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REVIEW

Epigenetic regulation of hypoxia inducible factor in diseases and therapeutics

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Abstract Hypoxia-inducible factors (HIFs) are master regulators of angiogenesis and cellular adaptation in hypoxic microenvironments. Accumulating evidence indicates that HIFs also regulate cell survival, glucose metabolism, microenvironmental remodeling, cancer metastasis, and tumor progression, and thus, HIFs are viewed as therapeutic targets in many diseases. Epigenetic changes are involved in the switching 'on' and 'off' of many genes, and it has been suggested that the DNA hypermethylation of specific gene promoters, histone modifications (acetylation, phosphorylation, and methylation) and small interfering or micro RNAs be regarded epigenetic gene targets for the regulation of disease-associated cellular changes. Furthermore, the hypoxic microenvironment is one of the most important cellular stress stimuli in terms of the regulation of cellular epigenetic status via histone modification. Therefore, drug development and therapeutic approaches to ischemic diseases or cancer for targeting HIFs by modulation of epigenetic status become an attractive area. Here, the authors provide a review of the literature regarding the targeting of HIF, a key modulator of hypoxic-cell response under various disease conditions, by modulating histone or DNA using endogenous small RNAs or exogenous chemicals.

Keywords Hypoxia-inducible factor · DNA methylation · Histone modification · Epigenetics · microRNAs

Abbreviations

5-aza-dC	5-Aza-deoxycitidine
ARD1	Arrest-defective-1
CBP	CREB binding protein
CpG	Cytosine-preceding-guanosine
DNMT	DNA methyltransferases
FIH	Factor inhibiting HIF-1
HDAC	Histone deacetylases
HIF	Hypoxia-inducible factor
HRE	Hypoxia response element
MAPK	Mitogen-activated protein kinase
MBD	Methyl-CpG-binding domain proteins
miRNA	Micro-RNA
mTOR	Mammalian target of rapamycin
mtROS	Mitochondrial reactive oxygen species
ncRNA	Non-coding RNA
ODDD	Oxygen dependent degradation domain
PHD	Prolyl hydroxylase domain protein
pVHL	von Hippel-Lindau protein
RCC	Renal cell carcinoma
RISC	RNA-induced silencing complex
RNAi	RNA interference
siRNA	Small interfering RNA
TSA	Trichostatin A
VEGF	Vascular endothelial growth factor
GLUT	Glucose transporter

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Introduction

Hypoxia-inducible factors (HIFs) are upregulated under hypoxia, in inflammatory microenvironments, and by growth factors usually at the posttranscriptional level by protooncogenes and tumor suppressors. Recent reports suggest that various cellular microenvironments regulate epigenetic status to turn on or off genes responsible for cellular responses. DNA methylation of the gene promoter for HIF-mediated transactivation can be inhibited by DNA methyltransferase (DNMT) inhibitors, and HIF-modifying genes can be re-expressed or suppressed by histone modifying enzymes, micro RNAs (miRs), or by small interfering RNAs (siRNAs). Here, we provide a summary of HIF regulators and the mechanisms responsible for regulating HIF stability and expression. We suggest that epigenetics be applied to regulate HIF expression, stability, and HIF-mediated transactivation by DNA promoter hypermethylation, histone modification, and the use of small nuclear RNAs. Furthermore, we propose that regulation of HIF stability and modulation of the epigenetic statuses of HIF regulating genes or of *HIF* gene expression be utilized to control HIF-mediated angiogenesis, inflammation, apoptosis, cell survival, and cancer metastasis.

Hypoxia inducible factors

The HIFs (HIF-1, HIF-2, HIF-3) are heterodimeric transcription factors composed of two subunits, HIF-α and HIF-β (Tian et al. 1997; Wang and Semenza 1995; Gu et al. 1998). HIF-β is the aryl hydrocarbon receptor nuclear translocator, whereas HIF-1α, HIF-2α, and HIF-3α are closely related homologs that control different biological processes. HIF-3α is a dominant negative regulator of HIF, and forms a transcriptionally inactive heterodimer with HIF-1β (Makino et al. 2001). On the other hand, HIF-1α and HIF-2α both activate hypoxia response element (HRE)-dependent gene transcription (Wenger 2002), but play different, critical roles in the mediation of cellular responses to hypoxia (Semenza 2004; Ratcliffe 2007).

