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Epigenetics, the epicenter of the hypoxic response

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It is becoming increasingly apparent that epigenetics plays a crucial role in the cellular response to hypoxia. Such epigenetic regulation may work hand in hand with the hypoxia-induced transcription factor (HIF) family or may contribute in a more substantial way to the maintenance of a hypoxia-adapted cellular phenotype long after HIF has initiated the immediate response pathways. In this article we discuss the current research implicating epigenetic mechanisms in the cellular response to hypoxic environments. This includes; the role of epigenetics in both the stabilization and binding of HIF to its transcriptional targets, the role of histone demethylase enzymes following direct HIF transactivation, and finally, the impact of hypoxic environments on global patterns of histone modifications and DNA methylation.

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It is widely accepted that the transcriptional responses, which are crucial for cells to adapt to hypoxic conditions, are predominantly driven by the hypoxia-induced transcription factor (HIF) family. However, recent literature increasingly suggests that hypoxia-induced transcription factors require the cooperation of epigenetic events in order to fulfill their role in initiating hypoxic response pathways and also in the maintenance of the post-hypoxic phenotype.[1]

Hypoxia occurs when the metabolic demand for oxygen exceeds the supply and can arise under many pathological states, including inflammatory, fibrotic, ischemic and tumorigenic processes. Under these conditions, hypoxia-responsive pathways are activated and play an important role in cellular responses that promote tissue survival. In some situations, such as myocardial infarction, a positive adaptive response to acute ischemia is crucial for patient survival, whereas in others, such as growth of a solid tumor, adaptive responses to chronic hypoxia that promote survival of the tumor are detrimental to the patient. Elucidating the mechanisms that control cellular responses to acute, chronic and intermittent hypoxia will potentially provide therapeutic opportunities to manipulate cell fate in these conditions.

The classical adaptive responses to hypoxia that aim to restore oxygen homeostasis in tissues are predominantly regulated by the HIF family. The HIF transcriptional complex is a heterodimer composed of one of three oxygen sensitive α-subunits (HIF-1α, HIF-2α or HIF-3α) and a constitutively expressed β-subunit (ARNT; also known as Aryl Hydrocarbon Receptor Nuclear Translocator). In the presence of oxygen, HIF-α is hydroxylated by prolyl hydroxylase enzymes (PHD1, PHD2 and PHD3) enabling interaction with the Von Hippel Lindau (VHL) protein and subsequent ubiquitination and proteasomal degradation.[2,3] In the absence of oxygen, PHD activity is inhibited, which results in the stabilization and nuclear translocation of the HIF-α subunits enabling them to bind to nuclear ARNT and form transcriptionally active HIF. HIF regulates gene expression through interaction with specific hypoxia response elements (HRE) present in pro-

moter and enhancer regions of hypoxia-responsive genes.

In addition to HIF, adaptive responses to hypoxia can be regulated by the transcriptional activity of other factors such as NFκB, CREB and EGR-1. The relative contribution of specific transcription factors may depend upon the intensity and duration of the hypoxic insult. Understanding the temporal relationship of transcription factor activation is crucial to elucidating cellular responses to hypoxia, but perhaps what is more important is understanding what regulates the availability of gene regulatory regions to hypoxia-inducible transcription factors.

There is increasing evidence that the activity of hypoxia-induced transcription factors, such as HIF-1, is superimposed upon a background of epigenetic changes that are essential for determining the hypoxic response. Specifically, epigenetic modifications both at the DNA and histone level have the ability to dictate HIF binding to target gene promoters. Moreover, hypoxia itself is a potent inducer of chromatin remodeling via the regulation of enzymes that modulate DNA methylation and histone modifications. In addition, we propose that long term adaption to chronic hypoxia requires significant modification of chromatin structure in order to maintain the hypoxic phenotype in the absence of HIF-1. It is also important to note that chronic hypoxia may induce changes in gene expression that are independent of the classical HIF pathway. This may occur through alterations in the methylation status of gene sequences or modification of the normoxic histone code, possibly through the prolonged alterations in epigenetic modifying enzymes.

There are four current opinions on the interaction of epigenetics and hypoxia:

(1) HIF stabilization is influenced by the epigenetically controlled expression of VHL and PHD3.

(2) Epigenetic mechanisms regulate HIF binding by maintaining a transcriptionally active chromatin confirmation within and around HIF binding site regions. This may occur through the action of the HIF-1α coactivation complex or through direct modifications of the HRE binding sites which prevent HIF binding.

(3) A significant number of histone demethylase enzymes are direct HIF-1 target genes and therefore play a role in the regulation of transcription during the hypoxic response.

(4) Significant global changes in histone modifications and DNA methylation occur in response to hypoxic exposure.

