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Epigenetic regulation of the hypoxic response

Hye Jin Nam[1] and Sung Hee Baek[2]

Hypoxic signaling occurs under several physiological and pathological conditions, such as in solid tumors. Hypoxia inducible factor 1 (HIF-1) mediates many hypoxic responses, and regulates hundreds of genes involved in many biological processes including tumor angiogenesis, invasion, and metabolism. Although the components of hypoxia that initiate and maintain the hypoxic responses have been well-studied, epigenetic regulation of the hypoxic responses is poorly understood. Thus, it would be useful to summarize current status of the field and discuss future directions. In this review, we will focus on the current understandings of epigenetic regulation in hypoxic response and discuss currently available epigenetic drugs to treat hypoxia-driven or related diseases.

Addresses
[1] Bio Platform Technology Research Center, Therapeutics and Biotechnology Division, Korea Research Institute of Chemical Technology, Daejeon 34114, South Korea
[2] Creative Research Initiatives Center for Epigenetic Code and Diseases, Department of Biological Sciences, Seoul National University, Seoul 08826, South Korea

Corresponding author: Baek, Sung Hee (sbaek@snu.ac.kr)

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Introduction

Hypoxia is a state in which the oxygen concentration is relatively lower than that of homeostasis under normoxic conditions. Hypoxic responses are mediated by hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor that is composed of an oxygen-regulated α subunit (HIF-1α or HIF-2α) and a constitutively expressed β subunit (HIF-1β) [1]. In hypoxic conditions, stabilized HIF-1 translocates to the nucleus and binds to hypoxia response elements (HREs) to activate its target genes. These genes regulated by HIF-1 control cellular oxygen homeostasis such as angiogenesis, oxygen consumption, erythrocyte production, glucose metabolism, and mitochondrial metabolism [2].

Epigenetic regulation is mainly controlled by DNA methylation and histone modification. Eukaryotic DNA is packaged with histone H2A, H2B, H3, and H4 octamer to form chromatin. Post-translational modifications (PTMs) of histone tails alter chromatin structure and induce recruitment of coactivator or corepressor complex depending on upstream signals. Therefore, gene expression is controlled by various PTMs such as methylation, ubiquitination, phosphorylation, and SUMOylation conferred by various epigenetic enzymes [3]. In addition, these epigenetic enzymes control gene expression by modulating PTMs of non-histone proteins.

DNA methylation mediated by DNA methyltransferases (DNMTs) is responsible for repression of gene expression. DNA methylation coordinately regulates repressive chromatin status through interaction with various other epigenetic modifications such as H3K9 or H3K27 methylation [4–6]. Since patterns of DNA methylation and histone or non-histone protein modifications differ between normal and cancer cells, restoring epigenetic modifications is considered as a promising method of cancer therapy [7]. Interestingly, hypoxia signals modulate the expression levels or enzymatic activities of responsible epigenetic enzymes to control target gene expression. Therefore, epigenetic enzymes can be attractive therapeutic targets in hypoxia-driven or related diseases.

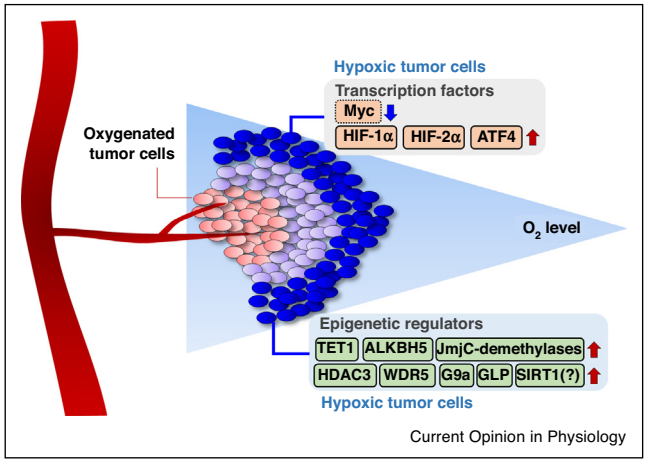
Epigenetic enzymes that are affected and regulated by the hypoxic responses

Since hypoxia threatens the survival of the cells or organisms, a variety of changes occur to overcome hypoxic conditions. Among them, we will focus on recently identified epigenetic enzymes induced or reduced by hypoxic stress for dynamic transcriptional control (Figure 1 and Table 1).