HIF-β is constitutively expressed regardless of oxygen availability (Kallio et al. 1997), whereas HIF-α is regulated by oxygen level (Salceda and Caro 1997). In particular, under normoxic conditions, HIF-1α is rapidly degraded via the ubiquitin–proteasome pathway, which is triggered by oxygen-dependent hydroxylation at two conserved proline residues (Pro402 and Pro564 in HIF-1α) in its oxygen dependent degradation domain (ODDD) by prolyl hydroxylase domain proteins (PHDs). The PHDs are family of 2-oxoglutarate dependent dioxygenases that require oxygen for hydroxylation, and which are inactivated in the absence of oxygen. All PHDs are able to hydroxylate HIF-α, and PHD2, which has most activity, is considered critical for HIF-α hydroxylation (Huang et al. 2002; Berra et al. 2003). Furthermore, PHD2 and PHD3 have been reported to be expressionally up-regulated under hypoxic conditions (Epstein et al. 2001; Metzen et al. 2003). In addition, in the presence of oxygen, PHDs hydroxylate HIF-α and trigger the HIF-α degradation pathway (Jaakkola et al. 2001), and therefore, they are called oxygen sensors.

Hydroxylated prolyls are recognized by von Hippel-Lindau protein (pVHL), the substrate recognition component of E3 ubiquitin complex, and binding of HIF-α to this complex leads to polyubiquitination of HIF-α, and ultimately, to its degradation in 26S proteasomes (Ivan et al. 2001; Jaakkola et al. 2001; Yu et al. 2001). Under hypoxic conditions, HIF-1α accumulates and translocates to the nucleus, where it dimerizes with HIF-1β and associates with coactivator to form an active complex that bind to a DNA sequence called hypoxia-responsive element (HRE) (Semenza et al. 1996). By activating HRE-harboring promoter, HIF-1 regulates the transcriptions of hundreds of gene in response to hypoxia (Manalo et al. 2005). On the other hand, in cells lacking pVHL function, HIF-1α and HIF-2α are stable, and hypoxia-inducible genes are induced under normoxic conditions (Iliopoulos et al. 1996). In addition to pVHL E3 complex, murine double minute 2 (an oncogenic E3 ubiquitin ligase) can ubiquinate HIF-1α in a p53-dependent manner (Ravi et al. 2000). Furthermore, interaction between HIF-1α and pVHL can be facilitated by acetylation of Lys532 in the ODDD domain of HIF-1α (Jeong et al. 2002). Acetyltransferase arrest-defective-1 (ARD1) has been reported to acetylate this lysine residue, and therefore, to favor HIF-α degradation (Jeong et al. 2002). On the other hand, the Lys-Agr mutation at this residue has been reported to increase HIF-1α stability (Tanimoto et al. 2000).

To bind DNA and activate transcription, HIF-1 requires cofactors, such as, p300/CREB binding protein (CBP) (Lando et al. 2000; Sang et al. 2002), redox factor 1 (Ref-1) (Lando et al. 2000; Carrero et al. 2000), steroid receptor coactivator 1 (SRC-1) (Lando et al. 2000; Carrero et al. 2000), and transcriptional mediators/intermediary factor 2 (TIF2) (Carrero et al. 2000). The interaction between HIF and p300/CBP is regulated in an oxygen-dependent manner by factor inhibiting HIF-1 (FIH-1), an Fe(II)-dependent asparaginyl hydroxylase. FIH prevents HIF binding to p300/CBP by hydroxylating asparagine residues in its C-terminal activation domain (C-TAD) in the presence of molecular oxygen (Mahon et al. 2001; Lando et al. 2002). Furthermore, the productions of mitochondrial reactive oxygen species (mtROS) are required for the hypoxic activation of HIF-1 (Chandel et al. 1998). It was suggested that oxidant signal from mtROS leads to inhibition of PHDs and therefore, in stabilization of HIF-1α and HIF-2α

(Guzy et al. 2005; Mansfield et al. 2005). Recently, Guo et al. suggested that mtROS-dependent accumulation of HIF is mediated by Akt and mitogen-activated protein kinase (MAPK) signaling pathways (Guo et al. 2012).

Hypoxia-inducible factors are induced by the activations of growth factors or cell adhesion pathways, like the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway. Inhibition of this pathway reduces the hypoxia-induced expression of vascular endothelial growth factor (VEGF) (Mazure et al. 1997), a direct transcriptional target of HIF with a HIF-binding site in 47 bp-HRE (Forsythe et al. 1996), whereas the activation of Akt increases HIF activity (Zelzer et al. 1998; Jiang et al. 2001; Zundel et al. 2000). mTOR is involved in the Akt-dependent induction of HIF (Majumder et al. 2004; Hudson et al. 2002), and HIF-1α activity increases when it is phosphorylated, for example, by p42/p44 MAPK (Wang et al. 2004; Richard et al. 1999). Moreover, inhibition of p42/p44 MAPK activity leads to the inhibition of HIF, but does not affect HIF stability or DNA binding ability (Hur et al. 2001).

