2α, more so than high number of HIF-2α^{+} cells, correlates positively to distant metastasis and negative overall survival (Holmquist-Mengelbier et al. 2006; Noguera et al. 2009). In contrast, HIF-1α protein expression did not correlate to aggressive disease or negative outcome (Noguera et al. 2009). As will be discussed below, we postulated that a fraction of the cells staining intensely for HIF-2α are the neuroblastoma tumor-initiating or stem cells, which could explain why presence of such cells so strongly associates with unfavorable disease (Pietras et al. 2008).

Breast carcinoma. HIF-1α protein is not expressed in normal breast tissue or ductal hyperplastic lesions, but is detected in ductal carcinoma *in situ* (DCIS) and invasive breast cancers (Bos et al. 2001; Helczynska et al. 2003). In cell lines, both HIFs are expressed and hypoxia-regulated. Similar to the situation in neuroblastoma, HIF-1α is acutely and transiently upregulated, whereas HIF-2α protein is still present after prolonged hypoxia and appears to mediate a sustained hypoxic response, including expression of VEGF (Helczynska et al. 2008). Expression in tumors and association of HIFs to breast cancer aggressiveness appear to be a complex issue. Early studies on HIF-1α protein expression in various subgroups of breast cancers link high levels of the protein to poor outcome, although several of these reports contradict each other. In more recent studies, the overall relationship between HIF-1α protein and breast-cancer-specific death is meager, and HIF-1α associates positively rather than negatively to favorable disease (Tan et al. 2007; Helczynska et al. 2008). However, there are early reports correlating high HIF-1α protein expression with shorter overall and disease-free survival time in patients with lymph node-positive breast cancer, whereas this association was not significant in lymph-node negative patients (Schindl et al. 2002; Kronblad et al. 2006). In contrast to these findings, two reports show association between high HIF-1α protein expression and poor outcome in node-negative but not in node-positive subgroups of patients (Bos et al. 2003; Generali et al. 2006). In addition, significant associations between HIF-1α protein expression and outcome without subgroup divisions (Dales et al. 2005) and unfavorable outcome in node-positive tumors, although restricted to T1/T2 tumors (Gruber et al. 2004), have been published. There are several putative explanations as to why these reports differ in predicting outcome and range from small or poorly defined clinical material to technical explanations. Our own experience is that commercial HIF antibodies vary in quality, also at the batch level, implying that immunohistochemical stainings have to be interpreted with some caution. In summary, we conclude from published data that the

prognostic impact of HIF-1α protein expression in breast cancer is, at best, restricted to subgroups of patients, which in such cases need to be verified in large prospective studies. Most studies, however, have in common that multi- and univariate analyses fail to reveal HIF-1α protein level as an independent prognostic factor.

HIF-2α and association with outcome in breast cancer patients has been far less studied, but published immunohistochemical data suggest that HIF-2α correlates to high metastatic potential and is an independent prognostic factor associated with breast cancer specific death (Helczynska et al. 2008). In two cohorts of breast cancer patients, both HIF-1α and HIF-2α correlated with increased VEGF expression, but only high HIF-2α protein exhibited a significant correlation to reduced recurrence-free and breast cancer-specific survival, and was an independent prognostic factor. Importantly, high HIF-2α protein expression correlated to presence of distal metastasis, but to no other clinical feature analyzed (Helczynska et al. 2008). In another report, HIF-2α protein was analyzed in a small subset of infiltrating ductal breast carcinomas, which showed a significant relationship between high HIF-2α protein expression and increased vascular density as well as secondary deposits to multiple axillary lymph nodes. Multivariate analysis revealed HIF-2α as an independent factor relating to extensive nodal metastasis (Giatromanolaki et al. 2006).