The ten-eleven translocation 1 (TET1)

TET1 was originally discovered as a form of fusion protein with MLL by translocation occurring between chromosomes 10 and 11 in acute myeloid leukemia (AML) [8]. Prolyl hydroxylases (PHD1, PHD2 and PHD3) hydroxylate HIF-1 and HIF-2 on specific proline residues using O_2 and 2-oxoglutarate as substrates and trigger their degradation under normoxic condition [9]. Interestingly, TET1 belongs to a family of nonheme Fe^{2+}/2-oxoglutarate-dependent dioxygenases (2-OGDOs) including PHDs, indicating that TET1 enzymatic activity is sensitive to O_2 levels [10]. TET1 is responsible for converting 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) by oxidation reaction. 5-hmC is an intermediate in the process of converting 5-mC to unmethylated cytosine, providing the first step in a

Figure 1



Epigenetic regulators or transcription factors that are regulated by hypoxia.

pathway for active DNA demethylation [11]. Missense and truncating mutations in the TET genes are observed in many solid tumors [12]. Furthermore, reduced TET expression levels and 5-hmC levels are generally observed in many solid cancer types including gastric, prostate, liver, lung, breast, brain, and skin cancers [13–15]. Since the rapid proliferation of cancer cells results in hypoxic environments, it has been questioned whether conversion of 5-mC to 5-hmC by TET is regulated by hypoxic stress in cancer. Neuroblastoma cells exposed to hypoxia show both global increase of 5-hmC levels and HIF-1 binding site-specific gains of 5-hmC levels with transcriptional increase of TET1 and hypoxic target genes [16]. Although the enzymatic activity of TET1 in neuroblastoma is critical, it seems dispensable for HIF-α protein stabilization. Both *Tet1*-null zebrafish and mouse model show more susceptibility to hypoxic conditions compared to WT. *Tet1*-null MEFs display unstable HIF-α proteins [17].

G9a and GLP

Hypoxic conditions induce the expression of both G9a (also known as EHMT2) and GLP (also known as EHMT1) methyltransferases, which are responsible for histone H3K9 methylation. Although the catalytic activity of G9a and GLP on histone H3 proteins is almost identical, functional effects of hypoxia-induced G9a and GLP depend on the different non-histone substrates. Hypoxia-induced G9a and GLP methylate Reptin (also known as RUVBL2) and Pontin (also known as RUVBL1). Reptin and Pontin are chromatin-remodeling factors that possess AAA+ ATPase and DNA helicase activities and usually form a complex to work together [18]. In the initial hypoxic state, G9a or GLP methylates Pontin to activate transcription of a subset of hypoxia-responsive genes by recruiting p300 acetyltransferase to the target promoters [19]. Pontin methylation contributes to the enhancement of proliferative and invasive potential of breast cancer cells. On the contrary to Pontin methylation, Reptin methylation occurs late in hypoxia and contributes to binding to HDAC1 for the negative regulation of hypoxia-responsive genes [20]. Introduction of methylation-defective mutant of Reptin to the breast cancer cells results in more aggressive cancer phenotypes including increased cell proliferation, invasion, migration, and *in vivo* tumorigenesis. In addition to Pontin and Reptin, HIF-1α is also methylated by G9a and GLP [21]. G9a/GLP-mediated HIF-1α methylation inhibits

Table 1

Epigenetic enzymes induced or reduced by hypoxic stress.