Upon activation, HIF-1α promotes many biological processes, such as, angiogenesis, cell survival, invasion, and migration, by activating the transcriptions of its target genes, many of which are important for tumor progression, for example, VEGF, stem cell derived factor 1 (angiogenesis), c-myc, transforming growth factor α, insulin-like growth factor 2 (cell survival and proliferation), glucose transporter 1,3, pyruvate dehydrogenase kinase 1 (hypoxic metabolism), E-cadherin, matrix metalloproteases (metastasis) (reviewed in Ke and Costa (2006)). In solid tumors, high metabolic activity and poor access to a blood supply reduce oxygen levels and induce the activation of HIF. Actually, HIF-1α and HIF-2α have been reported to be expressionally up-regulated in most human tumors, and to be conservatively up-regulated within hypoxic regions in tissues (Talks et al. 2000). In turn, HIF-α induces the expressions of various hypoxia-inducible genes and enhances tumorigenesis.

However, HIF-α can also be activated in tumors under normoxic conditions due to impairment of the oxygen-sensing pathway. For example, the genetic or epigenetic silencing of key factors in this pathway, such as, of pVHL or PHDs, leads to HIF stabilization even under normoxic conditions. pVHL is an important tumor suppressor and regulates HIF-α in normoxia. Furthermore, loss of pVHL expression (usually due to a germ-line mutation) has been reported in renal cell carcinoma (RCC) and other cancers (Latif et al. 1993; Gnarra et al. 1993; Sprenger et al. 2001). Mutations in other tumor suppressor genes, such as, in phosphatase and tensin homolog (PTEN) or tuberous sclerosis protein 2 (TSC2), have been reported to induce HIF activity by activating the Akt pathway (Zundel et al. 2000; Brugarolas et al. 2003). In addition, loss of p53 function, which is observed in the majority of human cancers, enhances HIF-α levels (Ravi et al. 2000), and HIF-1 expressional levels have also been reported to be correlated with poor prognosis and treatment resistance (Birner et al. 2001, 2000, Schindl et al. 2002; Vordermark 2002; Sivridis et al. 2002). Accordingly, the inhibition of HIF-1 might provide an effective therapeutic strategy in cancer. On the other hand, ischemic conditions, such as, those associated with stroke or a heart attack, might be improved by promoting HIF activity, because the induction of VEGF and of other angiogenic factor by HIFs promotes angiogenesis, and thus, provide oxygen to ischemic tissues.

Epigenetics

DNA methylation

DNA methylation involves the addition of a methyl group to the 5′-position of cytosine residues in cytosine-preceding-guanosine (CpG) dinucleotides (Bird 1986). DNA methylation is associated with gene repression (Miranda and Jones 2007), and plays an essential role in genomic imprinting, cell type specific expression, X-chromosome inactivation, and in the suppressions of repetitive elements (viral genes) and testis specific genes (Li et al. 1993; Walsh et al. 1998). CpG-islands are CpG-rich regions with a cytosine/guanosine content of about 50 % and an CpG excess of 0.6 (Gardiner-Garden and Frommer 1987). Most CpG Islands are sites of transcription initiation and are found in the promoter regions of 50–60 % of human genes (Antequera and Bird 1993). Furthermore, they are typically unmethylated (Saxonov et al. 2006; Gardiner-Garden and Frommer 1987). Cytosine methylation is catalyzed by the DNMT family, which has four members; DNMT1, DNMT2, DNMT3a, and DNMT3b (Weber and Schubeler 2007). DNMT1 is the "maintenance methyltransferase", and is responsible for the preservation of existing methylation patterns by adding methyl groups to hemi-methylated CpG sites following DNA replication (Newell-Price et al. 2000). Moreover, loss of DNMT1 function can lead to global hypomethylation in cancer cells (Robert et al. 2003). DNMT2, which has weak DNA methylation activity, appears to be involved in the methylation of RNA (Jurkowski et al. 2008; Goll et al. 2006), whereas DNMT3a and DNMT3b are *de novo* methyltransferases that target unmethylated CpGs to initiate methylation (Okano et al. 1999).

Methylated CpG islands are important for gene silencing and chromosomal stability (Grewal and Moazed 2003). In particular, gene promoter methylation interferes with the binding of transcriptional proteins to promoter regions, and thereby silences transcription. Furthermore, it has been

shown that methylation recruits methyl-CpG-binding domain proteins (MBDs), which recruit additional proteins, such as, histone deacetylases and other chromatin remodeling proteins, which can modify histones to form compact silent chromatin (Bogdanovic and Veenstra 2009). There are six different MBDs, namely, MECP2, MBD1, MBD2, MBD3, MBD4, and Kaiso (Clouaire and Stancheva 2008), each of which can recruit different chromatin-remodeling proteins and transcription-regulatory complexes to methylated DNA targets in the genome (Bogdanovic and Veenstra 2009).