Renal cell carcinoma. During normal kidney development, HIF-1α is expressed in most cell types whereas HIF-2α is mainly found in renal interstitial fibroblast-like cells and endothelial cells. In the fully developed normal kidney, HIF-1α expression is maintained, while HIF-2α expression has disappeared. The role of HIF-signaling during development is largely unclear, but the cell type- and stage-specific expression distribution of HIF-α subunits correlates with the expression of critical angiogenic factors such as VEGF and endoglin (Freeburg and

Abrahamson 2003; Bernhardt et al. 2006). Conditional knockouts in renal proximal tubule cells of either HIF-1α or HIF-β alone do not generate an abnormal phenotype, whereas conditional knockout of pVHL results in HIF-dependent development of tubular and glomerular cysts (Rankin et al. 2006).

Clear cell renal cell carcinoma (CCRCC) is characterized by extensive neovascularization. This is generally explained by impaired HIF-α subunit degradation due to mutation or hypermethylation of the *VHL* gene, found in approximately 60-70% of all CCRCCs (Gnarra et al. 1994; Herman et al. 1994). At normoxia, pVHL constitutes the recognition subunit of a larger E3 ubiquitin ligase complex that targets the HIF-α subunits for proteasomal degradation (Kaelin 2002). Thus, in CCRCCs where pVHL function has been lost, the HIF-α subunits are constitutively expressed and a pseudo-hypoxic phenotype, including increased vascularization, is present. Intriguingly, there seems to be a bias towards HIF-2α expression as compared to HIF-1α expression in these *VHL*-deficient carcinoma cells (Maxwell et al. 1999; Krieg et al. 2000). The abundance of *VHL*-deficient RCC cell lines expressing HIF-2α but not HIF-1α (Maxwell et al. 1999) is also interesting as this contrasts with normal renal epithelial cells, where HIF-2α expression is absent during ischemia (Rosenberger et al. 2003). Furthermore, the HIF signaling pathways are activated early in the development of neoplastic lesions in VHL disease, with the HIF-1α isoform being expressed even in earliest foci while the HIF-2α protein is detected first in more advanced lesions (Mandriota et al. 2002). In pVHL-defective CCRCC, HIF-1 positively regulates BNIP3, an autophagy marker, but has no profound effect on cyclin D1, TGF-α and VEGF expression, whereas HIF-2 negatively regulates BNIP3 but promotes cyclin D1, TGF-α and VEGF expression (Raval et al. 2005). Thus, these differences in regulation of autophagy versus cell growth and angiogenesis might be understood in light of HIF-2α being expressed mainly during late CCRCC progression and

in more advanced lesions. siRNA-mediated knockdown of HIF-2α represses tumor growth in pVHL-deficient CCRCC (Kondo et al. 2003; Zimmer et al. 2004), and overexpression of HIF-2α in the *VHL* wild type 786-O cells resulted in enhanced tumor formation (Raval et al. 2005). In contrast, overexpression of HIF-1α in 786-O cells diminished tumor xenograft growth (Raval et al. 2005). Finally, and in agreement with the HIF-1α overexpression xenograft data, in clinical RCC material, HIF-1α has been reported to be an independent prognostic factor predicting favorable outcome (Lidgren et al. 2005).

Non-small cell lung carcinoma. HIF protein expression is virtually absent in normal lung tissue at normoxia, whereas both isoforms are accumulated during hypoxic conditions (Giatromanolaki et al. 2001). In corresponding normal lung tissue examined from lung cancer patients, bronchial and alveolar epithelium adjacent to the tumor site shows weak to intense cytoplasmic staining of the HIF proteins, whereas all other lung tissue components are negative for HIF expression (Giatromanolaki et al. 2001).

Intratumoral hypoxia in lung cancers correlates with decreased overall survival (Swinson et al. 2003; Le et al. 2006). Both HIF-1α and HIF-2α are frequently expressed in NSCLC, also during early progression of disease; but whereas HIF-2α causes, or is a surrogate marker for poor clinical prognosis (Giatromanolaki et al. 2001), the role of HIF-1α in predicting outcome is debated. Some reports demonstrate that HIF-1α expression has no impact on patient overall survival (Giatromanolaki et al. 2001; Kim et al. 2005), but potentially contradictory data exist (Volm and Koomagi 2000; Yohena et al. 2009).