Epigenetic enzymes	Enzymatic activities in hypoxia	Phenotypic changes	Refs.
TET1	DNA demethylation	Hypoxic-target gene induction	[16]
G9a	Reptin methylation	Negative regulation of hypoxia	[20]
	Pontin methylation	Positive regulation of hypoxia	[19]
	HIF-1α methylation	Inhibition of HIF-1α transcriptional activity	[21]
GLP	Pontin methylation	Enhanced proliferation and invasiveness	[19]
	HIF-1α methylation	Inhibition of HIF-1α transcriptional activity	[21]
PRMT2	Not addressed	Increased asymmetric dimethyl-arginine	[28]
SIRT1	HIF-1α deacetylation	Repression of HIF-1α target genes	[35]
SIRT1	HIF-2α deacetylation	Activation of erythropoietin genes	[37]
SIRT6	H3K9 deacetylation	Regulation of glucose homeostasis	[33]
SIRT7	Independent of deacetylase activity	Inhibition of HIF-1/2 protein levels	[34]
HDAC3	H3K4 deacetylation	Epithelial-Mesenchymal Transition	[38]
WDR5	H3K4 methylation		
ALKBH5	m^6A-demethylation	Breast cancer stem cell phenotype	[41•]
JARID1B (KDM5B)	H3K4me1/me2/me3	Histone methylation homeostasis	[24,25•,72],
JMJD1A (KDM3A)	H3K9me1/me2		[73]
JMJD2A (KDM4A)	H3K9me2/me3, H3K36me2/me3		[25•,74,75]
JMJD2C (KDM4C)	H3K9me3, H3K36me3		[25•,26,27]
JMJD3 (KDM6B)	H3K27me2/me3		[25•,76]

HIF-1α transcriptional activity and impairs migration of glioblastoma mutiforme (GBM) cells. The expression levels of G9a and GLP are decreased during long-term hypoxia along with reduction in HIF-1α methylation. The decreased expression levels of G9a and GLP under hypoxia are thought to be due to GBM cell type-specific mechanism and are observed when exposed to hypoxic conditions for more than 48 h. Although HIF-1α methylation levels have not been compared GBM patient samples with normal counterpart, clinical significance of HIF-1α methylation is indirectly demonstrated by showing a positive correlation between G9a expression level and the survival rate of GBM patients. In hypoxic conditions, G9a and GLP epigenetically modulate various substrates and subsequent target gene expressions.

Jumonji C (JmjC)-domain containing demethylases

The enzymatic activity of JmjC-domain containing demethylases can be reduced by low oxygen levels because JmjC-domain demethylases require oxygen and 2-oxoglutarate for their activity [22,23]. The decreased availability of oxygen could affect the demethylation activity of JmjC-domain containing demethylases. However, a considerable number of JmjC-domain containing demethylases are transcriptionally upregulated by hypoxia [24]. Therefore, specific studies are needed on how JmjC-domain containing demethylases regulate global or promoter-specific demethylation leading to transcriptional control under hypoxia. Interestingly, both active (H3K4me3) and repressive (H3K9me3 and H3K27me3) histone methylation in human adipose-derived stem cells (hADSCs) are globally increased under hypoxic conditions [25•]. However, increased patterns of H3K4me3, H3K9me3, and H3K27me3 by hypoxia in chromatin are differentially distributed within gene structures, which correlates with mRNA expression. Reduced enzymatic activity of JmjC-domain containing demethylases by hypoxia is the main cause of the changes in histone tri-methylation, but not the altered expression levels of JmjC-domain containing demethylases.

The induction of JmjC-domain containing demethylases by hypoxia is critical for tumor progression. For example, JDMD2C is a HIF-1 target gene and is upregulated under hypoxia [26]. JMJD2C has been shown to stimulate HIF-1α-mediated transcription through demethylating H3K9me3 in breast cancer cells [27]. In other words, JMJD2C-mediated histone H3K9 demethylation occurs actively under hypoxic conditions to promote breast tumor growth and metastasis.

Protein arginine methyltransferase 2 (PRMT2)

PRMT2 is induced by chronic hypoxia. Mice exposed to three weeks of hypoxia show increased mRNA and protein levels of PRMT2, even though the functional significance of it is not yet elucidated clearly [28]. Further research on the biological and transcriptional effects of arginine methylation under hypoxia is still required. Recently, it is reported that PRMT4 also known as CARM1 is stabilized by autophagy signal and subsequently increases in H3R17me2 for activating autophagy-related and lysosomal genes [29••]. In nutrient-rich conditions, CARM1 is degraded by SKP2-containing SCF (SKP1-cullin1-F-box protein) E3 ubiquitin ligase in the nucleus. In nutrient-starved conditions, SKP2 is transcriptionally repressed by FOXO3a transcription factor leading to stabilization of CARM1 in the nucleus. Since hypoxia induces autophagy through HIF-dependent BNIP3 and BNIP3L induction [30–32], there might be a close correlation between hypoxia and CARM1-associated autophagy.

