GENOME INTEGRITY

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Tumor hypoxia as a driving force in genetic instability

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Abstract

Sub-regions of hypoxia exist within all tumors and the presence of intratumoral hypoxia has an adverse impact on patient prognosis. Tumor hypoxia can increase metastatic capacity and lead to resistance to chemotherapy and radiotherapy. Hypoxia also leads to altered transcription and translation of a number of DNA damage response and repair genes. This can lead to inhibition of recombination-mediated repair of DNA double-strand breaks. Hypoxia can also increase the rate of mutation. Therefore, tumor cell adaptation to the hypoxic microenvironment can drive genetic instability and malignant progression. In this review, we focus on hypoxia-mediated genetic instability in the context of aberrant DNA damage signaling and DNA repair. Additionally, we discuss potential therapeutic approaches to specifically target repair-deficient hypoxic tumor cells.

Keywords: Hypoxia, Genetic instability, DNA damage, DNA double-strand breaks, DNA repair

Introduction

The tumor microenvironment is characterized by sub-regions of nutrient deprivation, low extracellular pH, high interstitial fluid pressure, and hypoxia. Hypoxic areas arise when oxygen consumption exceeds that of supply [1]. In normal tissues, the oxygen supply matches the metabolic requirements of the cells. However, in locally advanced solid tumors, the oxygen consumption increases significantly, resulting in inadequate oxygen supply to some regions of the tumor. In addition, the blood vessels within a tumor microenvironment are usually chaotic, dilated and irregularly organized [1]. In normal tissues, the oxygen tension (pO_2) ranges from 10 to 80 mmHg (or 1.25% to 10% O_2). However, tumors often contain regions where the oxygen concentration can significantly decrease to less than 5 mmHg (or < 0.6% O_2) [2,3]. Clinical studies using pO_2 electrodes, hypoxia imaging (positron emission tomography (PET)), and immunohistochemistry (IHC) have demonstrated that hypoxia is a characteristic of all solid tumors [4]. Hypoxic regions within tumors can be measured by IHC assessment of intrinsic and extrinsic hypoxic cell biomarkers. Intrinsic biomarkers of hypoxic response include hypoxia inducible factor 1 (HIF1α), vascular endothelial growth factor (VEGF), carbonic anhydrase IX (CAIX), osteopontin and glucose transporters 1 and 3 (GLUT1, GLUT3) and the extrinsic biomarkers include drugs that specifically accumulate or become bio-reduced to form adducts within hypoxic cells such as pimonidazole (PIMO), EF5 and CCI-103 F [5]. Increased levels of hypoxia correlates with genetic instability, tumor progression, local and systemic resistance; all leading to poor clinical outcome following treatment [6-12].

Tumor cells that lie beyond the diffusion distance for oxygen (> 70 μm away from blood vessels) can quickly outstrip blood supply and are exposed to chronically low oxygen tensions [13]. These diffusion-limited conditions for duration of days are referred to as "prolonged" or "chronic hypoxia" [14]. The cells in these regions are believed to remain hypoxic until they die (due to lack of oxygen or nutrients) or are reoxygenated [15]. Hypoxia can also be transient or "cycling" due to acute perfusion changes in the tumor vasculature. The blood vessels formed during unregulated angiogenesis contain severe structural and functional abnormalities and can temporarily close and re-open, leading to cycles of acute hypoxia/anoxia (from minutes to hours) followed by

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reoxygenation (hence, cycling hypoxia) [14]. Both acute and chronic hypoxia co-exist within a tumor resulting in significant gradients of oxygen consumption leading to intratumor heterogeneity [16].

In an experimental setting, cellular hypoxia can be induced by placing cultured tumor cells in complete media in environmentally-controlled chambers in which oxygen levels in the gas phase are maintained at 0.01-3% [17]. These hypoxic conditions may not be lethal nor growth inhibitory to selected tumor cell lines when cultured in the presence of excess glucose and nutrients. However, when cells are placed in the complete absence of oxygen (anoxia), most cells will stop proliferating due to the activation of anoxia-mediated intra-S phase arrest mediated by the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3-related (ATR) kinases [18-21]. If prolonged, this arrest of DNA replication becomes irreversible leading to cell death mechanisms [22]. Hence, a permanent anoxic microenvironment (e.g. close to 0% O_2) eventually leads to cell death whereas tumor cells that exist in hypoxic microenvironments (e.g. 0.2 to 1% O_2) could adapt and continue to proliferate with altered biology [12,14]. Tumor cells that adapt to low oxygen conditions gain an overall advantage for growth and leads to treatment resistance following chemotherapy or radiotherapy [14]. Therefore, the study of proliferating hypoxic cells is important as it represents a clinically-challenging, sub-population of resistant cells with the potential of clonal expansion and metastatic spread.

