Therapeutic targeting of hypoxia and hypoxia-inducible factors in cancer

Caroline Wigerup, Sven Påhlman *, Daniel Bexell

Translational Cancer Research, Medicon Village 404C3, Lund University, Lund, Sweden

A R T I C L E   I N F O

Available online 29 April 2016

Keywords:
Cancer
Hypoxia
Hypoxia-inducible factor (HIF)
Neuroblastoma
Pheochromocytoma
Paraganglioma

A B S T R A C T

Insufficient tissue oxygenation, or hypoxia, contributes to tumor aggressiveness and has a profound impact on clinical outcomes in cancer patients. At decreased oxygen tensions, hypoxia-inducible factors (HIFs) 1 and 2 are stabilized and mediate a hypoxic response, primarily by acting as transcription factors. HIFs exert differential effects on tumor growth and affect important cancer hallmarks including cell proliferation, apoptosis, differentiation, vascularization/angiogenesis, genetic instability, tumor metabolism, tumor immune responses, and invasion and metastasis. As a consequence, HIFs mediate resistance to chemo- and radiotherapy and are associated with poor prognosis in cancer patients. Intriguingly, perivascular tumor cells can also express HIF-2α, thereby forming a "pseudohypoxic" phenotype that further contributes to tumor aggressiveness. Therefore, therapeutic targeting of HIFs in cancer has the potential to improve treatment efficacy. Different strategies to target hypoxic cancer cells and/or HIFs include hypoxia-activated prodrugs and inhibition of HIF dimerization, mRNA or protein expression, DNA binding capacity, and transcriptional activity. Here we review the functions of HIFs in the progression and treatment of malignant solid tumors. We also highlight how HIFs may be targeted to improve the management of patients with therapy-resistant and metastatic cancer.

© 2016 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

An adequate oxygen supply to cells and tissues is crucial for the function and survival of aerobic organisms. Hypoxia occurs when oxygen supply fails to meet demand. The definition of hypoxia is somewhat ambiguous since normal oxygen pressure (P_{O2}) varies between different tissues. For instance, the normal P_{O2} in arterial blood is ~100 mm Hg (~13%) but ~40 mm Hg (~5%) in the liver (Koh & Powis, 2012). No clear-cut threshold separates normoxia from hypoxia, so oxygen levels must be regarded in context. Likewise, definitions of mild, moderate, and severe hypoxia are not clearly defined. Nevertheless, 8–10 mm Hg...
(~1%) is estimated to represent a critical \( P_{O_2} \) since lower \( P_{O_2} \) values are associated with adverse effects caused by reduced O2 consumption (Hockel & Vaupel, 2001). For in vitro studies, 1% oxygen is commonly used to mimic a hypoxic environment. Solid malignant tumors commonly contain hypoxic areas, and direct measurement of tissue oxygenation using O2-sensitive microsensors has shown that the tumor microenvironment is characterized by much lower \( P_{O_2} \) values than their normal counterparts (Vaupel et al., 2004).

A major cause of tumor hypoxia is the formation of non-functional blood vessels in neoplastic tissue, particularly in rapidly growing tumors. Hypoxia is not merely epiphenomenal but is of clinical importance since tumor hypoxia is associated with aggressive tumor phenotypes, treatment resistance, and poor clinical prognosis (reviewed in Bertout et al. (2008)). The cellular response to hypoxia is mainly mediated by the hypoxia-inducible factor (HIF) family of transcription factors, which regulate the expression of multiple genes involved in processes that drive the adaptation and progression of cancer cells. Therefore, tumor hypoxia and HIFs affect most of the cancer “hallmarks” including cell proliferation, apoptosis, metabolism, immune responses, genomic instability, vascularization, and invasion and metastasis. HIFs also appear to contribute to chemo- and radiotherapy resistance via multiple mechanisms. In clinical tumor samples, HIF expression is associated with poor prognoses and relapse on treatment. HIFs appear to be crucial molecular targets that can be exploited to improve on the current treatment of metastatic and treatment-resistant cancer.

However, HIF biology is complex. Alternative HIF isoforms differentially regulate tumor growth; consequently, optimal HIF targeting depends on the specific tumor type and HIF-1 and HIF-2 selectivity. Strategies to inhibit HIFs in cancer include drugs with indirect effects on HIF signaling, but direct HIF inhibitors also exist. In this review we describe HIF regulation and function, their role as master regulators of solid tumor hallmarks (Fig. 1), the pseudohypoxic phenotype, and the different therapeutic strategies that have been developed to inhibit HIF activity (Fig. 2).

### 2. Regulation and function of hypoxia-inducible factors

HIFs are proteins that sense and respond to oxygen deficiency by acting as transcription factors (Fig. 2). Each HIF transcription factor is composed of two subunits: the \( \alpha \)-subunit and the \( \beta \)-subunit (HIF-1\( \beta \) or ARNT), both of which belong to the basic helix–loop–helix (HLH)–PER-ARNT-SIM (bHLH-PAS) protein family (Bersten et al., 2013). The \( \alpha \)-subunit is oxygen sensitive, while the \( \beta \)-subunit is ubiquitously expressed. HIF-1\( \beta \) and HIF-2\( \beta \) are inducible by hypoxia, and their stabilization is oxygen dependent. HIF activity is regulated by oxygen-dependent degradation (ODD) and oxygen-independent degradation (OID). ODD is mediated by prolyl hydroxylases (PHDs), whereas OID is mediated by von Hippel-Lindau protein (VHL).

Fig. 1. Hallmarks of cancer regulated by hypoxia and HIFs. Hypoxia and HIFs regulate multiple cancer phenotypes. Targeting hypoxia/HIFs will thus inhibit several traits of tumor progression, metastasis, and treatment resistance. Abbreviations: VEGF, vascular endothelial growth factor; PDGF-\( \beta \), platelet-derived growth factor-\( \beta \); ANGPT2, angiopoietin 2; oxphos, oxidative phosphorylation; SSB, single-strand break; TAM, tumor-associated macrophage; CTL, cytotoxic T-lymphocyte; Treg, regulatory T-cell; MDSC, myeloid-derived suppressor cell; DC, dendritic cell; MDR, multi-drug resistance; ROS, reactive oxygen species; IR, ionizing radiation.

C. Wigerup et al. / Pharmacology & Therapeutics 164 (2016) 152–169

153
expressed. In the presence of oxygen, conserved proline residues on the α-subunit are hydroxylated, promoting its degradation (Ivan et al., 2001; Jaakkola et al., 2001). This hydroxylation is mediated by prolyl-4-hydroxylases (PHDs), a set of oxygen-, iron-, and ascorbate-dependent enzymes belonging to the 2-oxoglutarate-dependent oxygenase superfamily (Schofield & Ratcliffe, 2004). The α-subunit hydroxylation serves as a recognition signal for the von Hippel–Lindau (pVHL) tumor suppressor protein, which subsequently targets the α-subunit for degradation by adding ubiquitin. However, under hypoxic conditions, PHDs cannot hydroxylate the α-subunit. This results in HIF-α protein stabilization, nuclear translocation, and dimerization with HIF-1β/ARNT to form the HIF transcription factor. Together with the co-activator CBP/p300, HIFs induce transcription of a plethora of target genes during hypoxia. The small molecules that can modulate HIF activity at different levels are also shown.

Fig. 2. Regulation of HIF-α under normoxic and hypoxic conditions. Under normoxic conditions, HIF-α undergoes hydroxylation at conserved residues, a process mediated by prolyl-4-hydroxylases (PHDs) and factor inhibiting HIF-1 (FIH-1) enzymes. Hydroxylation by PHDs promotes destabilization of the HIF-α protein, while hydroxylation by FIH-1 inhibits transcriptional activity by preventing interaction with CBP/p300. Degradation of HIF-α is mediated via an ubiquitin-dependent process executed by the VHL-complex. Inactivation of PHDs and FIH-1 under hypoxic conditions leads to HIF-α stabilization followed by translocation into the nucleus and dimerization with HIF-1β/ARNT to form the HIF transcription factor. Together with the co-activator CBP/p300, HIFs induce transcription of a plethora of target genes during hypoxia. The small molecules that can modulate HIF activity at different levels are also shown.

There are three different α-subunit isoforms: HIF-1α, HIF-2α, and HIF-3α. HIF-1α and HIF-2α have been most comprehensively studied, with less known about HIF-3α. Semenza and colleagues first described HIF-1α in the nineties (Wang et al., 1995), while Tian et al. (1997) described the second isoform (initially called EPAS1; endothelial PAS protein 1) soon after, findings that were quickly confirmed by several other groups. The α-subunits show conserved sequence homology (Fig. 3), mostly in the bHLH- and PAS-A and PAS-B domain-containing N-terminal region that mediates DNA binding and interaction with HIF-1β. Two transactivation domains (TADs) located in the α-subunit N- and C-termini regulate HIF transcriptional activity: the N-TAD is located in the region where hydroxylation takes place, known as the oxygen-dependent degradation (ODD) domain. HIF transcriptional activity is also regulated by a second oxygen-sensitive hydroxylation event mediated by factor inhibiting HIF-1 (FIH-1) (Mahon et al., 2001; Lando et al., 2002). FIH-1 is a 2-oxoglutarate-dependent oxygenase (similar to the PHDs), and it catalyzes hydroxylation of an asparagine residue in the C-TAD of HIF-1α, thereby preventing interaction with the p300/CBP co-activator. Of note, HIF-1α is more sensitive to FIH-1-mediated inhibition than HIF-2α (Khan et al., 2011) due to a specific amino acid difference between the two HIFs (Bracken et al., 2006). The affinity of PHDs and FIH-1 for oxygen differs and, since FIH-1 has a higher affinity (lower Km) for oxygen, PHDs are thought to be more rapidly inhibited in low oxygen conditions (Pouyssegur et al., 2006). Thus, when PHDs are inactive, the HIF-1α subunit is stabilized but FIH-1 can still inhibit transcription of genes that require HIF-1α C-TAD activity. Depending on the oxygen tension, different gene sets might be regulated according to their N-TAD or C-TAD requirement. Hypoxia-associated factor (HAF) is an E3 ubiquitin ligase that switches cells from HIF-1- to HIF-2-dependent signaling by targeting HIF-1α for degradation and increasing HIF-2α transactivation (Koh et al., 2011).