Sirtuins (SIRTs)

Sirtuins, which are NAD^+-dependent protein deacetylases, regulate a variety of cellular processes including aging, apoptosis, inflammation, circadian rhythm, and stress resistance. In particular, SIRT1, SIRT6, and SIRT7 are highly associated with HIF-α dependent signaling pathway. SIRT6 functions as a corepressor of HIF-1α by deacetylating H3K9 [33]. SIRT7 inhibits HIF-1 and HIF-2 transcriptional activity independent of its deacetylase activity [34]. The role of SIRT1 in hypoxia is controversial. Lim *et al.* [35] reported that the level of mRNA and protein expression of SIRT1 decreases over hypoxic time course, whereas Chen *et al.* [36] published that SIRT1 protein expression peaks at 4 h under hypoxia in a HIF-dependent manner. According to Lim *et al.*, both decreased expression level and catalytic activity of SIRT1 under hypoxia lead to acetylated HIF-1α and HIF-1α-dependent transcriptional activation. Chen *et al.* claims that HIF-1α-dependent induction of SIRT1 by hypoxia contributes to HIF-2α deacetylation [37]. Clarifying this issue using *in vivo* mouse models will be the future direction of research to validate the importance of this regulation.

Histone deacetylase 3 (HDAC3)

HDAC3 is induced by HIF-1α transcription factor upon hypoxia and deacetylates histone H3K4Ac [38]. WDR5, a histone H3K4 methyltransferase, is also induced by HIF-1α and HIF-2α. Induced HDAC3 and WDR5 under hypoxia interacts on chromatin to increase H3K4 methylation and activate mesenchymal gene expression. Investigation of a crosstalk between deacetylation by HDAC3 and methylation by WDR5 on same histone residue reveals the mechanism on how epigenetic control dynamically modulates epithelial–mesenchymal transition (EMT) under hypoxia.

Alkylation repair homolog protein 5 (ALKBH5)

Recently, explosive studies on RNA methylation have been published after discovering two mammalian RNA demethylases, the fat mass- and obesity-associated protein (FTO) and ALKBH5 [39,40]. In addition,

advancement of next-generation sequencing technology allows us to see when and where RNA methylation occurs. In hypoxic conditions, ALKBH5 RNA demethylase is induced in HIF-1α/β-dependent manner and mediates m^6A-demethylatin at 3′-UTR of NANOG mRNA in breast cancer [41•]. NANOG mRNA is stabilized by its demethylation, thereby promoting breast cancer stem cell phenotypes.

