

FOCUS: 50 YEARS OF DNA REPAIR: THE YALE SYMPOSIUM REPORTS

Hypoxia and DNA Repair

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Hypoxia is a characteristic feature of solid tumors and occurs very early in neoplastic development. Hypoxia transforms cell physiology in multiple ways, with profound changes in cell metabolism, cell growth, susceptibility to apoptosis, induction of angiogenesis, and increased motility. Over the past 20 years, our lab has determined that hypoxia also induces genetic instability. We have conducted a large series of experiments revealing that this instability occurs through the alteration of DNA repair pathways, including nucleotide excision repair, DNA mismatch repair, and homology dependent repair. Our work suggests that hypoxia, as a key component of solid tumors, can drive cancer progression through its impact on genomic integrity. However, the acquired changes in DNA repair that are induced by hypoxia may also render hypoxic cancer cells vulnerable to tailored strategies designed to exploit these changes.

THE TUMOR MICROENVIRONMENT AND THE MALIGNANT PHENOTYPE

It is well established that solid tumors constitute a unique tissue type, characterized by hypoxia, low pH, and nutrient deprivation [1]. Although decreased oxygen tensions are potentially toxic to normal human cells, cancer cells acquire genetic and adaptive changes allowing them to survive and proliferate in a hypoxic microenvironment.

Clinically, studies have established hypoxia as an independent and adverse prognostic variable in patients with carcinomas of the head and neck and the cervix, as well as soft tissue sarcomas [2-4]. These correlations do not simply reflect the resistance of hypoxic cells to radiotherapy [5], as correlations have also been seen in surgically treated cases [6]. These findings underscore the importance of elucidating the effects of hypoxia at the mo-

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†Abbreviations: HDR, homology-dependent repair; MMR, mismatch repair; UTRs, untranslated regions; miR, microRNA; HDAC, histone deacetylase.

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lecular level and the mechanisms by which such conditions can exacerbate the malignant phenotype.

HYPOXIA AND GENE EXPRESSION

Hypoxia induces the expression of many genes that play important roles in tumor angiogenesis, progression, and metastasis, including glycolytic enzymes, growth factors, and transcription factors [1,7-9], in many cases via the action of the transcription factor, HIF-1 α [7,10]. However, many genes are also suppressed under hypoxic stress, often via HIF-independent mechanisms. Hypoxia induces the expression of histone deacetylases, accounting for decreased expression of numerous genes [11]. However, identification of hypoxia-induced repressors such as DEC1 [12] and NC2 α/β [13] suggests that promoter-specific mechanisms also exist. Gene expression patterns in hypoxic tumor cells do not simply conform to global pro-survival or pro-apoptotic transcriptional programs *per se*, as studies have established that hypoxia induces the expression of genes involved in both pro-apoptotic and pro-survival pathways [14,15].

GENETIC INSTABILITY IN THE TUMOR MICROENVIRONMENT

In early work, we demonstrated that the hypoxic tumor microenvironment constitutes a cause of genetic instability in cancer cells [16,17]. Studies in our lab and others demonstrated that cells grown in tumors have higher mutation frequencies compared to matched cell lines grown in culture [16,18,19]. We went on to show that hypoxia, in particular, is responsible for this increased genetic instability [20]. Subsequent studies have shown that hypoxic stress is associated with increased DNA damage (from reoxygenation), enhanced mutagenesis, and functional impairment of DNA repair pathways [17,18,20-31]. With regard to DNA damage, hypoxia and subsequent reoxygenation can induce DNA strand breaks and oxidative base damage, such as 8-oxoguanine and thymine glycol [21,32]. Exposure of cells in culture to

hypoxia yields increased frequencies of point mutations at reporter gene loci [16]. Hypoxia-reoxygenation cycles are also associated with gene amplification and DNA over-replication, although the mechanism by which they occur has not been fully elucidated [33,34]. Collectively, these phenomena represent forms of genetic instability induced by hypoxia, thereby accelerating the multi-step process of tumor progression.