Histone modification

DNA within eukaryotic cells is condensed in chromatin, the structure of which is influenced by various histone modifications as well as by DNA methylation. Nucleosome is composed of an octamer of core histone proteins (two of H3, H4, H2A, and H2B) wrapped by 147 base pairs of DNA (Zhang et al. 1999), through which flexible 'tails' of core histones protrude. These histone tails can be post-translationally modified by methylation, acetylation, phosphorylation, sumoylation, ubiquitination, ADP-ribosylation, deamination, or by proline isomerization (Berger 2007), and these modifications determine whether chromatin adopts the euchromatin conformation, in which DNA is accessible for transcription, or the heterochromatin conformation, in which DNA is inaccessible. More than 60 different residues on histones have been reported to be modified (Kouzarides 2007), usually at lysine, arginine, and serine residues within histone tails. Furthermore, these modifications are reversible and are controlled by groups of enzymes, of which histone deacetylases (HDACs) and acetyltransferases are the most important (Thiagalingam et al. 2003). In particular, the acetylation of lysine residues neutralizes their positively charged side chains, decreases affinity between histone tails and negatively charged DNA, and loosens the chromatin structure, thus facilitating transcription (Gibney and Nolan 2010). The HDACs are divided into four classes. Classes I, II, and IV are zinc-dependent enzymes (Gao et al. 2002; Gregoretti et al. 2004), whereas class III HDACs, generally called sirtuins, are uniquely NAD^+-dependent (Michishita et al. 2005).

Regulatory RNAs

Although approximately 90 % of genomic DNA genome is transcribed, only 1–2 % of genes encode proteins. The majority of transcripts are non-coding RNAs (ncRNAs) (ENCODE-Project-Consortium, 2004), such as, housekeeping ncRNAs (small nuclear and nucleolar RNAs and ribosomal RNAs) and regulatory RNAs (microRNAs, long ncRNAs, Piwi-interacting RNAs, and siRNAs).

MiRNAs repress the expressions of target genes via the post-transcriptional degradation of their mRNAs or by inhibiting translation. Transcripts, called primary miRNAs, are processed into precursor miRNAs with a hairpin structure by a protein complex (Gregory et al. 2004; Lee et al. 2003). Precursor miRNAs are then cleaved by Dicer to mature miRNAs and incorporated into RNA-induced silencing complex (RISC) (Forstemann et al. 2005). RISC–miRNA complex then specifically targets mRNAs by inhibiting translation or causing mRNA degradation (Valencia-Sanchez et al. 2006; Guo et al. 2010). To date, more than eight hundred human miRNAs have been identified.

Small interfering RNAs are 20–25-nucleotide, double-stranded RNAs produced by the splicing of double-stranded RNA by Dicer (Bernstein et al. 2001; Hamilton and Baulcombe 1999). Usually, they completely match their target mRNA sequences and direct them for degradation. siRNAs can also regulate gene transcription by recruiting DNMTs and HDACs to genomic regions corresponding to their sequences (Grewal 2010; Lippman and Martienssen 2004).

Long non-coding RNAs of >200 nucleotides are categorized as sense, antisense, bidirectional, intronic, or intergenic, and their transcriptions regulate gene expressions via a variety of mechanisms (reviewed in (Ponting et al. 2009)).

Epigenetic regulation of HIF

Regulation of HIF expression

HIF-1α promoter harbors a hypoxia response element containing a CpG dinucleotide, which is normally repressed by methylation. However, aberrant demethylation has been observed in colon cancer. This enables HIF-1α to bind to its own promoter, which results in its autoregulation and in the amplification of hypoxia-associated signals (Koslowski et al. 2011). In addition, in these tumor cells, 5-aza-deoxycitidine (5-aza-dC, a DNMT inhibitor) increased hypoxia-induced gene expression (Koslowski et al. 2011). In another study, promoter methylation-dependent suppression of HIF-1α was observed in a hematopoietic cell line (Walczak-Drzewiecka et al. 2010).

HIF-1α expression is also regulated by SWI/SNF complex (a chromatin-remodeling complex), which is involved in both the enhancement and repression of gene transcription, and BAF57, a critical component of this complex is essential for the hypoxia-induced expression of HIF-1α, and has also been reported to be required for hypoxia-induced cell cycle arrest (Kenneth et al. 2009).