Regulation of HIF-1 stability by lysine methylation

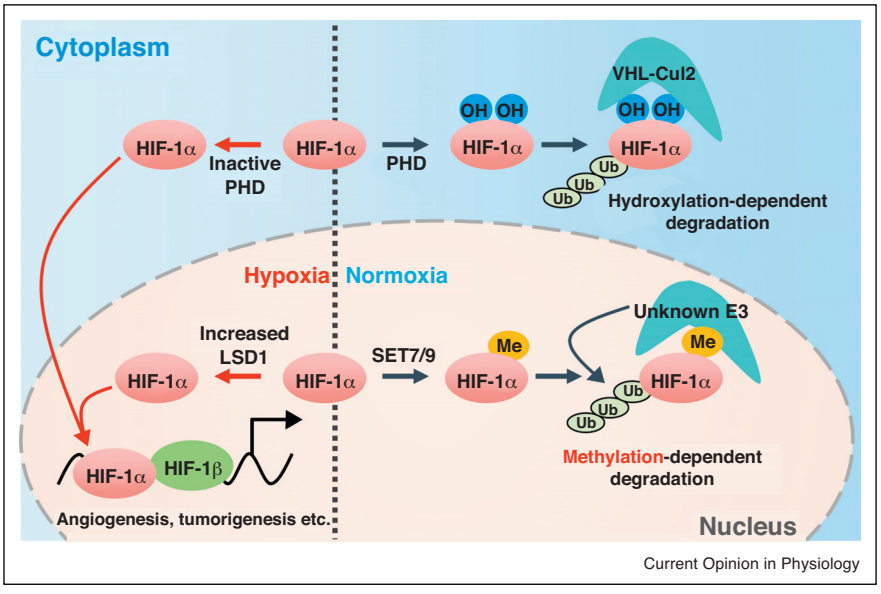
As mentioned in the introduction part, the major transcription factor in hypoxic response is HIF-1. HIF-1α protein levels are tightly regulated by PTMs such as hydroxylation, SUMOylation, acetylation, and phosphorylation [42–44]. Since these PTMs primarily occur in the cytoplasm, it is expected that there might be a mechanism to eliminate remaining HIF-1α in the nucleus under normoxic conditions. Interestingly, HIF-1α methylation by SET7/9 leads to HIF-1α degradation in the nucleus under normoxic conditions, which is independent of the hydroxylation-dependent degradation [45••,46]. In addition, HIF-1α methylation is dynamically reversed by a demethylase, LSD1, under hypoxia. LSD1-dependent demethylation of HIF-1α is critical for its stabilization in hypoxia. Knock-in mice bearing HIF-1α methylation defective mutant show elevated retinal angiogenesis and tumorigenesis due to increased expression of HIF-1α protein. In addition, mutations near the HIF-1α methylation site are detected in cancer patients, which are resistant to methylation by SET7/9 and methylation-dependent degradation. Identification of the molecular mechanism of HIF-1α methylation and demethylation provided new insights on fine-tuning the regulation of HIF-1α stability and a new therapeutic strategy for the development of anti-cancer drug (Figure 2).

Epigenetic control of hypoxic response

Like HIF-1α, HIF-2α is also stabilized under hypoxic condition by escaping from hydroxylation-dependent degradation. Although HIF-1α and HIF-2α share high sequence homology and have common target genes, they have distinct function depending on physiological and pathological conditions [47,48]. Peroxisome plays a role in the breakdown of long chain fatty acids and polyunsaturated fatty acids, and the enzymes within peroxisome require oxygen for proper functioning [49,50]. In *Vhl*-liver-specific knockout (KO) mice, peroxisome abundance is reduced, and this phenotype is rescued by *Vhl/Hif-2α* double KO, but not by *Vhl/Hif-1α* double KO mice [51]. Stabilized *HIF-2α* in *Vhl* KO mice mediates autophagy induction, which selectively degrades peroxisome by pexophagy and results in reduction of peroxisome in *Vhl* KO mice.

Solid tumors such as glioblastoma multiforme (GBM) suffer from an insufficient supply of oxygen during rapid tumor growth. The stem cell marker CD44 is specifically expressed in the perivascular niche (PVN) of GBM and its cleavage (intracellular domain of stem cell marker CD44, CD44ICD) is enhanced upon hypoxia [52•,53]. Specific interaction between CD44ICD and HIF-2α, but not HIF-1α, leads to enhanced HIF target activation under hypoxia or even pseudo-hypoxia, a condition in which

Figure 2



Regulation of HIF-1α stability by lysine methylation in the nucleus.

cells activate hypoxic response in the presence of oxygen. CD44ICD promotes GBM stemness by enhancing HIF-2α-dependent transcription with co-activator CBP/p300.

Expression of intestinal HIF-2α, but not HIF-1α, is increased during obesity and HIF-2α-dependent target genes are activated [54]. Intestinal-specific *Hif-2a* KO mice fed with a high-fat diet (HFD) exhibit less body weight gain and attenuated hepatic steatosis compared to WT mice. Oral administration of a specific HIF-2α inhibitor, PT2385, to mice fed with a HFD also results in reduced body weight and improved steatosis. Although the precise mechanism how a HFD activates intestinal HIF-2α signaling remains unclear, these data show that HIF-2α functions as a novel target to treat hepatic steatosis.