The fact that there is less sequence homology in the HIF-1α and HIF-2α transactivating domains than the DNA-binding and
Abbreviation: NLS, nuclear localization signal.

The heterodimerization domains suggests that HIF-1 and HIF-2 have unique target genes. Early studies showed that HIF-1 (but not HIF-2) induced expression of several glycolytic genes in various cell types, while unique HIF-2 target genes seemed to be cell type specific (Hu et al., 2003; Raval et al., 2005). The studies were mainly performed in VHL-defective renal cell carcinoma (RCC) cell lines with unique HIF expression patterns, i.e., expressing only HIF-1α or HIF-2α. HIF transcriptional target specificity was strikingly different in VHL-defective RCC cells compared to other cell types, and the HIF isoforms also showed a mutually dependent suppressive function in RCC (Raval et al., 2005). Several investigators have used chromatin immunoprecipitation together with microarrays (ChIP-chip) to directly identify HIF target genes (Mole et al., 2009; Xia & Kung, 2009; Xia et al., 2009). These studies confirmed the core A/GCGTG HIF-binding motif and, in agreement with earlier findings, showed that HIF-1 targets genes important in glycolytic metabolism. Furthermore, genes encoding histone demethylases were identified as HIF-1 target genes, suggesting that HIFs participate in epigenetic homeostasis during hypoxia. These studies also identified considerable overlap between HIF-1 and HIF-2 binding sites, yet HIF-2 seemed to contribute very little to the transcriptional changes during hypoxia (Mole et al., 2009). Integrating data from ChIP-chip analyses with gene expression profiles over a hypoxia time course showed that HIF-1 stabilization causes HIF-1 to preferentially bind to loci already transcriptionally active prior to the onset of hypoxia, partially explaining why most hypoxia-induced transcriptional changes are cell type specific (Xia & Kung, 2009). A study using chromatin immunoprecipitation followed by next-generation high-throughput sequencing (ChIP-seq) showed that HIF binding sites exist outside of promoter sequences and that HIF binds to other sites as well as the core A/GCGTG motif (Schodel et al., 2011). This was particularly true for HIF-2α, for which as many as 70% of the identified HIF-2 binding sites lay outside promoter regions. Consistent with other studies, glucose metabolism pathways were targeted by HIF-1, while HIF-2 targeted genes were involved in Oct4-regulated stem cell pluripotency. A representative list of shared and unique target genes regulated by HIF-1 and HIF-2 can be found in Keith et al. (2012). Moreover, while HIF-1 is thought to mediate the acute response to hypoxia, HIF-2 stabilizes over longer time frames and also at normal physiological oxygen levels (Holmquist-Mangelbier et al., 2006).

In addition to its role as a transcription factor subunit, HIF-2α is also part of a hypoxia-regulated translation initiation complex. Hypoxia induces the formation of a HIF-2α complex with the RNA-binding protein RBM4 and cap-binding protein eIF4E2, which is then recruited to a wide variety of mRNAs to promote active translation at polysomes (Unniece et al., 2012). This function is independent of HIF-1α dimerization.

As well as direct gene targeting via HRE binding, both HIF-1α and HIF-2α can indirectly influence gene expression by interfering with the activity of other transcription factors such as MYC (Koshiji et al., 2004; Gordan et al., 2007a), p53 (Chen et al., 2003; Bertout et al., 2009), and Notch (Gustafsson et al., 2005; Hu et al., 2014). Importantly, for many of these interactions, HIF-1α and HIF-2α seem to have completely opposing effects, a difference that needs to be considered when targeting HIFs for therapeutic benefit. Further adding to HIF complexity, α-subunits are differentially regulated at the transcriptional, translational, and posttranslational modification levels. A comprehensive description of HIF-α regulation and the conflicting roles of HIF-1α and HIF-2α are given in Keith et al. (2012).

Although a major mode of HIF stabilization is via proline hydroxylation and VHL-mediated degradation, it is important to mention that several non-hypoxia-driven stimuli also regulate HIFs such as growth factors, cytokines, hormones, and various stressors. Several growth factors and their cognate receptors that signal through the phosphoinositide 3-kinase (PI3K) or Ras/MAPK pathways induce HIF expression, and both basal and mitogen-induced HIF expression is abrogated upon PI3K pathway inhibition (Zhong et al., 2000; Beppu et al., 2005; Calvani et al., 2008; Mohlin et al., 2015). HIFs are direct substrates of a variety of protein kinases that regulate HIF stabilization, nuclear translocation, and activation (Kietzmann et al., 2016). Interestingly, several factors also use reactive oxygen species (ROS) as intermediate signaling molecules, and it has been shown that ROS directly regulate HIFs at different levels (Gorlach & Kietzmann, 2007).

3. Hypoxia and hypoxia-inducible factors in cancer

3.1. Measuring tumor hypoxia

In early key findings, O2-sensitive microsensors were used to show that tumor hypoxia was associated with tumor progression (Vaupel & Mayer, 2007). Patients with hypoxic head and neck tumors (Gatenby et al., 1988; Nordsmark et al., 2005), cervical cancer (Hockel et al., 1993; Hockel et al., 1996), and soft tissue sarcoma (Nordsmark et al., 2001) had a worse prognosis, although some results are conflicting (Nordsmark et al., 2006). Endogenous biomarkers associated with hypoxia in vitro (e.g., HIF-1α, glucose transporters, and carbonic anhydrase...
IX (CAIX)) have also been used in tumor hypoxia experiments; perhaps surprisingly, low O2 measured by microsensors did not correlate with expression of these biomarkers in patient tumors (Mayer et al., 2008a). Thus, even if endogenous “hypoxic” biomarkers are associated with poor clinical prognosis (as described below), it is worth remembering that a direct association between biomarker expression and intratumoral hypoxia is unproven, and tumor cells can also express “hypoxia” markers such as HIF-2α under normoxic conditions (discussed below and reviewed in Pietras et al. (2010)). Different methods for measuring hypoxia in patients are reviewed in Vaupel et al. (2004).

3.2. Patient prognosis

The HIF-1α protein is overexpressed in various common solid malignant tumors including breast, colon, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas compared to their respective normal tissues. In contrast, many benign tumors show very little or no HIF-1α expression (Zhong et al., 1999; Daponte et al., 2008; Simiantonaki et al., 2008; Mayer et al., 2008b). Importantly, HIF-1α or HIF-2α overexpression is associated with poor clinical outcomes in patients with various cancers. HIF-1α expression is associated with poor survival in cervical cancer (Birner et al., 2000), endometrial carcinoma (Sivridis et al., 2002), oligodendroglioma (Birner et al., 2001a), ovarian cancer (Birner et al., 2001b), and different breast cancer subtypes (Bos et al., 2001; Schindl et al., 2002; Bos et al., 2003; Generali et al., 2006; Kronblad et al., 2006). The prognostic value of HIF-1α in breast cancer has, however, been questioned in conflicting follow-up studies, but HIF-2α appears to be an independent prognostic factor in breast cancer (Helczynska et al., 2008). HIF-2α expression is also a marker of poor prognosis in glioblastoma (Li et al., 2009), neuroblastoma (Holmquist-Mengelbier et al., 2006), head and neck squamous carcinoma (Koukourakis et al., 2002), and non-small cell lung cancer (Giromatolakos et al., 2001). In ccRCC, VHL loss impairs degradation of HIF-α proteins, leading to HIF accumulation irrespective of oxygen levels (Gnarra et al., 1994; Krieg et al., 2000). The pathways involved in the pseudo-hypoxic phenotype in ccRCC are further reviewed in Bratslavsky et al. (2007). High nuclear expression of HIF-1α and high cytoplasmic expression of HIF-2α indicate poor prognosis in ccRCC patients (Fan et al., 2015). Thus, while intratumoral hypoxia in general is associated with poor prognosis, the prognostic role of HIF-1α and HIF-2α differs between tumor types. In addition, HIF-1α overexpression is associated with improved prognosis in patients with head and neck cancer (Beasley et al., 2002), non-small cell lung cancer (Vollm & Koomagi, 2000), and favorable outcomes in neuroblastoma (Noguera et al., 2009). Thus, similar to their molecular functions, HIF-1α and HIF-2α can have opposite clinical effects depending on the tumor type.

3.3. Tumor cell viability

Hypoxia can result in decreased cell proliferation, cell cycle arrest, and/or apoptosis. Giaccia and co-workers demonstrated that hypoxia can induce tumor cell apoptosis, an effect that was reduced by loss of p53 tumor suppressor function or overexpression of anti-apoptotic Bcl-2 (Graeber et al., 1996). Therefore, hypoxia can exert a selective pressure that leads to expansion of tumor cells with reduced apoptotic capacity due to, for example, TP53 mutations (Graeber et al., 1996). It has been suggested that hypoxia induces a physical interaction between HIF-1α and p53, resulting in stabilization of p53 and increased apoptosis (An et al., 1998). The interaction between HIF-1α and p53 is, however, also mediated by Mdm2, and HIF-1α can increase p53 levels by inhibition of Mdm2-mediated degradation (Chen et al., 2003). Intriguingly, p53 can also promote Mdm2-mediated degradation of HIF-1α (Ravi et al., 2000). HIF-2α seems to have an opposing effect on p53 (at least in ccRCC) since HIF-2α inhibits p53 in an Mdm2-independent manner, thereby providing therapeutic opportunities via HIF-2α inhibition (Bertout et al., 2009; Roberts et al., 2009). The interplay between HIFs and the p53 family is reviewed in Amelio and Melino (2015).

c-Myc is an essential cell cycle regulator and oncogene, and HIFs can either interfere with or promote c-Myc activity. Hypoxia-induced HIF-1α inhibits c-Myc transcription and suppresses proliferation (Koshiji et al., 2004), and HIF-1α can also promote c-Myc degradation (Zhang et al., 2007). In contrast, HIF-2α can enhance c-Myc activity and promote cell cycle progression (Gordan et al., 2007a). HIF-1α can further inhibit Myc by interfering with its partner protein Myc-associated protein X (Max), while HIF-2α can stabilize the Myc–Max complex and increase Myc activity. The complex interplay between HIFs and Myc is reviewed in Dang et al. (2008). These opposing effects on Myc function further highlight the need to develop specific inhibitors that target either HIF-1α or HIF-2α. The effects of hypoxia and HIFs on tumor cell proliferation, survival, and apoptosis are reviewed in Gordan et al. (2007b) and Keith et al. (2012).