Over the past 15 years, we have tested the hypothesis that hypoxia causes altered DNA repair. We found that hypoxia induces down-regulation of the DNA mismatch repair (MMR) factors, MLH1 and MSH2, at the transcriptional level. We also discovered that hypoxia induces down-regulation of the homology-dependent repair (HDR) factors, RAD51 and BRCA1. Interestingly, Francia et al. also found that MLH1 expression is reduced in cells grown as spheroids, consistent with the decreased oxygen tensions found within these cellular conglomerates [35].

Mechanistically, our work has identified roles for Myc and related factors in the co-regulation of *MLH1* and *MSH2* [27] and for E2F1 and E2F4/p130 complexes in the co-regulation of *RAD51* and *BRCA1* [28,29,36]. We have also found that hypoxia stimulates activation of the checkpoint kinase, CHK2, in an ATM-dependent manner and that CHK2, in turn, phosphorylates BRCA1 on Serine 988 [37,38]. The finding of CHK2 activation in response to hypoxia suggests that CHK2 may regulate *RAD51* and *BRCA1* expression in hypoxia by activating the phosphatase, PP2A (a known target of CHK2) [39,40]. The activation of PP2A may then dephosphorylate p130, thereby promoting the formation of repressive p130/E2F4 complexes.

In other work, we confirmed the hypothesis that unbalanced expression of MMR factors in mammalian cells can cause genetic instability and altered DNA damage responses [41]. We also carried out a comprehensive analysis of genetic instability in mice deficient in selected MMR factors [42], and we examined induced mutagenesis and carcinogenesis by diet-associated carcinogens in these mice [43,44].

DOWN-REGULATION OF *MLH1* AND *MSH2* GENE EXPRESSION IN HYPOXIA

Via a candidate-based approach, we initially found by western blot analyses that hypoxia specifically causes decreased expression of the MMR factors, *MLH1* and *PMS2* [23]. We determined that *MLH1*, but not *PMS2*, is down-regulated at the level of transcription [23], whereas *PMS2* protein levels drop in hypoxic cells because *PMS2* is destabilized in the absence of its heterodimer partner, *MLH1*. In further studies, we also found that hypoxia induces the down-regulation at the mRNA level not only of *MLH1* but also of *MSH2* following exposure to severe hypoxia [27].

We also carried out immunofluorescent image analysis of experimental tumors formed in mice from xenografts of human cancer-derived cell lines [23]. We found that regions of hypoxia (as judged by staining with the hypoxia marker, EF5) show reduced levels of *MLH1*.

ROLE OF MYC IN THE REGULATION OF MMR GENE EXPRESSION IN HYPOXIA

We have observed that hypoxia induces substantial down-regulation of Myc levels in MCF7, SW480, Caco-2, RKO, and HeLa cells. These expression decreases were correlated with a functional decrease in the transcriptional regulatory activity of Myc. As such, we considered the hypothesis that hypoxia-induced changes in Myc expression and/or transcriptional activity may directly play a role in the regulation of both *MLH1* and *MSH2* gene expression in hypoxia. We used quantitative chromatin immunoprecipitation (qChIP) to assess Myc binding to the respective promoters. We were able to localize Myc binding specifically to the proximal promoter region of both the *MLH1* and *MSH2* genes. Taking the next step, we also detected substantial decreases in Myc promoter occupancy in hypoxic versus normoxic cells at both the *MLH1* and *MSH2* proximal promoters.

BINDING OF MULTIPLE MYC-RELATED FACTORS TO THE PROXIMAL MMR GENE PROMOTERS

There are multiple repressive and activating factors within the Myc/Max network that can bind at both canonical E-boxes and non-canonical sites in Myc-target genes [45]. We found that several factors in the Max network bind with high specificity to the proximal *MLH1* and *MSH2* promoters in normoxic cells, including Max, Mad1, and Mnt [27]. Hence, hypoxia induces a dynamic shift in MMR promoter occupancy between activating and repressive members of this network.