Clinical observations, supported by pre-clinical data, have demonstrated that hypoxia is associated with an increased capacity for metastasis [23]. Metastasis is a multi-step process that involves disruption of cell adhesion to the neighboring cells and to the basement membrane, migration through the extracellular matrix, penetration of vessel walls and circulation exit, and finally initiation of angiogenesis to allow tumor growth in the target tissue [24]. Hypoxia can lead to altered expression of many proteins involved in this process by regulating the expression of E-cadherin (cell-cell contact), urokinase-type plasminogen activator receptor (uPAR; degradation of extracellular matrix proteins), hepatocyte growth factor (HGF; cellular motility) and vascular endothelial growth factor (VEGF; angiogenesis and vascular permeability) [14,24,25]. Hypoxia also limits the effectiveness of many anti-cancer therapies. The efficiency of ionizing radiation to create lethal DNA breaks is strongly associated with oxygen tension and creation of free radicals. Oxygen can react with the damaged DNA bases created by free radicals to yield a more stable adduct and this reaction chemically "fixes" the damage [2]. Indeed, oxygenated cells can be two to three times more sensitive to radiation than hypoxic or anoxic cells [12,26]. However, ionizing radiation under anoxic
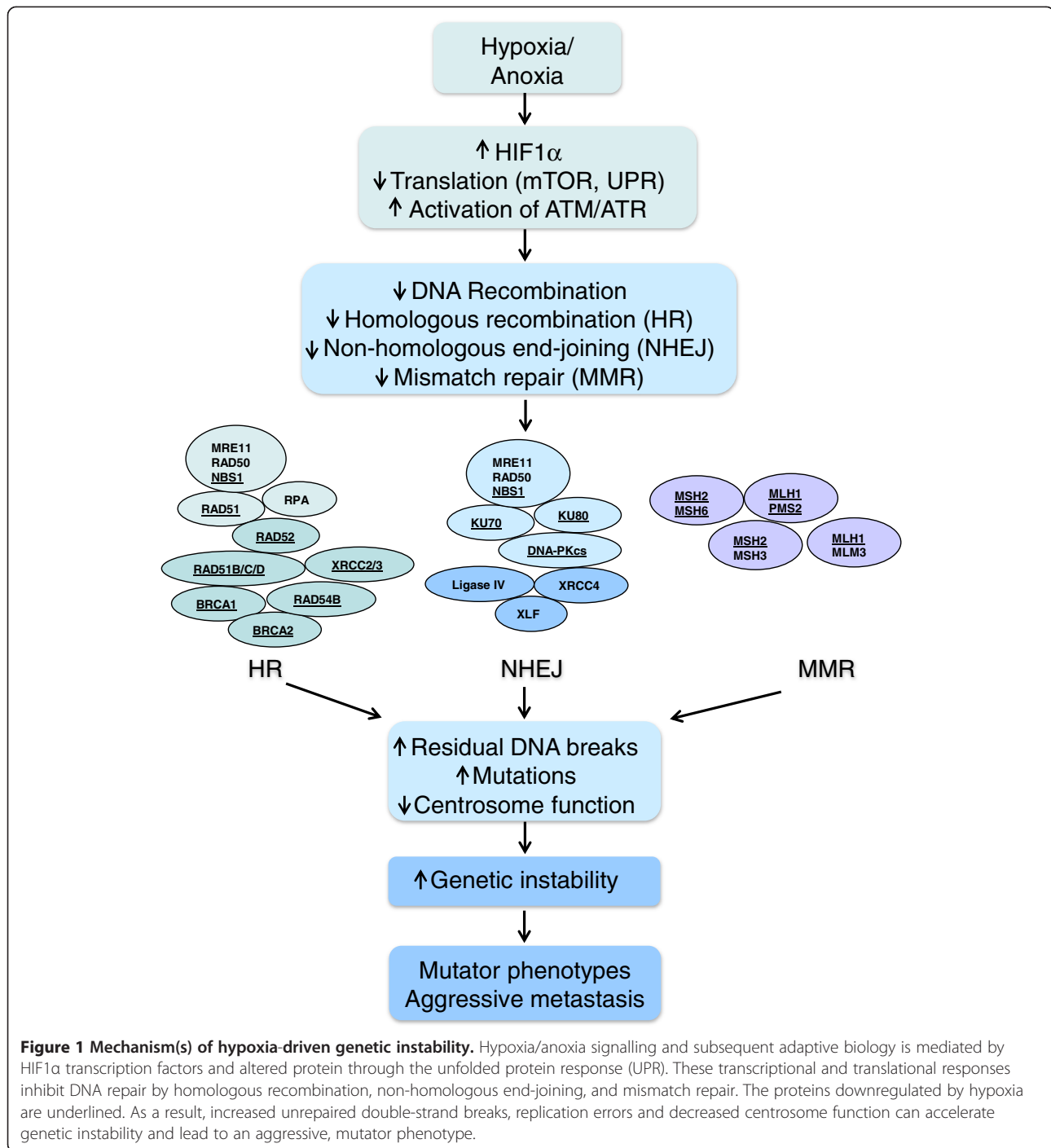
conditions has been shown to increase the levels of DNA-protein crosslinks [27,28]. Moreover, poor drug distribution and decreased proliferation can decrease the efficacy of many chemotherapy drugs [12,14]. Thus, the cells in hypoxic regions can adapt to become resistant to radiotherapy and chemotherapy and ongoing selection of increasing aggressiveness [29]. Therefore, two main clinical entities are associated with hypoxic tumors: increased local tumor cell resistance and development of systemic metastasis. Despite these data, hypoxia-targeted therapy is still not a standard of current cancer treatments [30]. Therefore, the study of hypoxic cells is important in order to gain a further understanding of the consequences of the hypoxic microenvironment for the development of genetic instability as a precursor to tumor progression and therapy-associated resistance.