In lung adenocarcinomas, mutations in *KRAS* are common, and the presence of *KRAS* mutations predicts poor outcome (Huncharek et al. 1999). Mice conditionally expressing a

non-degradable HIF-2α and mutated *Kras* (*Kras*^{G12D}) in the lungs display severe tumor burden and decreased survival, compared to mice expressing *Kras*^{G12D} only, suggesting that HIF-2α play a pivotal role in lung cancer pathogenesis (Kim et al. 2009b). In agreement with a role of HIF-2α in NSCLC tumorigenesis, HIF-2α in clinical material was an independent prognostic marker with high protein expression correlating to poor outcome (Giatromanolaki et al. 2001).

Glioblastoma. Glioblastoma multiforme (GBM) is characterized by a rich vascular network (Hossman and Bloink 1981; Blasberg et al. 1983; Groothuis et al. 1983) and intratumoral necrosis (Raza et al. 2002). Both HIF-1α and HIF-2α proteins are expressed in human glioblastomas (Jensen 2006; Li et al. 2009), with HIF-1α expression being mostly concentrated to areas of necrosis and at the tumor margin (Zagzag et al. 2000). Studies on a small set of brain tumors have suggested HIF-1α protein to correlate positively to brain tumor grade and vascularity (Zagzag et al. 2000).

Based on published data, the role of HIF-2α in glioblastoma formation and aggressiveness is not fully clear, as HIF-2α has been attributed a tumor-suppressor role (Acker et al. 2005), as well as being a marker for poor prognosis (Li et al. 2009). Overexpression of HIF-2α protein in rat glioblastomas suppressed tumor growth despite overall enhanced vascularization. This was in part explained by increased tumor cell apoptosis, and knockdown of HIF-2α in hypoxic human glioblastoma cells reduced the apoptotic rate of these cells (Acker et al. 2005). Recent work on GBM has focused highly on the small fraction of tumor cells with stem cell characteristics that are thought to initiate and maintain tumor growth (Hemmati et al. 2003; Singh et al. 2003; Galli et al. 2004; Singh et al. 2004). Several markers identifying a glioma stem cell population have been proposed, including CD133, Nestin (Singh et al.

2003), and A2B5 (Ogden et al. 2008). HIF-2α was recently shown to be expressed at high levels in $CD133^+$ glioma stem cells grown *in vitro* (McCord et al. 2009), and to co-localize with stem cell markers in tumor specimens (Li et al. 2009), suggesting that HIF-2α is an independent marker for glioma stem cells. Interestingly, HIF-2α is specifically expressed in brain tumor stem cells but not in neural progenitor cells, in contrast to HIF-1α, which is expressed in both cell types. As in neuroblastoma, a proportion of the HIF-2α positive cells are located adjacent to blood vessels in the tumor specimens, indicating that HIF-2α is expressed by a small but significant number of tumor cells, also in non-hypoxic regions (Pietras et al. 2008; Pietras et al. 2009; Li et al. 2009). Finally, analyzing gliomas at the mRNA level, *HIF2A*, but not *HIF1A* expression, correlates with poor patient survival (Li et al. 2009).

Hypoxia and tumor cell differentiation

As mentioned above, hypoxia has profound effects on cellular phenotypes. One aspect of adaptation to hypoxia, which is of particular importance in tumor cells, is the effect on the tumor cell differentiation status and newly discovered links between HIF-2α expression and tumor initiating/stem cells. Initially described in cultured neuroblastoma and breast cancer cells and in breast tumor specimens, hypoxia can push tumor cells towards an immature, stem cell-like phenotype (Jögi et al. 2002; Helczynska et al. 2003). The phenomenon has recently also been observed in glioma (Heddleston et al. 2009) suggesting that the dedifferentiating effect of hypoxia could be general and not restricted to specific tumor forms. These observations have potentially direct clinical impact, since at least in neuroblastoma and breast carcinoma, immature stages of differentiation correlate to aggressive tumor behavior and unfavorable outcome. Thus, we have proposed that the hypoxia-induced immature, stem cell

features work in concert with other hypoxia-driven changes in establishing an aggressive tumor phenotype (Jögi et al. 2002; Helczynska et al. 2003; Axelson et al. 2005).