DECREASED EXPRESSION OF *RAD51* IN HYPOXIA

In a survey of gene expression changes in response to hypoxia by transcriptome profiling in MCF7 cells, we found that hypoxia specifically down-regulates the expression of the HDR-associated genes, *RAD51* and *BRCA1* [26,29]. Consistent with this, we demonstrated that hypoxia causes a functional impairment of homologous recombination [26]. Interestingly, we found that *RAD51* expression is low not only in hypoxic cells but also in post-hypoxic cells for at least 24h following reoxygenation. Hypoxia-mediated *RAD51* down-regulation *in vivo* was also confirmed via immunofluorescent image analysis of experimental tumors in mice [26].

SUPPRESSION OF *BRCA1* EXPRESSION IN HYPOXIC CELLS BY E2F4/P130

Decreases in *BRCA1* expression were observed in numerous human cell lines derived from a wide range of tissues, and these decreases persisted during the post-hypoxic period following reoxygenation (as we found for *RAD51*) [29]. We next localized control of *BRCA1* expression in hypoxia to two adjacent E2F sites (referred to as E2FA and E2FB) in the proximal promoter region via a collection of wild-type and mutant *BRCA1* promoter-luciferase constructs [29]. We went on to perform an extensive series of qChIP assays in MCF7 cells that revealed that *BRCA1* pro-

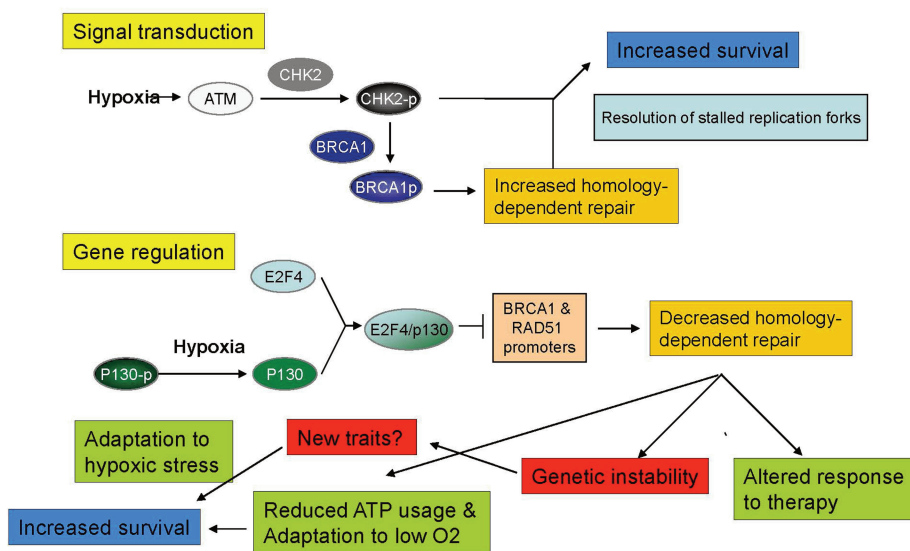


Figure 1. Regulation of homology-dependent repair in hypoxia and functional consequences.

motor occupancy by E2F1 decreases, whereas occupancy by E2F4 and the associated pocket proteins p130 and p107 increases [29], with similar results at the RAD51 promoter.

Mechanistically, we found that the increased E2F4/p130 occupancy at the *BRCA1* and *RAD51* promoters in hypoxia was correlated with p130 protein dephosphorylation and nuclear accumulation and increased formation of E2F4/p130 complexes [28]. Functionally, we found that the hypoxia-mediated decreases in HDR gene expression are also associated with functional changes in HDR at chromosomal sites, using a chromosomally based (DR-GFP/I-SceI) DSB repair assay [46], with production of GFP⁺ recombinants at frequencies of 0.53 percent (hypoxia) vs. 4.3 percent (normoxia) [29].

ACTIVATION OF CHK2 IN RESPONSE TO HYPOXIA IN AN ATM-DEPENDENT MANNER

In studying the cellular response to hypoxic stress, we found that CHK2 phosphorylation on threonine 68 is induced within a few hours after exposure to hypoxic conditions. We found that this induction is dependent on ATM [37], but not on the related kinase, ATR. Furthermore, we found that key downstream substrates of CHK2, including

p53, cdc25, and BRCA1, are modified under hypoxic conditions in a CHK2-dependent manner [38], indicating that hypoxia-induced phosphorylation of CHK2 leads to functional activation and downstream signaling. Finally, CHK2 was found to protect cells from hypoxia-induced apoptosis and, thus, appears to play a role in cell survival under hypoxic stress [37]. These results identified hypoxia as a new stimulus for CHK2 activation in an ATM-dependent manner and suggest a novel pathway by which tumor hypoxia may influence cell survival and DNA repair.