The first report to describe the negative regulation of HIF-1α by miRNA was issued by Taguchi et al. who found

that HIF-1α is a direct target of miR-17-92, which may mediate the c-myc mediated repression of HIF-1α (Taguchi et al. 2008). Subsequently, many other HIF-regulating miRNAs have been found, such as, miR-199a-5p, miR-20a, and miR-22 in colon cancer (Kang et al. 2012; Yamakuchi et al. 2011), miR-17-5p and miR-224 in RCC (Lichner et al. 2012), and miR-138 in clear cell RCC (Song et al. 2012). Importantly, HIF-targeting miRNAs are usually downregulated in hypoxia, and this establishes a positive feedback loop (Taguchi et al. 2008; Lei et al. 2009). The HIF-1β subunit of HIF is expressed regardless of oxygen concentration. However, Meng et al. found that HIF-1β is regulated by miR-107 in endothelial progenitor cells. Furthermore, miR-107 expression is increased under hypoxic conditions, and this partly suppresses hypoxia-induced endothelial progenitor cell differentiation. On the other hand, the down-regulation of miR-107 promotes endothelial progenitor cell differentiation (Meng et al. 2012).

Notably, Dicer-1 expression and activity are impaired by chronic hypoxia, and these impairments are associated with a global reduction in mature miRNA. Furthermore, HIF-2α is regulated by miR-185, which is Dicer-dependently down-regulated in hypoxia. This regulation of HIF-2α by miR-185 is actually an adaptive mechanism that maintains cellular hypoxic response controlled by HIFs and microRNAs (Ho et al. 2012). Furthermore, these relations suggests that Dicer-1 should be considered as a RNA-based therapies, because the effectiveness of Dicer-1-dependent RNA interference could be impaired in a background of chronic hypoxia (Ho et al. 2012).

Regulation of HIF stability

Epigenetic changes of components in the HIF-α degradation pathway can regulate HIF, for example, epigenetic silencing of the *VHL* gene results in the constitutive activation of HIF-1α. Hypermethylation of CpG in the *VHL* gene was first detected in 19 % of RCC cases (Herman et al. 1994), and although *VHL* is frequently mutationally inactivated in RCC, methylation of CpG has been suggested to be associated with the completed inactivation of *VHL* expression in heterozygostic germ-line mutants and in tumors homozygous for wild-type alleles (Herman et al. 1994). In one study, aberrant methylation in the *VHL* CpG island was detected in 33.3 % of patients with multiple myeloma, and found to be significantly associated with the development of bone disease (Hatzimichael et al. 2009). In fact, hypermethylation of *VHL* has been observed in retinoblastoma (Livide et al. 2012), non-small cell lung cancer (Dmitriev et al. 2012), pancreatic endocrine tumors (Schmitt et al. 2009), hematological neoplasia (Chim et al. 2002), squamous cell carcinoma (Stephen et al. 2009), acute myeloid leukaemia and myelodysplastic syndromes (Benetatos et al. 2008), and in esophageal squamous cell carcinoma (Kuroki et al. 2003). In another study, the silencing of *VHL* was associated with increase in nuclear translocation of HIF-1 and in expression of HIF-1 targets CA-9 and GLUT-1 (Schmitt et al. 2009).

Kim et al. reported that HDAC expression and activity were reduced by hypoxia in the HepG2 cell line, and the overexpression of HDAC1 in this cell line downregulated the expressions of p53 and *VHL* (both tumor suppressor genes). Furthermore, Trichostatin A (TSA; a HDAC inhibitor) significantly increased p53 and *VHL*, and reduced the hypoxia-induced up-regulations of the expression and activity of HIF-1α and of the expression of VEGF (Kim et al. 2001). Subsequently, another group showed HDAC regulates HIF-1α in a pVHL-independent manner (Qian et al. 2006).

VHL can also be regulated by miRNAs. Ghosh et al. found *VHL* is a direct target of miR-92-1 and that it is expressionally suppressed by overexpressing miR-92-1 in chronic lymphocytic leukemia B cells. Furthermore, this suppression led to the accumulation of HIF-1α and to the secretion of substantial amounts of VEGF (Ghosh et al. 2009). In 2011, a genome-wide profiling of microRNA was conducted in clear cell RCC, in which tumorigenesis is known to require *VHL* loss of function. It was found that of the microRNAs found in clear cell tumors, miR-92a was expressionally up-regulated and *VHL* mRNA was correspondingly down-regulated (Valera et al. 2011).