HIF-2α and tumor initiating/stem cells

HIF-2α is expressed during discrete periods of murine SNS development as determined by in situ hybridization (Tian et al. 1998), and the expression is both strong and selective as most other tissues either lack or only show week HIF-2α expression (Jögi et al. 2002), suggesting that HIF-2α in the developing SNS is regulated at the transcriptional level. By immunohistochemistry, we could further demonstrate HIF-2α protein in human SNS paraganglia at fetal week 8.5 (Nilsson et al. 2005), which developmentally corresponds to mouse embryonal day E16, a time point when mouse SNS paraganglia express HIF-2α as determined by in situ hybridizations (Tian et al. 1998; Jögi et al. 2002). Using the same immunohistochemical protocol that detects HIF-2α in developing human paraganglia, staining of human neuroblastoma specimens highlights small subsets of cells intensely expressing HIF-2α protein, and the presence of such cells strongly correlates to disseminated disease (high clinical stage) and tumor-related death (Holmquist-Mengelbier et al. 2006). Further immunohistochemical characterization of these cells reveals that they frequently are perivascularly located, lack the expression of SNS markers like TH and NSE found in the bulk of neuroblastoma tumor cells, but express neural crest and early SNS progenitor markers such as NOTCH-1, HES-1, Vimentin and HAND2 (Pietras et al. 2008). Histologically, these cells were classified as tumor cells, although ambiguous cases exist. However, in most cases it could be excluded that the HIF-2α^+ cells were tumor-associated macrophages, reported to express HIF-2α and contributing to adverse outcome when present in breast cancer specimens (Leek et al. 2002). To verify that the HIF-2α^+ cells indeed were tumor cells proper and not stromal cells, MYCN amplification was demonstrated by in situ FISH in perivascularly

located, strongly HIF-2α immunofluorescing cells in tumors harboring an amplified MYCN gene. We hypothesized that these immature, stem cell-like HIF-2α^{+} cells could be neuroblastoma stem or tumor initiating cells (Pietras et al. 2008).

Recently, David Kaplan's laboratory isolated neuroblastoma cells from patient bone marrows and showed that these cells grow and form neurospheres in neural stem cell promoting medium (Hansford et al. 2007). These cells are highly tumorigenic in an orthotopic xenograft mouse model (Hansford et al. 2007) and are, by this functional definition, tumor-initiating cells (TICs). The neuroblastoma TICs virtually lack expression of SNS markers but express neural crest markers including *NOTCH1*, *HES1*, *ID2* and *VIM* (Pietras et al. 2009). As the TICs also have high levels of HIF-2α at normoxic conditions, they strongly share phenotypic characteristics with the earlier identified HIF-2α^{+}, SNS marker^{-} and neural crest marker^{+} cells in neuroblastoma specimens (Pietras et al. 2008). Neither the relation between the isolated immature neuroblastoma bone marrow TICs and the phenotypically similar cells in neuroblastoma specimens, nor the relation between the immature stem cell-like cells in tumor specimens and the bulk of neuroblastoma cells expressing SNS markers have been established. However, down-regulation of HIF-2α in the cultured neuroblastoma TICs by an sh*HIF2A* approach releases the tumor cells from a differentiation block resulting in expression of the early SNS markers *ASCL1/HASH1*, *ISL1*, and *SCG10*. When removed from the stem-cell-promoting medium and grown *in vivo* as subcutaneous tumors, the shHIF-2α-transduced TICs develop into a more mature neuroblastoma phenotype with expression of classical SNS markers such as tyrosine hydroxylase and chromogranin A, thus acquiring a phenotype similar to that of the bulk cells of clinical neuroblastomas (Pietras et al. 2009). We conclude that HIF-2α keeps the neuroblastoma TICs in a stem cell-like state and that these cells have properties in keeping with what could be expected of a neuroblastoma stem cell.