In response to stressors such as hypoxia or nutrient deprivation, cells become dependent on increased autophagy for their survival. In autophagy – literally “eating of self” – autophagosomes are formed that sequester intracellular components prior to digestion by fusion with lysosomes. Basal levels of autophagy are needed for cellular homeostasis, but from the cancer perspective, autophagy is considered a double-edged sword since it can either be tumor suppressive or tumor promoting. Its suppressor effects arise because it prevents cells from being damaged by unfolded proteins or damaged organelles. However, it can also promote tumor growth by providing tumor cells with metabolites and by preventing the accumulation of malfunctioning mitochondria that would otherwise lead to increased ROS and cell death. HIF-1 is directly involved in regulating mitochondrial autophagy by inducing expression of the BH3-only proteins BNIP3 and BNIP3L, which promotes release of Beclin-1, a major autophagy regulator (Zhang et al., 2008a; Bellot et al., 2009). For a more detailed review on autophagy in cancer we refer interested readers to White (2012).

3.4. Tumor angiogenesis

A key feature of the hypoxic response is the upregulation of multiple genes that promote angiogenesis/vascularization to increase oxygen delivery. Cells grown under hypoxic conditions activate transcription of vascular endothelial growth factor (VEGF) (Shweiki et al., 1992; Forsythe et al., 1996), which is important for endothelial cell activation and proliferation. Other hypoxia-induced pro-angiogenic factors include platelet-derived growth factor-β (PDGF-β) (Kelly et al., 2003), angiopoietin-2 (ANGPT2) (Kelly et al., 2003), and stromal-derived factor 1α (SDF-1α) (Ceradini et al., 2004), all of which promote neoangiogenesis. In neuroblastoma, HIF-1 mediates acute hypoxia-induced VEGF expression, while HIF-2 regulates VEGF expression during prolonged hypoxia (Holmquist-Mengelbier et al., 2006). Furthermore, HIF-1α loss decreases tumor vascularization and impairs vascular function (Carmeliet et al., 1998), and HIF-2α knockdown in glioblastoma and neuroblastoma results in poorly vascularized tumors (Li et al., 2009; Pietras et al., 2009). Vascularization is severely impaired in HIF1A−/− (Carmeliet et al., 1998; Ryan et al., 1998) and HIF2A−/− (Peng et al., 2000) mice. Thus, both HIF-1 and HIF-2 play crucial roles in tumor vascularization, although their effect may vary depending on the temporal and cellular context.

3.5. Cancer stem cells

Hypoxia and HIFs regulate the proliferation and differentiation of different stem cell populations including embryonic, neural, and hematopoietic stem cells. The stem cell-like state is mediated by upregulation of OCT4, Notch, c-Myc, and telomerase (Keith & Simon, 2007). Certain tumor cells are known to possess cancer stem cell (CSC)-like features, although the presence of true CSCs in solid tumors remains to be
definitively proven. Irrespective of semantics and whether or not CSCs exist as a distinct sub-population of the cancer cell population, it is clear that hypoxia and HIFs regulate stem cell-like features in a proportion of cancer cells and make them more aggressive. In neuroblastoma, hypoxia downregulates neuronal and neuroendocrine differentiation markers and upregulates genes expressed during normal neural crest development (Jogi et al., 2002). HIF-2α is co-expressed with putative CSC markers in neuroblastoma and glioblastoma (Pietras et al., 2008; Li et al., 2009). Furthermore, HIF-2α silencing in glioblastoma decreases self-renewal, tumor cell proliferation in vitro, and tumor-initiating capability in vivo (Li et al., 2009). We and others have hypothesized that HIF inhibition promotes tumor cell differentiation and reduces tumor relapse after conventional therapy (Keith & Simon, 2007; Loftsted et al., 2007).

3.6. Metabolic reprogramming

Reprogramming of energy metabolism is another hallmark of cancer. In 1924, Otto Warburg observed that cancer cells metabolize glucose differently than untransformed cells. Instead of metabolizing glucose via the tricarboxylic acid (TCA) cycle and producing maximum amounts of ATP via oxidative phosphorylation, cancer cells redirect glucose metabolism away from these circuits. Instead of converting pyruvate generated from glycolysis into acetyl-CoA (for the TCA cycle), cancer cells convert pyruvate into lactate, the latter reaction only generating two ATP molecules per glucose molecule compared to 36 ATP molecules per glucose molecule during normal TCA cycling and oxidative phosphorylation. Under oxygen deprived conditions, untransformed cells also convert pyruvate into lactate, a process known as “anaerobic glycolysis”. However, cancer cells convert pyruvate into lactate even when oxygen is available, so their metabolism is often referred to as “aerobic glycolysis”, or “the Warburg effect” (Vander Heiden et al., 2009). Warburg proposed that cancer cells use aerobic glycolysis due to impaired mitochondrial function; however, later studies have shown that most cancer cells have functional mitochondria. Furthermore, studies on proliferating primary lymphocytes have shown that glucose is primarily converted to lactate, demonstrating that aerobic glycolysis is not only restricted to cancer cells (DeBerardinis et al., 2008). This raises the question: why would proliferating cells, including cancer cells, choose a pathway that produces less ATP? Even though this question has not been fully answered, one explanation could be that proliferating cells do not need optimized metabolism for maximal ATP production because the nutrient supply is not limited, so aerobic glycolysis still produces sufficient ATP. Another reason might be that cells require other molecules in addition to ATP to divide. Macromolecules such as amino acids, nucleotides, and fatty acids are needed for cellular replication and, consequently, pathways that support synthesis of these biomolecules are preferentially selected (DeBerardinis et al., 2008; Vander Heiden et al., 2009). Lactate is a byproduct of aerobic glycolysis and is considered a major player in tumor acidification. However, recent studies suggest that lactate also plays a role as a signaling molecule and can modulate the tumor microenvironment, providing a rationale to target lactic acid transporter complexes. Further reading on the therapeutic benefit of targeting lactate transporters can be found in Marchiq and Pouyssegur (2016).

Hypoxia promotes aerobic glycolysis by increasing both glucose uptake and expression of glycolytic enzymes (Iyer et al., 1998). Furthermore, Kim et al. provided the first evidence that HIF-1 actively participates in metabolic reprogramming from the TCA cycle to glycolysis by showing that HIF-1 directly targets the gene encoding pyruvate dehydrogenase kinase 1 (PDHK1) (Kim et al., 2006). PDHK1 inactivates pyruvate dehydrogenase (PDH), which catalyzes pyruvate into acetyl-CoA; therefore, by regulating PDHK1 expression, HIF-1 steers cells toward glycolysis and away from the TCA cycle. This knowledge has led to the testing of drugs that inhibit glycolysis such as dichloroacetate (DCA), which inhibits PDHK activity (Dunbar et al., 2014).

Although hypoxia and HIF signaling are important for glycolysis, other mechanisms regulate glycolytic metabolism. For instance, the oncogenic PI3K signaling pathway also promotes glucose uptake and metabolism. HIFs regulate a switch from glucose to glutamine metabolism to produce acetyl-CoA in fatty acid synthesis (Semenza, 2013). Cancer cells often consume glutamine, and those dependent on Myc signaling are highly sensitive to glutamine withdrawal (Yuneva et al., 2007). Furthermore, tumor suppressor pathways such as p53 and LKB1/AMPK signaling also regulate cellular metabolism (Vander Heiden et al., 2009). For further information on the role of hypoxia in metabolic reprogramming we refer readers to Masson and Ratcliffe (2014) and Semenza (2013).

3.7. Tumor immune response

Inflammation is another cancer hallmark, and the anti-tumor immune response plays a crucial role in many cancer types (Hanahan & Weinberg, 2011). Intratumoral hypoxia modulates the tumoral immune response in many ways, collectively pointing to an overall immunosuppressive effect (Palazon et al., 2012). For instance, tumor hypoxia and/or HIFs can attract myeloid-derived suppressor cells (Corzo et al., 2010), regulatory T-cells (Clambe et al., 2012), and tumor-associated macrophages (Doedens et al., 2010; Linty et al., 2010) with immunosuppressive functions. Hypoxia can also suppress dendritic cell function and inhibit infiltrating cytotoxic T-lymphocyte activity in a HIF-1α-dependent manner (Barsoum et al., 2014; Palazon et al., 2012). Adenosine accumulates in tumors during hypoxic stress and contributes to tumor progression via immune suppression and a direct effect on cancer cells. Adenosine is thus a crucial factor mediating the hypoxic inhibition of the anti-tumor immune response (Antonioli et al., 2013). In addition, hypoxia-induced HIF-1α drives expression of the immune checkpoint protein PD-L1, which contributes to immune suppression and evasion (Noman et al., 2014). Therefore, most evidence suggests that HIFs exert a tumor-promoting effect by immunosuppression. Immunotherapy has recently shown great promise in cancer patients, especially for the treatment of metastatic melanoma, and the combination of HIF inhibitors and immunotherapy may be a useful approach for clinical testing to further improve outcomes.