The Role of Epigenetics in Controlling HIF-α Stabilization

In the presence of oxygen, PHD-dependent hydroxylation of HIF-α confers susceptibility to VHL-dependent ubiquitination and degradation. Mutations of the VHL tumor suppressor gene predispose individuals to a variety of human tumors, including renal cell carcinoma, hemangioblastoma of the central nervous system, and pheochromocytoma. In addition to the relatively well studied VHL loss of function mutations it has been shown that expression of VHL can be blocked by promoter hypermethylation in renal cell carcinoma[4] and multiple myeloma.[5] Further epigenetic control of HIF stabilization is seen at the level of PHD expression. A recent study of approximately 80 patients with B-cell neoplasia showed that PHD3 (EGLN3) but not PHD2 (EGLN1) was frequently silenced by promoter methylation in multiple myeloma and B-cell lymphoma.[6] This loss of PHD3 and VHL expression, due to promoter hypermethylation, is potentially an important epigenetic mechanism that may exacerbate HIF activity.

The Role of Epigenetics in HIF-1 Binding and Regulation of Transcription of Hypoxia Response Genes

One of the first lines of evidence to suggest that the hypoxic response relies heavily on the cooperation of epigenetics was found when studying the HIF-1α coactivation complex, where several epigenetic modifying enzymes have been found in direct contact with HIF-1α during the initial cellular response to hypoxia. The histone acetyltransferase enzyme CBP/p300 is known to directly associate with HIF-1α and participate in the coactivation of a network of hypoxia-inducible genes.[7] This interaction can be disrupted by factor-inhibiting HIF (FIH) hydroxylation or the oxygen-dependent binding of VHL protein, which inhibits HIF-1α transactivation through the recruitment of histone deacetylase (HDAC) enzymes.[8] This has been demonstrated further in a study by Fath et al. showing that HDAC inhibitors repress the transactivation potential of HIF-1α under hypoxic conditions by targeting the HIF-1α/p300 complex.[9]

Other members of the HIF-1α coactivation complex SRC-1 and TIF2 have also been found to have histone acetyltransferase activity which enhance the hypoxia-inducible activity of HIF-1α both independently and in synergy with CBP/p300.[10] Histone deacetylase enzymes such as HDAC1, 3 and 7 have also been implicated in the actions of HIF-1α. Specifically, HDAC7 co-translocates to the nucleus with HIF-1α in response to hypoxia through a strong association between its C-terminal domain and the inhibitory domain of HIF-1α.[11] Moreover, HDAC1 and 3 are also direct binding partners of HIF-1α through an association with HIF's oxygen-dependent degradation domain, highlighting a role for these HDACs in the regulation of HIF-1α stability during hypoxic exposure.[12]

Further evidence that the hypoxic response relies on the epigenetic landscape comes from a number of recent studies revealing that hypoxic induction of gene expression often relies on HIF-1α binding sites being in an active chromatin configuration for binding to occur. This observation has been highlighted in both colorectal and pancreatic cancer where hypoxic induction of BCL-2/adenovirus E1B-19 kDa-interacting protein 3 (BNIP3), which is known to promote apoptosis, is blocked by promoter methylation.[13,14] Furthermore, it has been established that tissue specific expression and hypoxic induction of erythropoietin is controlled by high-density methylation of the 5'-untranslated region (5'-UTR).[15] Extending from these data, a recent publication by Xia et al. demonstrates that under acute hypoxia (0.5% oxygen, 4 hours), HIF-1 preferentially binds to gene loci and transactivates genes that are already in an 'active' configuration. In this study loci with an 'active' configuration have been

described to have; existing (pre-hypoxic) basal expression levels of mRNA, the presence of RNA polymerase II, and histone H3 lysine 4 trimethylation (H3K4me3).[16] Therefore if a gene is not defined in this way as being in an 'active' configuration HIF-1 is less likely to bind.

Direct control of HIF-1 binding has also been shown to be influenced by methylation of a specific CpG dinucleotide within the consensus HRE. This is evident in the erythropoietin and the class III beta-tubulin genes, where it has been shown that hypoxia-induced expression is dependent upon the tissue-specific methylation status of a HRE in the 3'-UTR.[17-19] As global changes in DNA methylation can occur under chronic hypoxic conditions, it is possible that this may impact on the methylation status of HRE sites,[1,20] and thus shape the HIF-dependent transcriptional profile according to the intensity and duration of the hypoxic insult. Gene specific DNA hypomethylation may reveal previously inaccessible HIF binding sites, thus exposing new active regions for HIF or other hypoxia-responsive transcription factors. Conversely, hypoxia-induced gene specific DNA hypermethylation may even mask previously active regions.