Our current view of the relation between neuroblastoma TICs, circulating neuroblastoma cells, tumor bulk and HIF protein expression is summarized in Fig. 2. The phenotypic similarities between bone marrow-derived TICs and the HIF-2α$^+$ tumor cells located adjacent to blood vessels in neuroblastoma specimens suggest that these cells are related and we postulate that circulating neuroblastoma cells are the connecting link as has been demonstrated in melanoma, breast and colon tumor model systems by Massague and co-workers (Kim et al. 2009a). In the tumors, we further postulate that the HIF-2α$^+$ neuroblastoma stem cells will spontaneously differentiate by unknown mechanisms into bulk cells expressing SNS markers such as *CHGA*, *TH* and *GAP-43*. The bulk cells have a reduced normoxic VEGF expression and thus reduced angiogenic capacity due to lowered HIF-2α protein levels, and when such cells experience hypoxia, they dedifferentiate and acquire stem cell, neural crest-like features (Jögi et al. 2002).

As briefly touched upon above, there is experimental support also from other cell systems for a role of HIF-2α during early development and maintenance of a (tumor) stem cell phenotype (reviewed in Keith and Simon 2007). In embryoid bodies, overexpression of HIF-2α results in maintained pluripotency and potentiation of tumorigenic growth (Covello et al. 2006). These effects were a result of direct transcriptional activation of the POU transcription factor *OCT4* by HIF-2α, as silencing of *OCT4* in HIF-2α knock-in cells reverted the stem cell phenotype and reduced tumor growth (Covello et al. 2006). In glioma, cell populations enriched for tumor stem cell properties have high HIF-2α protein levels, and, as in neuroblastoma, HIF-2α has been suggested to be a marker of glioma stem cells. In addition, downregulation of HIF-2α in such cells results in decreased tumor initiating capacity and glial differentiation (Li et al. 2009; Heddleston et al. 2009). In line with these findings are the observations that HIF-2α protein expression correlates to breast cancer specific death and distant metastasis, the latter

process most likely dependent on the presence of cells with tumor stem cell properties. Based on the published observations that HIF-2α is intimately and functionally linked to immature neural tumor stem cell phenotypes and appears to counteract early steps in SNS and glial differentiation, we hypothesize that HIF-2α might be a general marker of tumor stem cells, with a dedifferentiating function, similar to that in glioma and neuroblastoma stem/tumor initiating cells.

HIFs and vascularization

HIFs were early implicated in the tumor angiogenic process when it became clear that hypoxia promotes VEGF expression (Shweiki et al. 1992; Forsythe et al. 1996). With neuroblastoma as a model system, we showed that during hypoxia-driven VEGF expression there is a temporal shift in the usage of the HIFs; whereas the VEGF expression is HIF-1 dependent at an acute phase, the expression during prolonged hypoxia is primarily HIF-2 driven (Holmquist-Mengelbier et al. 2006). In a follow-up study using a large clinical neuroblastoma tissue microarray material immunohistochemically stained for HIF-1α, HIF-2α, VEGF and blood vessel endothelial cells (CD31), tumor cells staining intensely for HIFs correlated to VEGF positivity, while the HIF-1α and HIF-2α staining did not fully correlate to each other (Noguera et al. 2009). Furthermore, HIF-1α and VEGF, but not HIF-2α, showed a negative correlation to the number of blood vessels, in agreement with the observation that strongly HIF-2α positive tumor cells express VEGF and frequently locate adjacent to blood vessels. As opposed to HIF-2α, presence of HIF-1α positive cells did not correlate to tumor aggressiveness or disseminated disease (Noguera et al. 2009). We conclude from *in vitro* and *in vivo* neuroblastoma data that both HIF-1α and HIF-2α become stabilized at hypoxia and that the HIF-2α protein level can be positively regulated by additional, not fully explored mechanisms.

The HIF-2α$^+$ neuroblastoma and glioma stem/tumor initiating cells strongly express VEGF (Holmquist-Mengelbier et al. 2006; Bao et al. 2006; Calabrese et al. 2007; Pietras et al. 2008; Pietras et al. 2009; Li et al. 2009) and the perivascular location of phenotypically similar cells in tumor specimens might implicate that tumor stem cells actively take part in the process of tumor vascularization. The hypothesis is supported by the observation that knockdown of HIF-2α in neuroblastoma and glioma stem/tumor initiating cells results in poorly vascularized tumors. Moreover, vascularization is affected in mice with eliminated *Hif2a* (Peng et al. 2000; Rankin et al. 2008) and overexpression of *Hif2a* in embryoid bodies results in early and extensive formation of a vascular network (Covello et al. 2006). The underlying molecular mechanisms behind the perivascular localization of neural tumor stem cells is presently not understood and is a topic of future investigations.