3.8. Genomic instability

Intratumoral hypoxia promotes genomic instability, another hallmark of most cancers (Bristow & Hill, 2008). Early studies demonstrated that cancer cells cultured under hypoxic conditions contain an increased number of mutations and exhibit diminished DNA repair following UV irradiation-induced DNA damage compared to normoxic cells (Reynolds et al., 1996; Yuan et al., 2000). Furthermore, hypoxia downregulates DNA repair gene expression, and hypoxia/reoxygenation cycles can induce DNA strand breaks and oxidative base damage, gene amplifications, and DNA over-replication (Bristow & Hill, 2008). Hypoxia can also lead to the epigenetic regulation and silencing of the DNA repair gene BRCA1, thereby promoting genomic instability (Lu et al., 2011). HIF-1α is responsible for genetic instability in cancer cells by inhibiting the DNA mismatch repair genes MSH2 and MSH6, which leads to decreased levels of the MSH2-MSH6 (MutSα) base mismatch recognition complex (Koshiji et al., 2005). Hypoxia-induced HIF-1 upregulates microRNAs miR-201 and miR-373, thereby suppressing DNA repair pathways and causing additional genetic instability (Crosby et al., 2009). Conversely, HIF-2 does not have the same effects on genomic stability, in part due to Thr-324 phosphorylation in the PAS-B domain, which prevents HIF-2 from repressing DNA repair genes (To et al., 2006). Furthermore, HIF-2 regulates the expression of genes involved in protecting cells from DNA damaging ROS (Scorteccia et al., 2003).
3.9. Tumor cell invasion and metastasis

Metastasis is the main cause of cancer-related deaths. Intratumoral hypoxia is associated with metastasis in experimental tumors (Young et al., 1988; Brizel et al., 1996; Cairns et al., 2001; De Jaeger et al., 2001), and HIFs have been implicated in metastatic disease in various clinical studies (Zhong et al., 1998; Zhong et al., 1999; Dales et al., 2005; Holmqist-Mengelbier et al., 2006; Helczynska et al., 2008). Interestingly, HIF-1α was overexpressed in 29% of primary breast cancers but 69% of breast cancer metastases (Zhong et al., 1999). Insights from a transgenic mouse model of metastatic breast cancer (Liao et al., 2007) and xenograft models of breast cancer (Hiraga et al., 2007; Lu et al., 2010) also point to HIF-1α as a critical regulator of metastasis.

Mechanistically, hypoxia and HIFs are involved in many different steps of the metastatic process. Epithelial-to-mesenchymal transition (EMT) is an initial step in which tumor cells lose expression of the intercellular adhesion molecule E-cadherin (encoded by CDH1) and acquire a motile phenotype. Hypoxia and HIFs contribute to EMT by upregulating transcriptional repressors such as SNAIL (Imai et al., 2003), TWIST (Yang et al., 2008), TCF3, ZFHX1A, and ZFHX1B (Krishnamachary et al., 2006), whose gene products in turn repress CDH1. Loss of VHL in ccRCC also downregulates E-cadherin in a HIF-dependent manner (Esteban et al., 2006).

Hypoxia and HIFs can also induce tumor cell invasion and degradation of the extracellular matrix via various mechanisms including upregulation of cathepsin D, urokinase-type plasminogen activator receptor, and matrix metalloproteinases-1 and -2 (Krishnamachary et al., 2003; Munoz-Najar et al., 2006) and activation of Met (Pennacchietti et al., 2006). The RIOK3 kinase promotes re-organization of the actin cytoskeleton in a HIF-1α-dependent manner, which has been shown to increase breast cancer cell migration and invasion/metastasis (Singleton et al., 2015). HIF-1 activates transcription of collagen prolyl hydroxylases, which promote collagen deposition and breast cancer cell migration along collagen fibers, thereby enhancing invasion and metastasis (Gilks et al., 2013). HIF regulates lysyl oxidase, a critical mediator of hypoxia-induced breast cancer cell migration and metastasis, which mediates its action through increased focal adhesion kinase activity and extracellular matrix modulation (Erler et al., 2006).

Hypoxia-induced VEGF production promotes lymph- and angiogenesis, which facilitate tumor cell intravasation and dissemination (Claffey et al., 1996; Schoppmann et al., 2006). VEGF can also contribute to systemic intravasation by increasing microvascular permeability (Claffey et al., 1996; Dvorak et al., 1999). HIF-1α-dependent expression of angiopoietin-like 4 and the L1 cell adhesion molecule further modulate intra- and extravasation (Zhang et al., 2012).

Tumor cell homing to distant sites involves the HIF-regulated chemokine receptor CXCR4 (Staller et al., 2003), which binds to stromal-derived factor-1 (SDF-1α) in secondary organs and drives breast cancer metastasis (Muller et al., 2001). Primary tumor hypoxia can also promote metastatic spread by establishing a pre-metastatic niche in distant organs. Specifically, hypoxia-induced lysyl oxidase contributes to the pre-metastatic niche in a process that includes collagen crosslinking and recruitment of CD11b+ myeloid cells (Erler et al., 2009). Finally, HIF expression in tumor stromal cells can also affect metastasis: HIF-2 expression in tumor endothelial cells has been shown to increase tumor cell migration and metastasis, while endothelial HIF-1 reduces cell migration and metastasis (Branco-Price et al., 2012).

We refer readers to Rundqvist and Johnson (2010) for a detailed review of HIFs in breast cancer metastasis and to Johnson et al. (2015) for a review of the role of hypoxia and HIFs in bone metastasis.

3.10. Treatment resistance

Intrinsic resistance and acquired resistance to radiotherapy (ionizing radiation; IR) and chemotherapy are major reasons for treatment failure in cancer patients. It has been known since the 1950s that hypoxia is a crucial mediator of chemotherapeutic resistance (Gray et al., 1953; Roizin-Towle & Hall, 1978). HIF overexpression in clinical samples is associated with therapeutic resistance or decreased survival following IR or chemotherapy (Aebersold et al., 2001; Birner et al., 2001b; Koukourakis et al., 2002; Burrij et al., 2003; Generali et al., 2006; Vergis et al., 2008).

Under normoxic conditions, IR generates ROS that cause irreparable DNA damage and cell death. Intratumoral hypoxia prevents ROS formation, resulting in inefficient DNA strand breaks and IR resistance (Brown & Wilson, 2004). HIF-1α expression is temporarily and acutely decreased after IR due to re-oxygenation of surviving hypoxic tumor cells (Harada et al., 2009). ROS production later stabilizes the HIF-1α protein and increases expression of HIF-1α and its target genes (Moeller et al., 2004; Dewhirst et al., 2008; Harada et al., 2009). HIF-1α appears to exert both radiosensitizing (e.g., by promoting proliferation and p53-induced apoptosis) and radioreistant effects (via vascular radioprotection) (Moeller et al., 2005). HIF-1α-mediated alterations in tumor glucose metabolism may also affect cellular responses to IR (Meijer et al., 2012). Importantly, silencing or pharmacological inhibition of HIF-1α can increase the anti-tumor effects of IR (Zhang et al., 2004; Moeller et al., 2005; Harada et al., 2009; Harada et al., 2012). HIF-2α contributes to tumor cell survival after IR, and HIF-2α inhibition can increase the IR effect by activating the p53 pathway and increasing apoptosis (Bertout et al., 2009). Thus, HIF inhibitors can be used in combination with radiotherapy to target radiotherapy-resistant tumor cells. Furthermore, radiosensitizers such as misonidazole mimic the effect of oxygen and can be used to enhance IR efficiency in hypoxic tumors, particularly in head and neck cancers (Brown & Wilson, 2004).

A number of studies suggest that HIFs mediate chemoresistance. One main mechanism by which HIF-1 mediates chemoresistance is by activating the multidrug resistance 1 (MDR1) gene (Comerford et al., 2002), which encodes a glycoprotein belonging to the ATP-binding cassette (ABC) transporter family and functions as a drug efflux pump. In this way, HIF-1α and MDR1 can decrease the intracellular concentration of various chemotherapeutic drugs and contribute to hypoxia-induced drug resistance (Comerford et al., 2002). Similarly, HIF-2 can activate transcription of another ABC transporter ABCG2, suggesting a potential mechanism of HIF2-induced chemoresistance (Martin et al., 2008). Hypoxia and/or HIFs can mediate chemotherapy resistance through additional mechanisms including: a) hypoxia-induced decreases in tumor cell proliferation (Tannock, 1968); b) metabolic reprogramming; c) hypoxia-driven selection of p53-mutated tumor cells with less apoptotic capacity (Graeb et al., 1996); and d) inhibition of DNA damage (Sullivan & Graham, 2009). Taken together, these data on the role of HIFs in therapeutic resistance suggest that HIF1/2 inhibitors might be effective in combination with conventional chemotherapy (Unruh et al., 2003; Erler et al., 2004; Brown et al., 2006; Nardinocchi et al., 2009; Roberts et al., 2009; He et al., 2012; Zhao et al., 2014). For a comprehensive review of the mechanisms underlying HIF-induced chemotherapeutic resistance, we refer the reader to Rohwer and Cramer (2011).

4. Targeting hypoxia and hypoxia-inducible factors in cancer

Since hypoxia is a hallmark of solid tumors and mediates aggressive, metastatic, and resistant disease, it is arguably one of the most attractive therapeutic targets in cancer. Several approaches for targeting hypoxic tumor cells have been proposed including hypoxia-activated prodrugs, gene therapy, recombinant anaerobic bacteria, specific targeting of HIFs, or targeting pathways important in hypoxic cells such as the mTOR and UPR pathways (Melillo, 2007; Wilson & Hay, 2011; Semenza, 2012). We briefly discuss the mechanism of action of hypoxia-activated prodrugs before describing in detail the drugs that directly or indirectly modulate HIFs (see Fig. 2 and Table 1).
prodrugs, we refer the reader to Phillips (2016).