Of the three PHDs, PHD3, but not PHD2 or PHD1, is regulated by DNA methylation (Hatzimichael et al. 2010; Place et al. 2011). The relation between methylation of the CpG island of *PHD3* and its silencing has been reported in multiple myeloma, B cell lymphoma (Hatzimichael et al. 2010), and in carcinoma cell lines of diverse origins (melanoma, prostate, and breast carcinoma cell lines) (Place et al. 2011). Furthermore, in the latter study, PHD3 expression was rescued by the DNMT inhibitor 5-aza-dC. However, *PHD3* silencing by CpG methylation was not found to have a significant impact on HIF-1α or HIF-2α protein levels (Place et al. 2011).

Regulation of HIF transactivation activity

The DNA-binding activity of HIF is also dependent on binding site methylation status. *BNIP3* (an apoptotic regulator) is induced by HIF-1 binding to its promoter during hypoxia (Kothari et al. 2003; Bruick 2000), and hypermethylation of *BNIP3* promoter was blocked by *BNIP3* induction by hypoxia in pancreatic cancer (Okami et al. 2004) and colorectal cancer cell lines (Bacon et al. 2007). In addition, hypoxia-induced *BNIP3* expression was restored by 5-aza-dC (a DNA methylation inhibitor) (Okami et al. 2004; Ishiguro et al. 2007) or TSA (a HDAC

inhibitor) (Bacon et al. 2007), and the aberrant methylation of *BNIP3* associated with gene silencing was detected in 66 % of primary colorectal and 49 % of primary gastric cancers (Murai et al. 2005). Furthermore, silencing of *BNIP3* associates with resistance to cell death (Ishida et al. 2007; de Angelis et al. 2004) and a poor clinical outcome (Erkan et al. 2005; Heller et al. 2008). Methylation of the HIF-1α binding site of erythropoietin enhancer has been reported to block HIF binding and erythropoietin gene activation (Wenger et al. 1998). *STC2*, a glycoprotein that is overexpressed in many cancers and promotes cell proliferation (Law and Wong 2010a), invasion and epithelial-mesenchymal transition (Volland et al. 2009; Law and Wong 2010b), harbor HRE in its promoter (Law and Wong 2010a). Moreover, epigenetic changes in the promoter region of *STC2* adversely affect its induction of HIF-1 expression. In fact, the transcriptional silencing of *STC2* has been associated with CpG island promoter hypermethylation in some cancer cell lines, and treatment with 5-aza-dC has been reported to increase HIF-1α to *STC2* promoter binding significantly and to induce *STC2* expression (Law et al. 2008). Recently, S100A4 was reported to be regulated by HIF-1α when HRE within its first intron is hypomethylated, and this upregulation was correlated to ovarian cancer invasiveness (Horiuchi et al. 2012).

HIF transactivity is regulated by FIH under normoxic conditions. One group suggested that miR-31 targets the UTR of FIH, represses FIH expression, and thus, increases HIF transactivation activity in normoxia. In human head and neck squamous cell carcinomas, miR-31 up-regulation was correlated with FIH down-regulation in tumor tissues (Liu et al. 2010). HIF-1α regulation by epigenetics is summarized in Fig. 1.

Epigenetic therapy and the regulation of HIF

DNMT inhibitors

The best studied DNMT inhibitors are the nucleoside analogs 5-azacytidine (5-aza) and 5-aza-dC, which incorporate into DNA instead of cytosine during DNA replication (Pinto and Zagonel 1993). Once incorporated into DNA, these agents covalently trap and inactivate DNMTs, and cause DNA demethylation after several cell cycles (Pinto and Zagonel 1993). In particular, demethylating agents could be effective for treating clear cell RCC, which is typically associated with epigenetic loss of the tumor suppressor gene *VHL*, which is silenced by hypermethylation in up to 15–19 % of clear cell RCC cases (Herman et al. 1994; Clifford et al. 1998). In one study, zebularine, another DNMT inhibitor, and 5-aza-dC were examined for their abilities to cause *VHL* re-expression in vitro and in vivo (Alleman et al. 2004). It was found 5-aza-dC caused *VHL* re-expression in culture and in xenograft murine tumors, and that treated cells exhibited less invasiveness into Matrigel and lower VEGF and glucose transporter-1 expressions. Furthermore, in 5-aza-dC treated mice, *VHL* methylated clear cell RCC xenograft tumors were significantly reduced in size (Alleman et al. 2004).