HIF-2α and the pseudo-hypoxic phenotype – targets for tumor treatment

One striking feature of both neuroblastoma and glioma stem/tumor initiating cells is their pseudo-hypoxic phenotype due to high expression of active HIF-2α at physiological oxygen tensions (Holmquist-Mengelbier et al. 2006; Pietras et al. 2008; Pietras et al. 2009; Li et al. 2009). As a consequence, several genes considered to be hypoxia-regulated are highly expressed at non-hypoxic conditions in these HIF-2α$^+$ tumor cells, thus creating a pseudo-hypoxic phenotype, presumably similar to that in a majority of CCRCCs. Whether the pseudo-hypoxic phenotype is drastically different from a corresponding *bona fide* hypoxic phenotype is not known, but as discussed above, VEGF expression and presumably vascularization would be one important determinant of the high HIF-2α expression at non-hypoxic conditions. This conclusion, in addition to data indicating that HIF-2α appears to play a pivotal role in aggressive and disseminated growth of neuroblastoma, glioma, breast

carcinoma, non-small cell lung carcinoma and CCRCC, strongly suggests HIF-2α and the pseudo-hypoxic phenotype as attractive therapeutic targets in at least these tumor types. By pushing HIF-2α^{+} tumor stem cells toward a more mature, tumor bulk-like phenotype by interfering with the expression or activity of HIF-2, the tumor stem cell pool as well as the bulk of tumor cells, targeted by existing, efficient treatment protocols, would be reduced in numbers. The demonstration that both neuroblastoma and glioma stem/initiating cells differentiate *in vivo* when HIF-2α is knocked down (Pietras et al. 2009; Heddleston et al. 2009; Li et al. 2009) can be seen as proof of principle. Diminished HIF-2α activity also leads to reduced VEGF expression in these two tumor stem cell models, and as would be anticipated, neuroblastoma and glioma tumors with reduced HIF-2α expression are highly necrotic (Pietras et al. 2009; Li et al. 2009; Heddleston et al. 2009), suggesting that targeting HIF-2α will result in both an anti-angiogenic effect and a reduction of the tumor stem cell pool. As high HIF-2α protein levels associate with disseminated disease, targeting of HIF-2α or the HIF-2α-driven pseudo-hypoxic phenotype might also affect tumor spread and transition into advanced clinical stages.

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Figure 1. HIF-2α positive human paraganglia at fetal week 8.5. Paraganglia stained for tyrosine hydroxylase (TH) and HIF-2α in non-consecutive but adjacent sections (ethical approval LU 389-98, Lund University, Sweden). Arrows indicate distinct nests of immature paraganglia cells positive for both TH and HIF-2α. These structures have been further characterized in (Hoehner et al. 1996).

Figure 2. A putative interplay between neuroblastoma (NB) tumor-initiating cells (TICs), tumor bulk, HIF-2α, sympathetic nervous system (SNS) differentiation and oxygen status. We postulate that bone marrow-derived neuroblastoma TICs communicate with primary neuroblastomas as circulating tumor cells. In neuroblastoma tumors, TICs will spontaneously, by unknown mechanisms, lose their HIF-2α protein expression, differentiate and acquire expression of SNS markers. In hypoxic regions of neuroblastomas, tumor cells lose their differentiated phenotype and become stem cell-like (Jögi et al. 2002). In this model, HIF-1α protein expression is strictly linked to a hypoxic cellular environment.