Table 1

<table>
<thead>
<tr>
<th>Inhibitory mechanism</th>
<th>Drug</th>
<th>Target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>mRNA/protein expression</td>
<td>Wortmannin</td>
<td>PI3K</td>
<td>Jiang et al., 2001</td>
</tr>
<tr>
<td></td>
<td>LYS94002</td>
<td>PI3K</td>
<td>Jiang et al., 2001; Mohlin et al., 2015</td>
</tr>
<tr>
<td></td>
<td>GDC-0941</td>
<td>PI3K</td>
<td>Mohlin et al., 2015</td>
</tr>
<tr>
<td></td>
<td>PI-103</td>
<td>PI3K</td>
<td>Mohlin et al., 2015</td>
</tr>
<tr>
<td></td>
<td>Rapamycin</td>
<td>mTOR</td>
<td>Hudson et al., 2002</td>
</tr>
<tr>
<td></td>
<td>PP242</td>
<td>mTOR</td>
<td>Mohlin et al., 2015</td>
</tr>
<tr>
<td>Aminoflavone</td>
<td>unknown</td>
<td>unknown</td>
<td>Terzulli et al., 2010</td>
</tr>
<tr>
<td>Glycoconis</td>
<td>AKT/mTOR, Hsp90</td>
<td></td>
<td>Lee et al., 2015</td>
</tr>
<tr>
<td>Topotecan, PEG-SN38</td>
<td>Topoisomerase 1</td>
<td></td>
<td>Rapisarda et al., 2004; Pastoreno et al., 2010</td>
</tr>
<tr>
<td>EZN-2968</td>
<td>HIF-1α mRNA</td>
<td></td>
<td>Greenberger et al., 2008</td>
</tr>
<tr>
<td>2ME2, ENMD-1198</td>
<td>Microtubules</td>
<td></td>
<td>Malbueh et al., 2003; LaVallee et al., 2008</td>
</tr>
<tr>
<td>Geldanamycin and analogs</td>
<td>Hsp90</td>
<td></td>
<td>Issacs et al., 2002</td>
</tr>
<tr>
<td>Verinostat</td>
<td>HDAC</td>
<td></td>
<td>Hort et al., 2014</td>
</tr>
<tr>
<td>YC-1</td>
<td>unknown</td>
<td></td>
<td>Chun et al., 2001; Li et al., 2008</td>
</tr>
<tr>
<td>PX-478</td>
<td>unknown</td>
<td></td>
<td>Koh et al., 2008</td>
</tr>
<tr>
<td>PX-12, pleurin</td>
<td>Thioredoxin-1</td>
<td></td>
<td>Welsh et al., 2003</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>unknown</td>
<td></td>
<td>Zhang et al., 2008b</td>
</tr>
<tr>
<td>FM19G11</td>
<td>unknown</td>
<td></td>
<td>Moreno-Manzano et al., 2010</td>
</tr>
<tr>
<td>HIF-2α translational inhibitors</td>
<td>IRP1/IRE interaction</td>
<td></td>
<td>Zimmer et al., 2008</td>
</tr>
<tr>
<td>HIF-α/HIF-1α dimerization</td>
<td>Acriflavine</td>
<td>HIF-1α/2α PAS-B domain</td>
<td>Lee et al., 2009</td>
</tr>
<tr>
<td>HIF binding</td>
<td>Echinomycin</td>
<td>HRE</td>
<td>Scheurmann et al., 2013</td>
</tr>
<tr>
<td>DNA binding</td>
<td>Polyamides</td>
<td>HRE</td>
<td>Kong et al., 2005</td>
</tr>
<tr>
<td>Transcriptional activity</td>
<td>Chetomin</td>
<td>p300 recruitment</td>
<td>Kung et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Bortezomib</td>
<td>p300 recruitment</td>
<td>Kaluz et al., 2006; Shin et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Amphotericin B</td>
<td>HIF-1α interaction and p300 recruitment</td>
<td>Yeo et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Triptolide</td>
<td>Hsp70</td>
<td>Zhou et al., 2010</td>
</tr>
<tr>
<td></td>
<td>AM290, AW464</td>
<td>Thioredoxin-1</td>
<td>Jones et al., 2006</td>
</tr>
</tbody>
</table>

PI3K, phosphoinositide 3-kinase; mTOR, mechanistic/mammalian target of rapamycin; Hsp90, heat shock protein 90; HDAC, histone deacetylase; IRP1, iron-responsive element-binding protein 1; IRE, iron response element; HRE, hypoxia response element; FIH-1, factor inhibiting HIF-1; Hsp70, heat shock protein 70.

4.1. Hypoxia-activated prodrugs

A prodrug is an inactive compound that can, either spontaneously or via specific metabolic pathways, be converted to its pharmacologically active species. One way of exploiting tumor hypoxia has been to design hypoxic prodrugs that are activated in hypoxic tissue and thus selectively kill hypoxic tumor cells (Denny, 2000). Hypoxic prodrugs are activated by reduction of the prodrug by cellular reductases. One-electron reductases generate a prodrug radical that can be further reduced into the active toxic compound in hypoxic cells or be re-oxidized back to the prodrug in normoxic cells. Alternatively, some prodrugs are reduced by two-electron reductases, thus bypassing the formation of a prodrug radical.

The hypoxic prodrug tirapazamine has been extensively tested in clinical trials, but results have been disappointing (Rischin et al., 2010; DiSilvestro et al., 2014). A second-generation tirapazamine analog SN30000 has improved pharmacodynamic and pharmacokinetic properties and superior anti-tumor effects in xenograft models (Hicks et al., 2010). Some of the disappointment in clinical trials may have been due to a failure to adopt a “personalized” or precision approach: to optimize the potential therapeutic effect, it is crucial that patients are stratified based on tumor-specific predictive biomarker expression that indicates sensitivity to the drug being tested. However, predictive biomarkers for hypoxic prodrugs are poorly defined. Interestingly, P450 oxidoreductase was recently identified as a biomarker of sensitivity to the hypoxia prodrugs SN30000 and TH-302 (Hunter et al., 2015). Results from recently published phase II clinical trials for TH-302 in combination with gemcitabine in pancreatic cancer (Borad et al., 2015) or in combination with doxorubicin in soft tissue sarcoma (Chawla et al., 2014) are encouraging, and phase III studies have been initiated. The prodrug apaziquone (EO9), a mitomycin C derivative, showed efficacy in preclinical studies, but clinical trials were negative. An evaluation of why apaziquone failed showed that the drug had poor pharmacokinetics: the drug could not penetrate the tumor and was rapidly degraded on systemic delivery (Phillips et al., 2013).

![Fig. 4. Activation mechanism of hypoxic prodrugs. The prodrug can be converted into its active compound either by one-electron or two-electron reduction. One-electron reduction produces a prodrug radical that can be further reduced into the active toxic compound in hypoxic cells or be re-oxidized back to the prodrug in normoxic cells. Alternatively, some prodrugs are reduced by two-electron reductases, thus bypassing the formation of a prodrug radical.](image-url)
Therefore, loco-regional administration of apaziquone was proposed as a superior option to avoid problems with drug delivery and clearance. Therefore, loco-regional administration of apaziquone was proposed as a superior option to avoid problems with drug delivery and clearance. Examples of hypoxia prodrugs in preclinical and clinical trials.

<table>
<thead>
<tr>
<th>Prodrug</th>
<th>Clinical trial status</th>
<th>Cancer</th>
<th>NCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH-302 (Evofoxamide)</td>
<td>Phase III</td>
<td>Soft tissue sarcoma</td>
<td>NCT014400088</td>
</tr>
<tr>
<td></td>
<td>Phase III (active)</td>
<td>Pancreatic cancer</td>
<td>NCT01746979</td>
</tr>
<tr>
<td></td>
<td>Phase III (completed)</td>
<td>Bladder cancer</td>
<td>NCT00598806</td>
</tr>
<tr>
<td></td>
<td>Phase III (active)</td>
<td></td>
<td>NCT00461591</td>
</tr>
<tr>
<td></td>
<td>Phase III (completed)</td>
<td></td>
<td>NCT014010565</td>
</tr>
<tr>
<td></td>
<td>Phase III (completed)</td>
<td></td>
<td>NCT02563561</td>
</tr>
<tr>
<td></td>
<td>Phase III (completed)</td>
<td></td>
<td>NCT01469221</td>
</tr>
<tr>
<td>AO4N</td>
<td>Phase I/II</td>
<td>Multiple</td>
<td>NCT00394628</td>
</tr>
<tr>
<td></td>
<td>Phase I/II</td>
<td></td>
<td>NCT00109356</td>
</tr>
<tr>
<td></td>
<td>Phase I</td>
<td></td>
<td>NCT00090727</td>
</tr>
<tr>
<td></td>
<td>Phase I/II (completed)</td>
<td>Acute leukemia</td>
<td>NCT0103756</td>
</tr>
<tr>
<td>Tirapazamine (TPZ)</td>
<td>Phase III (completed)</td>
<td>Cervical cancer</td>
<td>NCT00262821</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Head and neck cancer</td>
<td>NCT00174837</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung cancer</td>
<td>NCT00094081</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NCT00174599</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NCT00064848</td>
</tr>
<tr>
<td>SN30000 (CEN-209)</td>
<td>Preclinical</td>
<td>Non small cell lung cancer</td>
<td>NCT02454842</td>
</tr>
<tr>
<td>TH-4000</td>
<td>Phase II (active)</td>
<td>Squamous cell carcinoma</td>
<td>NCT02440681</td>
</tr>
</tbody>
</table>

### 4.2. Drugs targeting hypoxic signaling

Strategies to target hypoxia include direct and indirect HIF targeting as well as targeting of downstream HIF signaling pathways. A classic example of the latter is anti-VEGF-therapy: monoclonal antibodies targeting VEGF (bevacizumab) or small molecule inhibitors targeting the VEGF receptor have shown clinical benefit in advanced cancer (Ellis & Hicklin, 2008). There are considerably fewer selective and specific HIF inhibitors compared to non-selective inhibitors that target multiple pathways including the HIF pathway. Nonetheless, the non-selective inhibition of HIFs might be an important anti-tumor mechanism in practice. In the following section (and Fig. 2), we summarize the drugs that have been shown to directly or indirectly target HIFs or their pathways.

#### 4.2.1. Inhibitors of hypoxia-inducible factor messenger ribonucleic acid or protein expression

**Aminoflavone** is an aryl hydrocarbon receptor ligand, has been shown to partially inhibit HIF-1α mRNA expression in breast cancer cells and almost completely inhibit HIF-1α protein accumulation and transcription of downstream target genes in an aryl hydrocarbon receptor-independent fashion (Terzuoli et al., 2010). However, its clinical utility has not been demonstrated, and several clinical trials using the prodrug AFP-464 have either been terminated or withdrawn prior to enrollment due to lack of efficacy.