SENSITIVITY OF HYPOXIC CELLS TO INHIBITION OF POLY(ADP-RIBOSE) POLYMERASE-1 (PARP-1)

Based on the finding that *BRCA1*- and *BRCA2*-deficient cells are hypersensitive to PARP-1 inhibitors [47,48], we tested whether that sensitivity may also occur in cells with transient and partial BRCA1 (or RAD51) deficiency due to hypoxia-induced down-regulation. We found that hypoxic cells are, indeed, sensitive to PARP inhibition. We also made the unexpected finding that PARP inhibition itself suppresses BRCA1 and RAD51 gene expression in a manner dependent on E2F4 [49]. This suppression of BRCA1 and RAD51 by PARP inhibition was also shown

to mediate increased radiation sensitivity [49], providing a mechanistic basis for the observation that PARP inhibitors serve as radiation sensitizers.

Figure 1 provides a diagram depicting the complex regulation of the homology-dependent repair pathway in response to hypoxia. The predicted functional consequences are illustrated, some of which have been experimentally validated, including genetic instability, decreased homology dependent repair, increased survival, and altered therapy response [16,26,29,37,38,50].

HYPOXIA-INDUCED microRNAS THAT IMPACT DNA REPAIR FACTORS

In addition to classic transcription factors like E2F and Myc, control of gene expression can be mediated by small non-protein-coding RNAs, or microRNAs (miRs), which target mRNA destabilization or suppress translation. In general, miRs bind to the 3' untranslated regions (UTRs) of target mRNAs via imperfect base-pairing complementarity leading to degradation or translational inhibition. The regulation of mRNAs by miRs can impact multiple cellular processes, including apoptosis, differentiation, and cell survival. We examined miR expression in response to hypoxic stress as another potential mechanism that might alter the factors involved in DNA repair. We found that two miRs, miR-210 and miR-373, are elevated in response to hypoxia in a pathway dependent on HIF-1 [51]. We found that miR-210 targets RAD52, a member of the HDR pathway, whereas miR-373 targets both RAD52 and RAD23B [51]. Mechanistically, levels of both RAD52 and RAD23B are down-regulated in hypoxic cells; in normoxic cells, the forced expression of miR-210 reduces RAD52 levels, while miR-373 suppresses both RAD52 and RAD23B. In hypoxic cells, the inhibition of miR-210 and miR-373 partially reverses the hypoxia-induced down-regulation of RAD52 and RAD23B, respectively. The suppression of RAD52 by miR-210 and by miR-373 offers an additional mechanistic explanation for the reduced HDR activity in hypoxic cells,

whereas the down-regulation of RAD23B by miR-373 provides a new mechanism for the previously unexplained reduction of NER in hypoxia [20]. These findings also highlight an important role for miRs in the regulation of DNA repair.

HYPOXIA-INDUCED miR-155 CONFERS RADIATION RESISTANCE

Another miR, miR-155, has emerged as a key regulator of numerous biological processes, including immune function and carcinogenesis. Interestingly, miR-155 is overexpressed in lung cancers [52,53], and its increased expression is associated with poor prognosis in lung cancer patients [53,54]. We identified miR-155 as another miR that is induced by hypoxia [55] and were able to show that elevated levels of miR-155 protect cancer cells from radiation; conversely, we further showed that inhibition of miR-155 radiosensitizes hypoxic lung cancer cells [55]. In recent work, it was shown that miR-155 targets MLH1 and MSH2 [56]. Based on this, we predicted that increased levels of miR-155 would drive genetic instability and mutagenesis by suppressing DNA mismatch repair. Preliminary experiments suggest that this is the case.