The proto-oncogene Myc is generally expressed at low levels in normal proliferative cells, but frequently overexpressed in cancer cells [55]. Interestingly, degradation of c-Myc occurs in HIF-1α and HIF-2α-dependent manner under hypoxia [56]. In hypoxic tumor microenvironment with rapid cell proliferation, ATP production is often reduced due to decreased oxidative phosphorylation. In order to survive under ATP deficient conditions, the energy consumption should be reduced until complete angiogenesis. For this reason, cancer cells under hypoxia are thought to degrade Myc protein, which is involved in high-energy-consuming proliferation [57]. In other words, cancer cells are willing to give up proliferation to survive under hypoxia. Activating transcription factor 4 (ATF4) is induced by severe hypoxia, endothelial reticulum (ER) stress, or oxidative stress. Severe hypoxia-induced ATF4 confers ER stress and ATF-dependent autophagy [58]. Further, elevated levels of ATF4 in hypoxia are positively correlated with M2-polarized macrophage infiltration in infantile hemangioma patients [59], suggesting that a new tumor environment can be created by macrophage infiltration in severely hypoxic areas. Diverse tumor microenvironments make it difficult to fundamentally cure cancer.

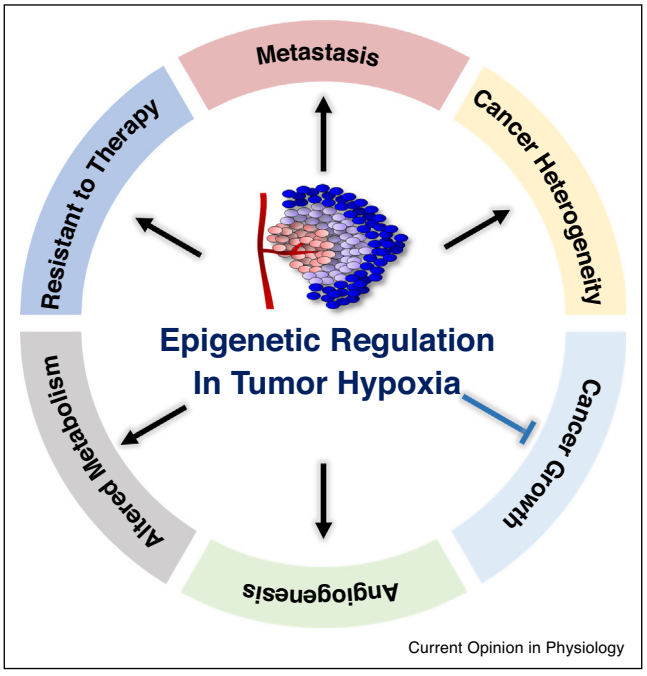
Functional consequences of epigenetic regulation upon hypoxia

Our current understanding of how dysregulations of epigenetic regulators or transcription factors (involved in hypoxia) are associated with hypoxia-driven or related diseases is summarized (Figure 3). Through basic research on how hypoxia-driven or related diseases such as cancer are initiated and progress, a functional link between hypoxia and epigenetics has been revealed.

Drug-resistant cancer cells can be driven by hypoxic tumor microenvironment. Disseminated tumor cells (DTCs) are detected in the peripheral blood, bone marrow or lymph nodes in cancer patients [60]. Although metastasis can be originated from DTCs, DTCs can remain dormant in patients with no evidence of disease for many years before reactivation [61]. A hypoxic microenvironment influences the fate of DTCs by upregulating the key dormancy (NR2F1, DEC2, p27) genes [62••]. Among dormancy inducing genes, upregulation of NR2F1, an orphan nuclear is epigenetically controlled. NR2F1 is highly expressed in dormant cells but not in proliferative tumor cells. Treatment with 5-aza-deoxycytidine, an inhibitor of DNA methylation (5-Aza-C) increases NR2F1 mRNA expression in proliferative tumor cells [63]. In addition, transcriptional activation markers H3K4me3 and H3K27ac are enriched on NR2F1 transcription start site in dormant tumor cells, whereas the transcriptional repressive mark H3K27me3 is enriched in NR2F1 promoter in proliferative tumor cells. Although hypoxic microenvironments induce NR2F1-dependent dormancy, primary tumors under hypoxic microenvironments give rise to a subpopulation of dormant DTCs which evade chemotherapy [62••]. These post-hypoxic dormant DTCs can be an origin of cancer recurrence or metastasis which is resistant to therapeutics, and this research suggests that hypoxic environment can give rise to various cancer heterogeneity.