However, in a study by Koslowski et al. 5-aza-dC appeared to affect negatively HIF-1α expression in colon cancer, in which the hypoxic activation of HIF-1α is controlled by a methylation-sensitive HRE in its promoter. In addition, the tumor-associated demethylation of genomic DNA was found to be associated with the positive autoregulation of HIF-1α and with the amplifications of the hypoxia-induced transactivations of HIF-1α target genes (Koslowski et al. 2011). Accordingly, it was concluded that 5-aza-dC treatment increases hypoxia-induced gene expression (Koslowski et al. 2011). In another study, zebularine was tested for antiangiogenic activity in oral squamous cell carcinoma, and found to inhibit VEGF secretion by enhancing the proteasome-dependent degradation of HIF-1α. This effect of zebularine was found to be independent of the Akt and p53 pathways, but the mechanism involved was not determined (Suzuki et al. 2008).

HDAC inhibitors

Initially, HDACs were found to remove acetyl groups from the acetylated lysine residues of histones, but it has since been established that they are a large family of enzymes that act in this manner on many substrates (Yang and Seto 2003; Choudhary et al. 2009). The acetylation statuses of proteins are reversible and regulated by a dynamic balance between the actions of acetyltransferases and HDACs. By disrupting this balance, and thus, the acetylation statuses of histones and transcription factors, HDACs inhibitors generally increase gene expression. The anticancer activities of these inhibitors have been well investigated, and some have been approved by the US Food and Drug Administration for cancer treatment. In tumor cells, HIF function is usually regulated by class I/II HDACs (Qian et al. 2006; Kato et al. 2004; Kim et al. 2001; Maltepe et al. 2005), and several HDAC inhibitors have been reported to repress HIF function and angiogenesis. In particular, TSA has a direct effect on angiogenesis and has been reported to upregulate p53 and pVHL, which are down-regulated in hypoxic hepatoblastoma, and thereby, to down-regulate HIF1a and VEGF (Kim et al. 2001). TSA was found to specifically inhibit hypoxia-induced angiogenesis in a Lewis lung carcinoma model (Kim et al. 2001), and in another study, TSA and sodium butyrate promoted the degradation of HIF-1α via its acetylation in a manner similar to acetyltransferase