**Inhibitors of phosphoinositide 3-kinase/mechanistic/mammalian target of rapamycin.** Apart from the well-defined oxygen-mediated regulation of HIF-α subunits, translation of HIF-α mRNA is controlled by growth factor signaling pathways such as the PI3K/AKT/mTOR pathway. Thus, molecules that target this pathway presumably also modulate HIF activity. Consistent with this, several studies have shown that mTOR inhibition decreases HIF-1α and HIF-2α levels under both normoxic and hypoxic conditions (Hudson et al., 2002; Majumder et al., 2004; Thomas et al., 2006; Mohlin et al., 2015). We recently showed that hypoxia-induced HIF-2α transcription in neuroblastoma cells is highly dependent on PI3K-mTORC2 but not PI3K-mTORC1, further supporting the hypothesis that targeting this pathway abrogates HIF activity (Mohlin et al., 2015). A novel group of HIF-1 inhibitors called glyceollins (isolated from soybeans) can also block HIF-1α translation by inhibiting the PI3K/AKT/mTOR pathway under hypoxic conditions (Lee et al., 2015).

**Topoisomerase 1 inhibitors.** Irinotecan and topotecan are FDA-approved topoisomerase I inhibitors and analogs of the naturally occurring cytoxic alkaloid camptothecin. Topotecan was identified as an inhibitor of HIF-1 activity (Rapisarda et al., 2002) and was later shown to inhibit HIF-1α translation (Rapisarda et al., 2004). More recently, the camptothecin-induced effects on HIF-1α levels were shown to occur as a result of changes in miRNA expression (Bortozzi et al., 2014). In a small pilot trial, topotecan decreased HIF-1α protein expression in 4 out of 7 paired tumor biopsies taken from patients before and after treatment (Kummar et al., 2011). In an ongoing phase II study of bevacizumab and irinotecan in young patients with brain tumors, one of the secondary outcomes is measurement of HIF-2α expression at baseline and association of HIF-2α expression with progression-free survival. SN-38 is the active metabolite of irinotecan, and attachment of polyethylene glycol (PEG) to SN-38 improves drug solubility: PEG-SN38 (or EZN-2208) has been shown to downregulate both HIF-1α and HIF-2α in a neuroblastoma model (Pastorino et al., 2010), and a phase I study of PEG-SN38 patients with neuroblastoma and other solid tumors showed low toxicity and supported further study of this drug in neuroblastoma (Norris et al., 2014). Furthermore, PEG-SN38 in combination with all-trans retinoic acid was synergistic in a mouse model of acute promyelocytic leukemia (Coltellia et al., 2015).

**Synthetic oligonucleotides.** EZN-2968 is a synthetic antisense oligonucleotide comprised of 16 nucleotide residues 100% complementary to residues in the mRNA coding sequence of human HIF-1α. EZN-2968 binding leads to HIF-1α mRNA downregulation in a dose-dependent manner and results in near complete inhibition at 5 nM concentrations (Greenberger et al., 2008). EZN-2968 has a three base pair mismatch with the HIF-2α sequence and, as a consequence, only downregulates HIF-2α mRNA by 20% at 25 nM. EZN-2968 treatment of tumor cells in vitro downregulated HIF-1α protein levels during hypoxia and reduced the growth rate. The in vivo effect of EZN-2968 has also been tested in a subcutaneous prostate cancer cell line xenograft model and, although treatment did not result in a significant reduction in tumor burden, pre-treatment of cells with EZN-2968 prior to injection resulted in a significant reduction in tumor size. EZN-2968 has also been evaluated in two phase I clinical trials: results from one trial showed reduced HIF-1α mRNA in post-treatment biopsies from 4 out of 6 patients with paired tumor biopsies, and HIF-1α protein and mRNA levels of target genes were reduced in biopsies from two patients. Although the trial provided preliminary proof of concept for modulation of HIF-1α mRNA and protein expression in response to EZN-2968, the trial closed prematurely due to suspended development of the compound (Jeong et al., 2014). A third phase I trial has recently been initiated in patients with hepatocellular carcinoma to establish proof-of-mechanism for EZN-2968 (R07070179).

2-Methoxyestradiol (2ME2) and analogs. 2ME2 is a microtubule-targeting estrogen metabolite that causes mitotic arrest. 2ME2 inhibits tumor growth and angiogenesis by downregulating HIF-1α protein expression, most likely through translational inhibition (Mahjeesh et al., 2003). A recent report has suggested that 2ME2 also downregulates HIF-2α to overcome sorafenib resistance in hepatocellular carcinoma cells (Ma et al., 2014). The safety and efficacy of 2ME2 (Panzem™)
have been evaluated in numerous phase I and phase II studies, and there have been efforts to generate more effective 2ME2 analogs. To this end, ENMD-1198 has been selected as a lead analog (LaVallee et al., 2008) and has been tested in a phase I dose-escalation study in advanced cancer patients (Zhou et al., 2011).

Heat shock protein inhibitors. Heat shock proteins (Hsp) are cellular chaperones that ensure that target proteins are correctly folded and localized. Geldanamycin (GA) is an anti-cancer antibiotic that promotes protein degradation by binding and inhibiting Hsp90. GA can induce proteosomal degradation of HIF-α in a VHL-independent manner both under normoxic and hypoxic conditions (Isaacs et al., 2002). The GA analogs 17-AAG (tanespimycin) and 17-DMAG (alvespimycin) have been evaluated in several phase I and phase II trials either alone or in combination with other chemotherapies. In a comparative analysis, a next-generation small molecule Hsp90 inhibitor EC154 more effectively inhibited HIF-1α and HIF-2α than 17-AAG (Bohonowycz et al., 2011). This study highlights the discordance between HIF activity and HIF protein expression, emphasizing that pharmaceutical inhibition of HIF activity is not necessarily equivalent to HIF tissue expression and, therefore, HIF biomarker analysis is not always a robust surrogate of HIF suppression.

Triptolide, isolated from a Chinese herb, mediates its anti-tumor effects by inhibiting Hsp70. Somewhat unexpectedly, triptolide increased HIF-1α levels under both normoxic and hypoxic conditions, although target gene transcription was reduced suggesting that triptolide inhibits HIF-1 activity (Zhou et al., 2010). The triptolide produg Minneld™ is currently being tested in a phase I trial of patients with advanced gastrointestinal tumors (NCT01927965).

Histone deacetylase (HDAC) inhibitors. HDACs remove acetyl groups from histones or non-histone proteins such as transcription factors. The HDAC inhibitors vorinostat (SAHA, Zolinza®), romidipin (Istodax®, previously known as FK228), panobinostat (Farydak®), and belinostat (Bleomdaq®, also known as PXD101) are FDA approved for the treatment of various cancers. HDAC inhibitors have been shown to promote HIF-1α degradation and, although the exact mechanism is not completely understood, transcriptional inhibition of HIF-1α has been proposed as vorinostat’s mechanism of action (Hutt et al., 2014). Another mechanism of HDAC inhibitor action might be via Hsp90 inhibition, as described above. Both HIF-1α and HIF-2α are themselves regulated by acetylation and deacetylation events but in opposing manners (Keith et al., 2012). Deacetylation of HIF-2α by SIRT1, a deacetylase, increases HIF-2α’s transcriptional activity, while deacetylation of HIF-1α results in transcriptional repression. This suggests that HDAC inhibitors can have direct but contrasting effects on HIF-α subunits.

YC-1 was originally discovered as an activator of soluble guanylate cyclase, an enzyme that increases cGMP levels and, as a result, inhibits platelet aggregation and vasconstriction. Additionally, YC-1 has anti-tumor effects by inhibiting HIF activity via an unknown non-guanylate cyclase-mediated mechanism. Treatment of hepatocellular carcinoma Hep3B cells with YC-1 decreased hypoxic induction of erythropoietin (EPO) and VEGF, decreased HIF-1α protein accumulation, and reduced HIF-1/ΔRNA binding (Chun et al., 2001). Bearing in mind that EPO is a specific HIF-2 target (Rankin et al., 2007), the observed downregulation of EPO in Hep3B cells by YC-1 suggests that YC-1 also inhibits HIF-2 activity. YC-1 is thought to inhibit HIF-1 activity by preventing its interaction with the p300 co-activator (Li et al., 2008), i.e., the same mechanism used by the endogenous FIH enzyme. However, empirically this mechanism was cell line dependent and was not observed in Hep3B cells. This suggests that EPO downregulation in Hep3B cells upon YC-1 treatment is not mediated by preventing HIF interaction with p300 but rather via another unknown mechanism. This is consistent with the observation that HIF-2 is not regulated to the same extent as HIF-1 by FIH. YC-1 also has anti-tumor and anti-angiogenic effects in several xenograft models (Yeo et al., 2003) and has also been shown to inhibit tumor cell invasion and metastasis (Shin et al., 2007), but there have been no YC-1 clinical trials to date. Based on YC-1’s structure, new analogs have been synthesized and screened for their HIF inhibitory effects (Masoud et al., 2015).

PX-478 is derived from the alkylating agent melphalan, a cytotoxic agent used in the treatment of multiple myeloma and some other cancers including ovarian cancer. PX-478 inhibits HIF-1α at multiple levels, primarily by translational inhibition and, to a lesser extent, by promoting protein degradation in an oxygen-, pVHL-, and p53-independent manner (Koh et al., 2008). PX-478 has shown anti-tumor activity in several xenograft models (Welsh et al., 2004), and a phase 1 dose-escalation study of PX-478 in patients with advanced solid tumors or lymphoma showed, at best, stable disease in 14 out of 36 evaluable patients (Ban et al., 2011).