RRx-001 catalyzes the reduction of nitrite to nitric oxide, which accumulates in poorly oxygenated tumor. RRx-001 is currently under Phase II clinical trials, alone or in combination, for the treatment of solid tumors (NCT02096354; NCT02489903; NCT02452970;

Figure 3



Epigenetic regulation in tumor hypoxia.

NCT02215512). Interestingly, RRx-001 significantly reduces expression and activity of DNMT1, DNMT3A, and DNMT3B and reduces global DNA methylation levels with apoptosis of cancer cells [64]. Since RRx-001 has a different mechanism of action compared to conventional DNMT inhibitors, RRx-001 can be a new hypoxia-selective epigenetic drug.

In sum, recent studies have shown how epigenetic enzymes including histone methyltransferases and demethylases can dynamically affect and regulate various hypoxia-driven or related diseases. However, clinical validation will be needed to confirm whether histone methylation-related enzymes are novel and potent targets of epigenetic drug.

Importance of epigenetics in tumor hypoxia and cancer immunotherapy

Cancer immunotherapy such as immune checkpoint inhibitors has shown promising clinical results. However, the efficacy of immunotherapy in solid tumors is not as effective as blood cancers. Therefore, applying and expanding cancer immunotherapy in more types of cancer including various solid tumors is considered to be an important breakthrough in cancer treatment. Hypoxic microenvironment of solid tumor might cause resistance to cancer immunotherapy. Therefore, studies on the effect of hypoxic microenvironment of solid tumor on immune suppression such as T cell exhaustion should be more actively conducted.

The degree of T cell activation is stronger in well-oxygenated environment, suggesting that T cell activation is inhibited in the oxygen-poor tumor microenvironment [65]. In addition, tumor hypoxia attracts immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) [66]. Hypoxia alters the function of MDSC in the tumor microenvironment and redirects their differentiation toward tumor-associated macrophage [67]. The expression levels of PD-L1 immune checkpoint are higher on tumor-infiltrating MDSCs as compared with splenic MDSCs [68]. As a HIF-1 direct target, PD-L1 is upregulated by hypoxia in MDSCs. Blockage of PD-L1 by PD-L1 antibody decreases MDSC-mediated T cell suppression under hypoxia, suggesting that combinatorial therapy targeting tumor hypoxia along with PD-L1 blockage might encourage the immune system in cancer patients.

Recently, two remarkable studies have reported that T cell exhaustion is highly associated with extensive changes in chromatin, especially enhancer and transcription factor binding regions [69••,70••]. Since hypoxic stress also causes epigenetic changes in cancer cells and immune cells, more research into the relationship between hypoxia and epigenetics is expected to enable the application of a broad spectrum of immunotherapies.

Concluding remarks

Hypoxia on tumor cells contributes to the therapeutic resistance and heterogeneity, making cancer treatment difficult. Hypoxia reduces the efficacy of cancer immunotherapy as well as conventional therapy. Abnormal alteration of epigenetic modification by hypoxia can be a one of the reasons why cancer treatment is difficult. The reversibility of epigenetic modifications makes epigenetic enzymes more attractive therapeutic targets of cancer. Drugs targeting DNA methylation (DNMT inhibitors) and histone acetylation (HDAC inhibitor) are currently in the clinical trials or United States Food and Drug Administration (FDA) approved, but their efficacies are very limited in mono-therapy [71]. Therefore, in order to achieve high efficacy, it is necessary to study the effects of combinatorial treatment of epigenetic drugs and HIF-targeting therapy.

Conflict of interest statement

Nothing declared.

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