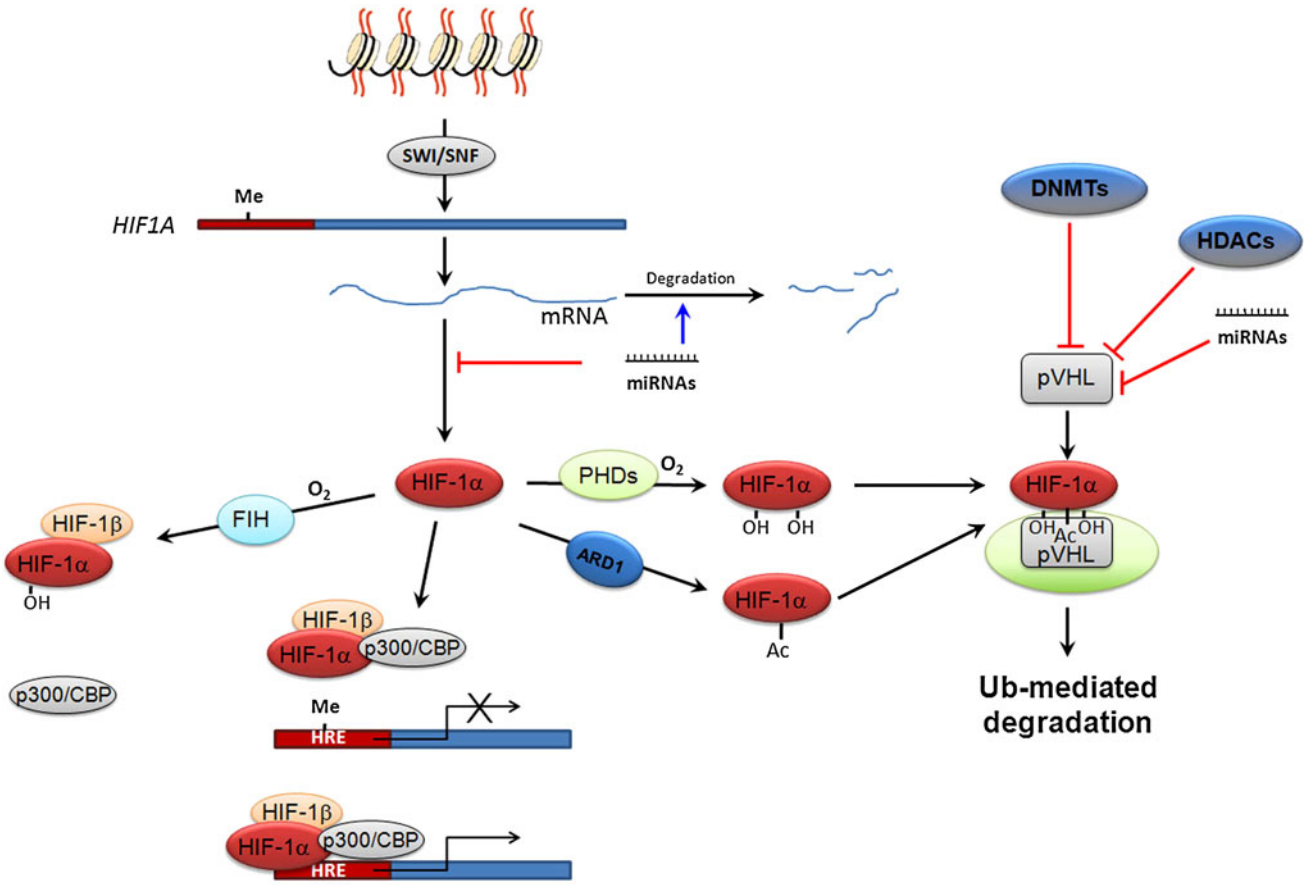

Fig. 1 Epigenetic regulation of HIF. Expression of HIF-1α requires chromatin-remodeling complex SWI/SNF and is inhibited by methylation of the CpG island in *HIF1A* promoter. HIF-1α mRNA is directly targeted by many microRNAs, and HIF-1α can be acetylated by ARD1 and oxygen-dependently hydroxylated by PHDs. These modifications are required for the HIF-1α degradation controlled by pVHL, the expression of which is also regulated by histone deacetylation, promoter methylation, and microRNAs. Hydroxylation of HIF-1α by FIH in the presence of oxygen blocks its association with cofactor p300/CBP, and methylation of CpG in the HREs of target genes inhibits HIF-1α binding and gene transcriptional activation

ARD1 (Jeong et al. 2002). In addition, FK228 (a HDAC inhibitor) was also found to inhibit HIF-1α activity, VEGF expression, and tumor angiogenesis (Sasakawa et al. 2003; Mie Lee et al. 2003). The suppressions of tumor suppressor genes that regulate HIF, such as, *VHL* and p53, were initially suggested to be action mechanisms of HDAC inhibitors. However, it was later shown that HDAC inhibitors target HIF by acetylating CBP and p300, and thus, inhibit the interaction between HIF-1α and p300/CBP (Fath et al. 2006; Stiehl et al. 2007).

RNA interferences

RNAi-based therapy offers a promising means of specifically targeting gene expression. Li et al. (2005) tested the efficacy of HIF-1α targeting by RNAi in vivo. Using an inducible RNAi targeting HIF-1α and a xenograft model, it was found that HIF-1α and some HIF targets were down-regulated in inducible knockdown cells, and that RNAi retarded tumor growth. It was also found that the efficiency of this approach was comparable to that of HIF-1α ablation. However, the inhibition of HIF-1α in more established tumors seemed to be less than in initial stage tumors (Li et al. 2005). Interfering RNA targeting HIF-1α was effective in suppression of tumor growth and angiogenesis of pancreatic and hepatobiliary cancers (Mizuno et al. 2006), and oral squamous cell carcinoma (Zhou et al. 2012). RNA interference of HIF-1α could also improve effectiveness of transcatheter arterial embolization (Chen et al. 2012), and of drugs by reversing hypoxia-induced drug resistance (Min et al. 2012).

Non-coding RNAs can also be targeted by drugs. M2, a pyrrolopyrazine metabolite of the cancer chemopreventive agent oltipraz, facilitates the maturations of miR-199a-5p and miR-20a from their primary to precursor forms. Furthermore, elevated levels of these miRNAs induced by M2 inhibit the *de novo* synthesis of HIF-1α, without effecting its mRNA level or protein degradation. In a study

by Kang et al. M2 inhibited the up-regulations of HIF-1α induced by insulin, hypoxia, $CoCl_2$, or hydrogen peroxide, and reduced HIF-1α target gene induction and cancer cell invasion and migration (Kang et al. 2012).

This review shows that HIFs are key participants in the processes of cancer initiation, invasion, metastasis, angiogenesis/vasculogenesis, and cancer cell survival, and that they can be targeted using epigenetic modification strategies based on the use of endogenous or exogenous small molecules or macromolecules. HIF is a pleiotropic factor with important roles in normal cells, thus direct targeting HIF may affect some physiological processes. Epigenetic defects observed in tumor cells are rarely detected in normal cells, and thus, the drugs used to correct abnormal epigenetic components could provide tumor-specific effects and be only minimally toxic to normal cells. Functional defects in cancer cells may be due to genetic or epigenetic changes, but are more commonly caused by epigenetic changes, which are also more easily reversed.

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