Thioredoxin inhibitors. PX-12 is an inhibitor of the redox protein thioredoxin (Trx-1). Trx-1 is found at high levels in many human cancers and is also associated with increased expression of HIF-1α and HIF-regulated genes (Welsh et al., 2002). Two Trx-1 inhibitors, PX-12 and pleurotin, were initially found to decrease HIF-1α and VEGF levels in vitro and in vivo (Welsh et al., 2003). However, a phase II trial of PX-12 in patients with advanced pancreatic cancer was terminated early due to lack of significant efficacy (Ramanathan et al., 2011). Two novel Trx-1 inhibitors, AJM290 and AW464, also inhibited HIF transcriptional activity and DNA binding but, unexpectedly and in contrast to other Trx-1 inhibitors, stabilized both HIF-1α and HIF-2α (Jones et al., 2006).

Cardiac glycosides were identified as HIF inhibitors in a cell-based screen (Zhang et al., 2008b). Both HIF-1α and HIF-2α protein expression were reduced during treatment with the cardiac glycosides ouabain, proscillaridin A, and digoxin. In contrast, HIF-1α mRNA levels increased during treatment. Of note, the in vitro doses used were much higher than circulating therapeutic doses of digoxin and would, therefore, be expected to induce severe toxicity (Lopez-Lazaro, 2009). Also, species-related differences in cardiac glycoside sensitivity might contribute to the anti-tumor effects of digoxin observed in mice harboring human malignant cells. A phase II breast cancer trial recently commenced in which changes in HIF-1α protein expression in tumor tissue will be evaluated in patients taking daily digoxin for two weeks prior to surgery compared to no drug prior to surgery (NCT01763931).

FM19G11 was identified as a novel, low cytotoxicity inhibitor of HIF transcriptional activity in a cell-based screen (Moreno-Manzano et al., 2010). Both HIF-1α and HIF-2α protein accumulation was inhibited during hypoxia independent of proteasome activity, and FM19G11 also promoted differentiation of hypoxic neural stem cells, possibly through downregulation of Sox2 and Oct4.

Hypoxia-inducible factor-2α translational inhibitors. HIF-2α translation is regulated by an iron-dependent mechanism involving an iron-responsive element (IRE) in the 5′-UTR of the HIF-2α mRNA (Sanchez et al., 2007). The IREs can bind iron-regulatory proteins (IRPs), which then repress mRNA translation. Binding of IRP1 to the IRE in the 5′-UTR of HIF-2α mRNA is impaired during hypoxia, leading to derepression of HIF-2α mRNA translation. However, when intracellular iron levels are low, IRP1 still binds to the IRE and represses HIF-2α mRNA translation. Zimmer et al. identified compounds that could repress HIF-2α activity by promoting IRP1 binding to the IRE in the HIF-2α 5′-UTR (Zimmer et al., 2008).
4.2.2. Inhibitors of hypoxia-inducible factor dimerization

The PAS domains in the HIF-α and HIF-1β/ARNT subunits mediate HIF complex assembly, and small molecules that target these domains have been screened to discover inhibitors of this protein–protein interaction.

Acriflavine is a potent inhibitor of HIF-α–HIF-1β dimerization by directly binding to the PAS-B domain of both HIF-1α and HIF-2α (Lee et al., 2009). Treatment with acriflavine reduced tumor growth and vascularization in prostate and hepatocellular xenograft models (Lee et al., 2009).

PT2385. Based on the crystal structures of the PAS–HIF-2α and PAS–HIF-1β heterodimers, the HIF-2α PAS-B domain was found to harbor a large internal cavity (Scheuermann et al., 2009). Interestingly, an allosteric ligand that binds in this cavity and inhibits HIF-2 transcriptional activity has recently been described (Scheuermann et al., 2013). The ligand did not affect HIF-2α mRNA and protein levels; thus, this inhibitor is unlikely to affect HIF-1–independent HIF-2α mechanisms such as translational activation. Based on this ligand, a novel compound PT2385 has now entered a phase 1 dose-escalation trial in patients with advanced ccRCC (NCT02293980). Since HIF-2α rather than HIF-1α is the major driver of VHL-mutated ccRCC (Kondo et al., 2003; Carroll & Ashcroft, 2006), specific targeting of HIF-2 in this tumor is likely to be more beneficial.

4.2.3. Inhibitors of hypoxia-inducible factor–deoxyribonucleic acid binding

Transcriptional activation of target genes by HIFs requires DNA binding. Inhibiting this step is another potential mechanism to inhibit HIF activity.

Echinomycin is a small antibiotic molecule isolated from Streptomyces echinatus that binds DNA in a sequence-specific manner. In cell-based screening, echinomycin inhibited binding of HIF-1 to HREs and thus prevented induction of hypoxia-induced VEGF expression (Kong et al., 2005). In a mouse model of relapsed acute myeloid leukemia, echinomycin cured mice without affecting normal hematopoietic stem cells (Wang et al., 2014). Nevertheless, clinical trials of echinomycin have been disappointing, and thus its therapeutic relevance remains to be established (Melillo, 2007).

Polyamides. Synthetic oligomers containing N-methylpyrrole and N-methylimidazole amino acids can be modified to target specific DNA sequences to modulate gene expression. A polyamide designed to target HREs inhibited binding of HIF-1 to the VEGF HRE and blocked VEGF synthesis (Olensyk et al., 2004). A recent study compared polyamide uptake in different tumor xenografts and showed that there was significant variability in compound uptake in xenografts of different origin (Raskatov et al., 2014). Polyamides are currently not in clinical use.

4.2.4. Inhibitors of hypoxia-inducible factor transcriptional activity

N-TAD and C-TAD, the two functional HIF domains, are responsible for mediating their transcriptional activity by interacting with co-activators such as p300/CREB. Therefore, transcriptional inhibition has been pursued as a therapeutic strategy by interfering with this HIF-p300 interaction.

Chetomin, a Chaetomium fungus metabolite, was identified as a potent disrupter of the HIF-p300 interaction in a high-throughput screening study of >600,000 substances (Kung et al., 2004). Chetomin inhibited binding of both HIF-1α and HIF-2α to p300 and in vivo antitumor effects were also demonstrated. In a later study, chetomin was shown to disrupt zinc-binding sites in the p300 CH1 domain, which hindered the HIF-p300 interaction (Cook et al., 2009). Glutoxin and chaetocin are other naturally occurring metabolites belonging to the same family as chetomin, and they have also been shown to block the HIF-p300 interaction (Rhee et al., 2014). However, due to chetomin’s toxicity, this drug has not been pursued clinically (Onnis et al., 2009).

Proteasome inhibitors. Bortezomib (Velcade™) is an FDA-approved proteasome inhibitor for the treatment of multiple myeloma and mantle cell lymphoma. Bortezomib blocked hypoxia-induced CAIX, EPO, and VEGF accumulation in cell lines by inhibiting HIF-1α C-TAD’s transcriptional activity (Kaluz et al., 2006; Shin et al., 2008). However, data were conflicting as to whether this was due to repression of the HIF-1–p300 interaction. Since bortezomib blocked hypoxic EPO production in Hep3B cells (Shin et al., 2008), HIF-2 activity was also presumably compromised, but this was not investigated. As a single agent in a phase II trial, bortezomib was inactive in patients with metastatic colorectal cancer even though tumor biopsies collected pre- and post-treatment showed HIF-1α accumulation and reduced CAIX levels, indicating that hypoxic signaling pathways were affected (Mackay et al., 2005). Falchok et al. hypothesized that bortezomib combined with bevacizumab would overcome anti-angiogenic therapy resistance by blocking HIF-1α upregulation, although the published study only recruited a small number of patients and a lack of paired biopsies precluded any robust analysis of changes in HIF-1α and downstream targets (Falchok et al., 2014).

Amphotericin B (AmB) is used to treat systemic fungal infections, but long-term treatment is associated with anemia, supposedly by EPO suppression. Yeo et al. showed that AmB treatment downregulated EPO mRNA and protein levels in rat kidneys and Hep3B cells (Yeo et al., 2006). Only HIF-1α protein levels were investigated in this study, and AmB affected neither protein levels nor HIF-1α localization, suggesting that reduced EPO was due to repressed HIF-1α C-TAD activity via enhanced interaction between HIF-1α and FIH-1. However, the downregulation of EPO implies that AmB could also inhibit HIF-2 activity.

5. Neural crest-derived tumors as model systems for targeting hypoxia-inducible factor-2 and the pseudohypoxic phenotype

The neural crest-derived sympathoadrenal stem cell gives rise to a sympathetic nervous system (SNS) composed of cells from three differentiation lineages, namely the ganglionic/neuronal, chromaffin, and SIF (small intensely fluorescent) lineages (Landis & Patterson, 1981). Several observations suggest that HIF-2 signaling has specific roles during SNS development. HIF-2α is transiently and specifically expressed in human embryonal and fetal SNS cells and during rodent SNS development (Tian et al., 1998; Nilsson et al., 2005; Mohlin et al., 2013). Furthermore, hypoxia and HIFs are important regulators of the synthesis (Schnell et al., 2003) and secretion (Kumar et al., 1998) of catecholamines, which are key SNS neurotransmitters. Consistent with these observations, mouse embryos lacking HIF-2α have severely reduced norepinephrine levels compared to wild type mice (Tian et al., 1998).

Human tumors develop from at least the ganglionic and the chromaffin SNS lineages. Pheochromocytomas and paragangliomas arise from chromaffin cells in the adrenal medulla and extra-adrenal sympathetic paraganglia, respectively, and affect adults and, rarely, children. Neuroblastomas arise from sympathetic neural precursor cells in the adrenal medulla or sympathetic ganglia and are bona fide childhood tumors. The vast majority of pheochromocytomas and paragangliomas are benign, in contrast to neuroblastomas that present with metastases at the time of diagnosis in about a half of patients. Interestingly, the tumorigenesis of these closely related tumors seems to involve aberrant HIF-2 signaling, resulting in pseudohypoxic phenotypes linked to disseminated disease (Eisenhofer et al., 2004; Pietras et al., 2008; Favier et al., 2009). Somatic gain-of-function mutations in HIF2A have been
discovered in pheochromocytomas and paragangliomas (Zhuang et al., 2012; Comino-Mendez et al., 2013; Lorenzo et al., 2013; Taieb et al., 2013; Toledo et al., 2013; Yang et al., 2013), while high HIF-2α expression is associated with poor survival in patients with neuroblastoma (Holmquist-Mengelbier et al., 2006). Interestingly, HIF-2α-positive neuroblastoma cells have been identified in perivascular niches (Holmquist-Mengelbier et al., 2006; Pietras et al., 2008), indicating that HIF-2α protein is stabilized in these cells despite sufficient oxygen and facilitating the pseudohypoxic phenotype in tumor cells. Thus, these SNS-derived tumors provide models to study HIF-2’s role in tumorigenesis and metastasis and to test novel treatment protocols that target HIF-2 directly or indirectly by interfering with upstream or downstream pathways (Richter et al., 2013; Mohlin et al., 2015).