HYPOXIA DRIVES SILENCING OF THE BRCA1 PROMOTER

As discussed above, we have shown that *BRCA1* and *MLH1* are down-regulated at the mRNA and protein levels in response to hypoxia via specific pathways of transcriptional regulation [57-60]. Intriguingly, *BRCA1* and *MLH1* are silenced in many sporadic cancers of multiple sites [61-63], similar to a number of other tumor suppressors. The silencing of *BRCA1* and *MLH1* initially was attributed primarily to promoter DNA hypermethylation at CpG sites [62]. However, further studies suggest that silenced promoters in cancer cells are also marked by characteristic histone modifications [64-66], and evidence is emerging that histone methylation may be a mediator of silencing that is independent of DNA methylation [67-69].

Relevant to our work, hypoxia-induced histone modifications have been reported, and these can be found in both hypoxia-activated and hypoxia-repressed genes [70]. The regulation of gene expression by hypoxia through covalent modification of histones is also supported by evidence that histone deacetylase (HDAC) activity plays a role in activation of many HIF-1-responsive genes [71]. In addition, certain histone demethylases and histone methyltransferases have been identified as hypoxia- or HIF-1-regulated genes, including JMJD1A, JMJD2B, JARID1B, and G9a histone methyltransferase [64,69,72-75].

Based on the above, we tested whether hypoxia might be a driving force in the silencing of tumor suppressor genes, particularly *BRCAl* and *MLH1*. Recent evidence has shown that silenced *BRCAl* alleles found in sporadic cancers are associated not only with promoter DNA hypermethylation [62,76,77], but also with histone modifications in the promoter region [78]. Hence, we hypothesized that hypoxia-induced down-regulation of *BRCAl* might cause epigenetic histone modifications that mark the locus for potential silencing. We found that hypoxia induces a set of repressive histone marks at the *BRCAl* promoter, including H3K4 demethylation, H3K9 methylation, and H3K9 deacetylation, with opposite changes in the histone code at the promoter of the hypoxia-inducible *VEGF* gene [79]. We further found that a key histone modification at the *BRCAl* promoter in response to hypoxia, H3K4 demethylation, is mediated by the histone demethylase, lysine-specific demethylase 1 (LSD-1) [79].

Importantly, using a reporter gene assay system in which the *BRCAl* promoter is inserted upstream of a selectable gene, we determined that prolonged exposure of cells to moderate hypoxia over the course of several weeks can promote the emergence of subclones in which the *BRCAl* promoter has undergone long-term silencing [79]. We further showed that the *BRCAl* silencing persists for weeks even when cells are no longer in hypoxic conditions. In these silenced clones, the *BRCAl* promoter is marked by H3K4 demethylation and H3K9 deacetylation [79]. In recent preliminary work, we have ob-

tained initial evidence that a related pathway drives silencing of the *MLH1* promoter in response to hypoxia.

CONCLUSIONS

Genetic instability is a hallmark of cancer, and our ongoing work has shown that the hypoxic tumor microenvironment is a key driver of this instability. Mechanistically, we have demonstrated that hypoxia down-regulates the expression of several DNA repair genes. We have also found that hypoxia induces post-translational modification of key DNA repair and damage response factors, including ATM and CHK2, thereby further altering the DNA repair capacity of hypoxic cells. In addition, we have identified hypoxia-induced microRNAs (miR-155, miR-210, and miR-373) that also impact DNA metabolism and DNA damage responses. In recent work, we have also discovered that hypoxic stress can bring about durable long-term silencing of the *BRCAl* and *MLH1* promoters by means of specific epigenetic factors, including LSD-1.

Overall, our work has begun to elucidate changes in DNA metabolism and in epigenetic regulation in response to hypoxia that underlie carcinogenesis and tumor progression. The thrust of our findings is that hypoxia causes both genetic and epigenetic instability. Moreover, based on acquired changes in DNA repair, we have begun to identify potential vulnerabilities of hypoxic cancer cells, but not normoxic, healthy cells, to selected anti-cancer agents. We expect that further characterization of the hypoxic cell phenotype with respect to DNA repair will offer the possibility of developing new therapeutic agents to which hypoxic cancer cells are particularly susceptible.

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