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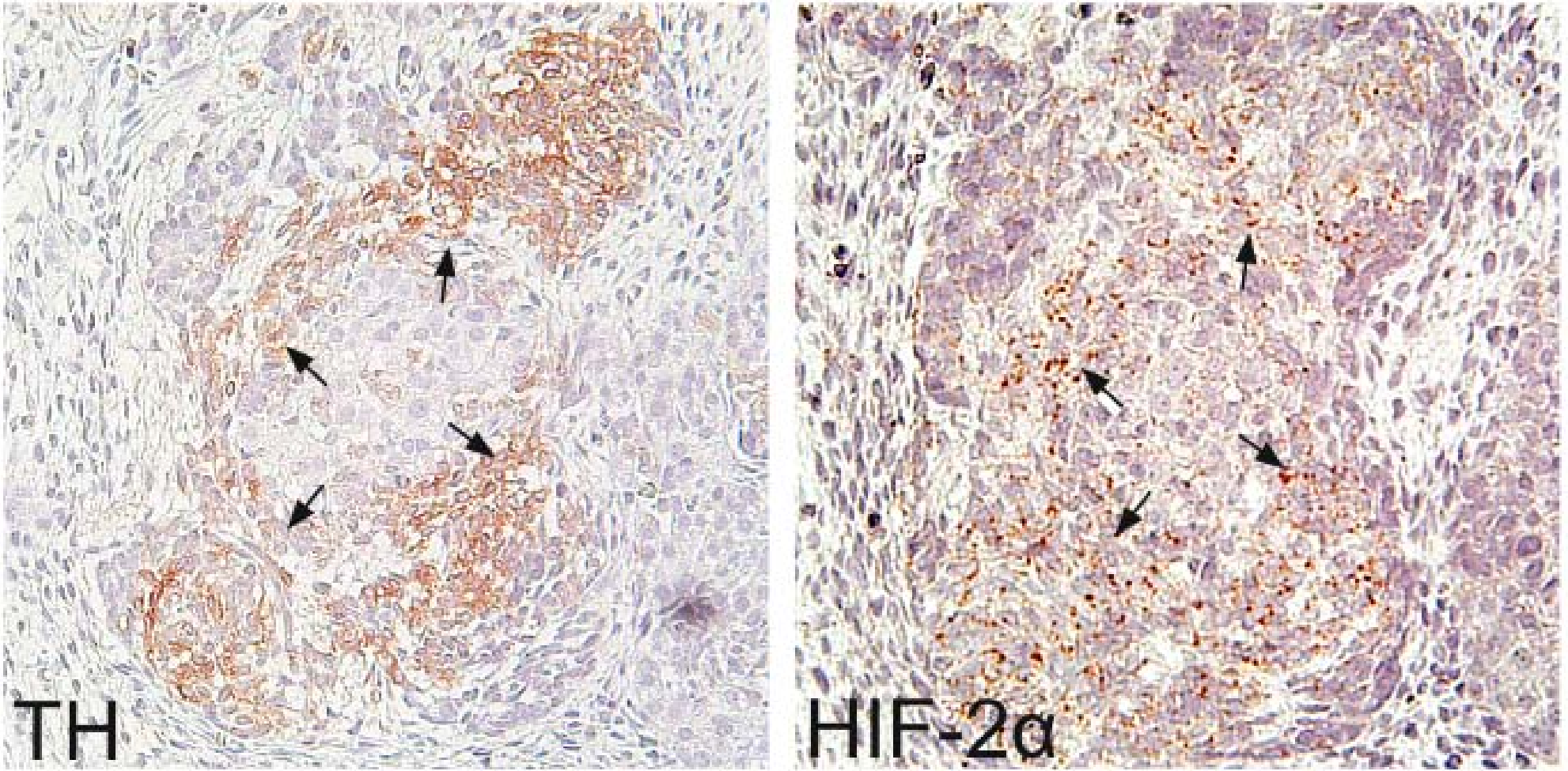
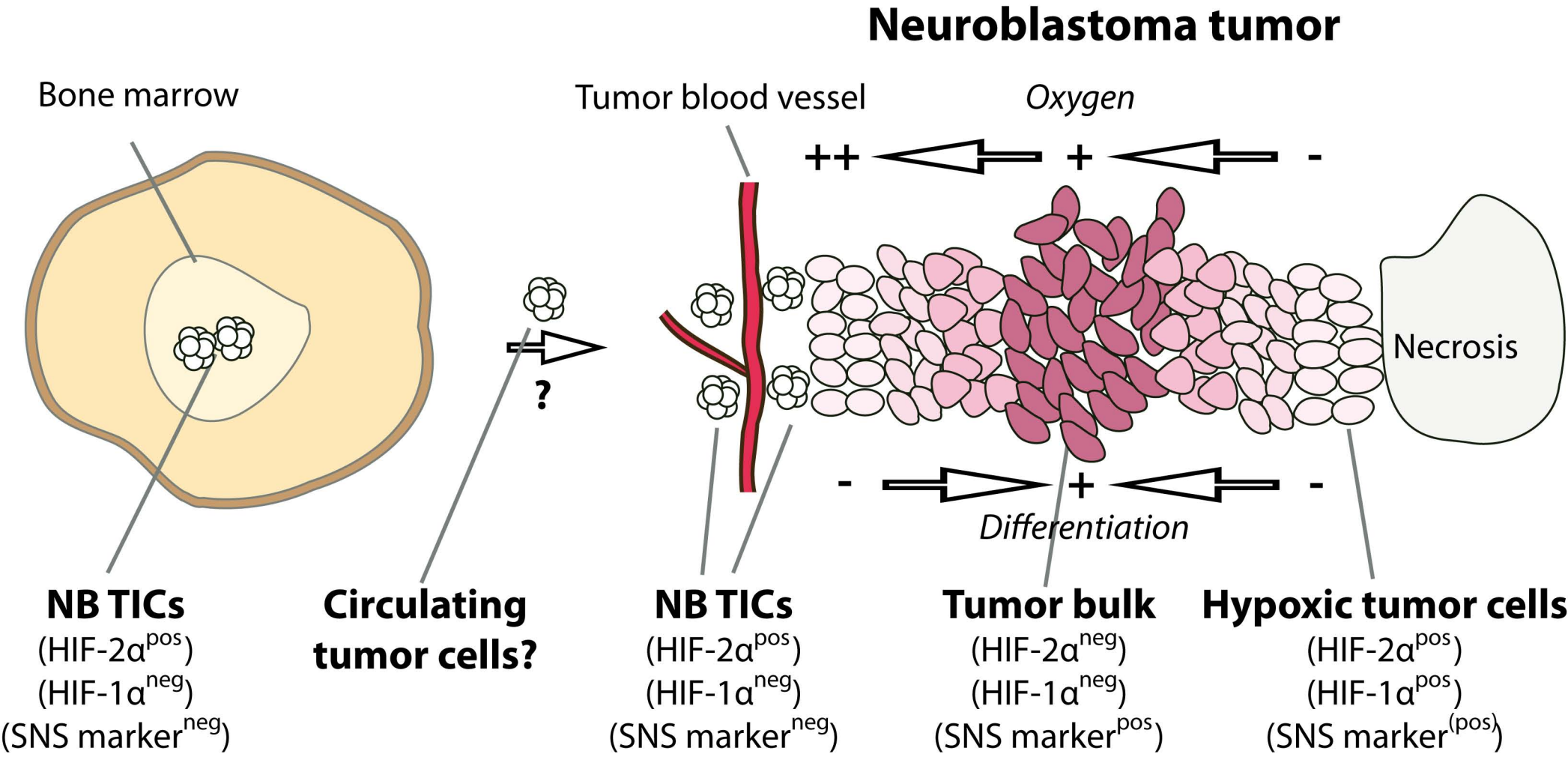

TH
HIF-2α


Neuroblastoma tumor
Bone marrow
Tumor blood vessel
Oxygen
++
+
-
?
Necrosis
- + -
Differentiation
NB TICs
(HIF-2α^pos)
(HIF-1α^neg)
(SNS marker^neg)
Circulating tumor cells?
NB TICs
(HIF-2α^pos)
(HIF-1α^neg)
(SNS marker^neg)
Tumor bulk
(HIF-2α^neg)
(HIF-1α^neg)
(SNS marker^pos)
Hypoxic tumor cells
(HIF-2α^pos)
(HIF-1α^pos)
(SNS marker^(pos))