Pheochromocytomas and paragangliomas have the largest hereditary component of all human tumors, and many of the associated germline mutations (such as those in VHL and succinate dehydrogenase (SDH)) result in pseudohypoxic phenotypes (Dahia, 2014). As noted above, VHL is a crucial mediator of HIF-α degradation at normoxia; thus, VHL loss stabilizes HIF-α and results in pseudohypoxia. Mutations in SDHx (SDHA, SDHB, SDHC or SDHD) lead to succinate accumulation, which acts as a competitive inhibitor of the 2-oxoglutarate-dependent dioxygenases such as the PHDs, again stabilizing HIF-α and causing pseudohypoxia.

Unsupervised hierarchical classification of gene expression profiles of pheochromocytomas and paragangliomas distinguishes two main clusters. Cluster 1 includes HIF2A-, VHL-, and SDHx-related tumors and is characterized by expression of genes involved in hypoxic signaling and high expression of HIF-2α itself (Eisenhofer et al., 2004; Lopez-Jimenez et al., 2010). Tumors with SDHB mutations seem to be more aggressive and metastatic (Amar et al., 2007; King et al., 2011), and although the molecular explanation for this phenomenon has not been fully established, a potential role for active HIF-2 signaling has been proposed (Guzy et al., 2008). HIF-2α, therefore, appears to be a highly relevant target in most, if not all cluster 1 pheochromocytomas and paragangliomas. Moreover, HIF-2α silencing in PC-12 rat pheochromocytoma cells led to increased expression of the catecholamine-synthesizing enzyme PNMT, while HIF-1α silencing had the opposite effect (Qin et al., 2014), again suggesting opposing roles for these two HIFs in SNS-derived tumor cells.

Clinically relevant in vitro and in vivo models are required to effectively evaluate drugs that target HIF-2 in neural crest-derived tumors. A number of xenograft models and genetically engineered mouse models (GEMMs) have been developed for neuroblastoma and pheochromocytoma (Table 3 and further reviewed in Korpershoek et al., 2012). GEMMs have contributed to our understanding of tumor initiation and growth; however, GEMM tumors are by definition of murine origin and the models may not necessarily accurately recapitulate the biological and clinical features of human disease. GEMMs carrying SDHx mutations have been created, but neither Sdhα+/− nor Sdhβ+/− mice develop pheochromocytomas or paragangliomas (Piruat et al., 2004; Bayley et al., 2009). This subject is further reviewed in (Maher et al., 2011).

Many conventional human neuroblastoma cell lines exist that have contributed to progress in understanding this disease. Similarly, the rat PC-12 pheochromocytoma cell line (Greene & Tischler, 1976) and the mouse MPC pheochromocytoma cell line (Powers et al., 2000) have been widely used. However, the degree to which conventional cancer cell lines resemble patient disease has been questioned due to the tumor cell differentiation and aberrant genetic deviations that arise following long-term culture in serum-containing media (Lee et al., 2006). There is thus a need to develop relevant mouse models with disease-specific genotypes to target HIF-2 and the pseudohypoxic phenotype.

Recently established neuroblastoma patient-derived orthotopic xenografts (PDXs), in which surgical resection specimens from patients are implanted to the para-adrenal space of immunodeficient mice, retain the geno- and phenotypes of patient tumors (Braekeveldt et al., 2015, 2016). Neuroblastoma PDXs also exhibit infiltrative tumor growth and spontaneous metastatic spread to clinically relevant locations including the bone marrow. Furthermore, PDX-derived human neuroblastoma cells cultured under serum-free stem cell-promoting conditions also retain the genotype and tumorigenic and metastatic capacity in vivo (Braekeveldt et al., 2015). These cells can be used for drug testing (Mohlin et al., 2015). A limitation of this model system is that the mice lack a fully functional immune system and efforts are therefore being made to reconstitute the human immune system in immunodeficient mice (Shultz et al., 2014). Nevertheless, neuroblastoma PDXs in vivo and in vitro will facilitate the identification of drugs targeting hypoxic tumor cells and the pseudohypoxic phenotype in aggressive neuroblastoma. Although these drugs are likely to also be effective in Cluster 1 pheochromocytomas and paragangliomas, establishing PDXs and human cell lines from metastatic pheochromocytoma/paraganglioma would nevertheless be highly desirable.

### 6. Summary and future perspectives

Tumor hypoxia and its main mediators the HIFs regulate many important biological hallmarks of cancer ranging from genetic instability and tumor cell differentiation to metabolic reprogramming and tumor vascularization. Experimental and clinical data from various tumor types suggest that HIFs also regulate metastasis and treatment resistance, which account for the majority of cancer-related deaths. Thus, HIF inhibitors are likely to target multiple important carcinogenetic processes. Intriguingly, HIF-1 and HIF-2 often have opposing effects on tumor growth, for instance on crucial cell cycle and apoptotic regulators like c-MYC and p53. Consequently, specific HIF-1 or HIF-2 inhibition, depending on the tumor type, is likely to be required for successful clinical exploitation of HIF action.

Precision-based targeting of specific growth factors/receptors and/or pathways is currently being tested in numerous preclinical and clinical trials and some personalized therapies, such as trastuzumab in breast cancer, are now standard care. Although the targeted approach has been effective in some tumor types, most if not all of the cancers treated with targeted drugs eventually become resistant. In addition, intratumoral heterogeneity and clonal evolution, which are common in solid tumors, are also likely to contribute to resistance to some targeted therapies. In contrast, targeting the hypoxic phenotype represents a more general approach to eradicate malignant cells. Importantly, dysregulated HIF-2 is common in various tumor types regardless of their genetic and molecular diversity, and HIF-2 inhibition could

### Table 3
Animal models of neuroblastoma and pheochromocytoma/paraganglioma.

<table>
<thead>
<tr>
<th>Model</th>
<th>Tumor</th>
<th>Metastasis</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xenografts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conventional cell-line derived</td>
<td>NB</td>
<td>Rare</td>
<td>Khanna et al., 2002</td>
</tr>
<tr>
<td>Orthotopic PDX</td>
<td>NB</td>
<td>Multiple incl. BM</td>
<td>Braekeveldt et al., 2015</td>
</tr>
<tr>
<td>GEMMs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TH-MYCN</td>
<td>NB</td>
<td>No</td>
<td>Weiss et al., 1997</td>
</tr>
<tr>
<td>ALK110;MYCN</td>
<td>NB</td>
<td>No</td>
<td>Berry et al., 2012</td>
</tr>
<tr>
<td>TH-MYCN;Gspase-8-deleted</td>
<td>NB</td>
<td>BM</td>
<td>Teitz et al., 2013</td>
</tr>
<tr>
<td>Lin28b overexpression</td>
<td>NB</td>
<td>Not reported</td>
<td>Molenaar et al., 2012</td>
</tr>
<tr>
<td>NFI-/+</td>
<td>Pheo</td>
<td>Not reported</td>
<td>Jacks et al., 1994</td>
</tr>
<tr>
<td>Ink4a Arf–/–</td>
<td>Pheo</td>
<td>Lungs</td>
<td>You et al., 2002</td>
</tr>
<tr>
<td>PSK-Cre;Pten-loxp/PloxP</td>
<td>Pheo</td>
<td>Lungs</td>
<td>Korpershoek et al., 2009</td>
</tr>
<tr>
<td>Mecl8;Thr</td>
<td>Pheo</td>
<td>No</td>
<td>Smith-Hicks et al., 2000</td>
</tr>
<tr>
<td>Germine</td>
<td>Cdkn1b–/–</td>
<td>Pheo/PGL</td>
<td>Not reported</td>
</tr>
</tbody>
</table>

PDDs in vivo and in vitro will facilitate the identification of drugs targeting hypoxic tumor cells and the pseudohypoxic phenotype in aggressive neuroblastoma. Although these drugs are likely to also be effective in Cluster 1 pheochromocytomas and paragangliomas, establishing PDXs and human cell lines from metastatic pheochromocytoma/paraganglioma would nevertheless be highly desirable.
potentially overcome the resistance associated with growth factor re-
ceptor inhibition (Franovic et al., 2009). In particular, glioblastoma, cccRCC, and neuroblastoma represent tumor types that might be par-
icularly vulnerable to HIF-2 inhibition. Phaeochromocytomas and para-
gangliomas are also interesting tumor types from the perspective of
HIF-2 inhibition due to their HIF2A mutations and mutations in VHL
and SDH causing a pseudohypoxic phenotype. To optimize their effects,
HIF inhibitors should be combined with conventional chemotherapy
and/or IR. Recent progress in clinical immunotherapy and discoveries
into how HIFs regulate the tumor immune response suggest that
combined immunotherapy and HIF inhibition is likely to be a powerful
therapeutic approach.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by grants from the Berta Kampafrad
Foundation, the Swedish Cancer Society, the Swedish Childhood Cancer Foun-
dation, the Swedish Research Council, VINNOVA, the SF Strategic Center for Translational Cancer Research-CREATE Health, the Strategic Cancer Research Program BioCARE, Craefood Foundation, The Royal
Foundation, and Region Skåne and Skåne University Hospital research
funds.

References

with malignant pheochromocytomas or paragangliomas. J Clin Endocrinol Metab 92, 3822–3828.
Anello, I., & Melino, G. (2015). The p53 family and the hypoxia-inducible factors (HIFs):
392, 405–408.
86, 1104–1111.
develop paraganglioma or pheochromocytoma. Expert Opin Ther Pat 21, 2258–2270.


genes is differently regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell 10, 413–423.