An additional mechanism by which epigenetics can impact on HIF signaling has recently been described by Kenneth et al. where they highlight the involvement of SWI/SNF chromatin remodeling complexes in the regulation of HIF-1α.[21] SWI/SNF, which can play a role in both enhancement and repression of gene transcription, was shown to directly bind the HIF-1α promoter and the BAF57 subunit of SWI/SNF was essential for hypoxia induced HIF-1α expression. Kenneth et al. also revealed that the SWI/SNF chromatin remodeling complex was required for hypoxia-induced cell cycle arrest.[21]

Histone Demethylase Enzymes as HIF-1α Targets

Histone methylation is an important epigenetic phenomenon that can result in both active and inactive chromatin states. These histone modifications are dynamically regulated by histone methyltransferases and histone demethylases. The impact of environmental factors on the regulation of these enzymes and the subsequent alteration in histone methylation status is a relatively novel field of investigation.

A collection of recent publications have investigated the impact of hypoxia on the regulation of a subset of Jumonji proteins that possess histone demethylase properties, specifically at lysine and arginine residues and contain a common catalytic Jumonji C domain. In particular, these articles highlight the direct involvement of HIF-1α in the transactivation of Jumonji proteins within a hypoxic environment, both in vitro and in vivo.[22-26] Furthermore, protein and mRNA levels of JMJD1A (jumonji domain containing 1A) JMJD2B, JMJ2C and JARD1A are increased under hypoxic conditions in vitro (0.2–1%, up to 24 h) in a variety of cell lines. JMJD1A is also upregulated in several rat organs in vivo (8%, up to 12 h).[22] All of these Jumonji proteins are direct targets of HIF, and their expression is induced as a consequence of HIF binding during hypoxic exposure.[22-25] The relevance of increased levels of Jumonji proteins in hypoxic environments was further investigated by Beyer et al. who acknowledged the importance of molecular oxygen for their demethylase activity and provided evidence that the demethylase activity of JMJD1A and JMJD2B was also increased in hypoxia, through studying H3K9me2 and H3K9me3 levels.[23]

Finally, the importance of hypoxic regulation of histone demethylases has been highlighted by the fact that hypoxic induction of some genes, such as adrenomedullin, are dependent on JMJD1A-induced changes in histone methylation status at the gene promoter region.[24]

Global Alterations in Histone Modifications and DNA Methylation in Hypoxia

The effect of hypoxia on chromatin remodeling is becoming increasingly evident with the observation of global changes in the normoxic histone code. Johnson et al. document a range of global histone modifications occurring in hypoxia (0.2% oxygen, 48 h), resulting in both activation and repression of gene transcription.[27] Importantly, they show the direct interaction of these modified histones with promoter regions of hypoxia-responsive genes. Of interest, analysis of the association of histone modifications and the promoters of these genes revealed that hypoxia increased H3K4me3 (usually associated with activation of gene transcription) and decreased levels of H3K27me3 (a transcriptional repressing event) in both activated and repressed hypoxia-responsive genes studied.[27] The authors suggest this may enable a more flexible chromatin response to transient hypoxic stimuli.

Additional evidence of global changes in histone methylation comes from interrogation of the levels of H3K4me2/me3, H3K9me2 and H3K36me3 which increase under hypoxic conditions (0.5–1% oxygen, up to 24 h).[25,28] One potential mechanism of increased H3K9me2 is believed to be mediated by the histone methyltransferase G9a, which has also been shown to increase in hypoxia.[28]

In addition to the emerging role of DNA methylation in regulating HIF binding to target genes, it has been shown that hypoxia can itself promote epigenetic changes in cancer cell lines. Using HPLC to quantify the global levels of 5'-methylcytidine in human colorectal and melanoma cell lines, Shahrzad et al. demonstrated that exposure to anoxia (≤0.1% oxygen, 24 hours) induces a 15–20% reduction in CpG methylation.[20] More recently our own data has shown significant alterations in the global levels of histone acetylation and DNA methylation in response to chronic hypoxic exposure where cells were permanently maintained at 1% oxygen. Specifically we found consistent and sustained global increases in both H3K9 acetylation and DNA methylation in the absence of HIF-1α and in association with significant increases in the expression of the DNA methyltransferase (DNMT) 3b enzyme.[1] The mechanism that promotes DNMT3b expression in hypoxia remains to be elucidated. Recent evidence suggests that microRNA's are important modulators of DNMT3a and DNMT3b expression with decreased expression of the miR29 family in lung cancer correlating with increased DNMT3 expression.[29] Given that miR29

expression drops significantly in the ischemic heart[30] it is tempting to speculate that hypoxic regulation of DNMT3b may be under the control of miR29.

Although many of the current opinions regarding the coordination of the hypoxic response with epigenetic mechanisms focus on the known interactions with HIF-1α, we believe that epigenetics has a further and more important role to play in the adaption and survival of cells growing in chronic hypoxia following the initial HIF-1α-induced cellular response. We have previously shown that epigenetic modifications are sustained in the absence of HIF-1 and suggest that they represent a newly established pattern of epigenetic marks, unique to this hypoxic response, which allow the cells to survive and function in this environment.

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