Hypoxia-mediated genetic instability
Tumor cells can acquire multiple adaptations in the selective pressure of the tumor microenvironment. Hypoxia inducible factor 1α (HIF1α) is a transcription factor, which is kept at low levels in the presence of oxygen by von Hippel-Lindau protein (VHL)-mediated degradation [31]. In hypoxic conditions, HIF1α is quickly stabilized and regulates a number of genes including those involved in vascularization, glycolysis and pH homeostasis [31]. HIF1α is crucial for hypoxic adaptation, and overexpression of HIF1α is associated with a poor disease outcome [32]. Loss of HIF1α control can promote the malignant phenotype and genomic instability via interplay with oncoproteins such as c-MYC [33-37]. Oncogene amplification, DNA replication stress, and deregulated DNA damage checkpoint signaling in hypoxic tumor cells, together with the ability to escape cell death, can allow cells to proliferate in the presence of damaged DNA and acquire further mutations [38,39]. The vicious cycle is accelerated by increased frequency of mutations and by the ability of hypoxic cells to downregulate DNA repair; therefore further driving genomic instability (see Figure 1) [14,40]. Moreover, when hypoxic cells become reoxygenated, they may acquire further DNA damage as a result of a sudden burst of free radicals [41,42]. We now discuss further hypoxia-mediated genomic instability in the context of the DNA damage signaling and inhibited DNA repair.

Hypoxia and the DNA Damage Response (DDR): checkpoints and DNA replication
Human cells maintain genetic integrity by detecting DNA damage and activating cell cycle checkpoints and DNA repair pathways [43]. The G1/S, intra-S, and the G2/M checkpoints, are mediated by ATM/ATR and checkpoint kinases 2 and 1 (CHK2)/(CHK1), respectively [18-21,43]. These kinases transmit signals to the effector

Figure 1 Mechanism(s) of hypoxia-driven genetic instability. Hypoxia/anoxia signalling and subsequent adaptive biology is mediated by HIF1α transcription factors and altered protein through the unfolded protein response (UPR). These transcriptional and translational responses inhibit DNA repair by homologous recombination, non-homologous end-joining, and mismatch repair. The proteins downregulated by hypoxia are underlined. As a result, increased unrepaired double-strand breaks, replication errors and decreased centrosome function can accelerate genetic instability and lead to an aggressive, mutator phenotype.

molecules p53, p21 (G1/S) and CDC25 (G1/S, intra-S and G2/M) to prevent cell cycle progression or to initiate programmed cell death [44,45]. Cycles of hypoxia followed by reoxygenation in tumors cyclically activates many DNA damage response (DDR) proteins. Furthermore, ATM, DNA-PKcs, H2AX, p53, CHK1, CHK2, 53BP1 and NBS1 are phosphorylated under conditions of severe hypoxia (<0.02%) in the absence of exogenous DNA damage [18,41,46-51]. Anoxia therefore leads to cell cycle arrests at G1 and intra-S in the absence of DNA damage, and in turn, reoxygenation causes CHK2-mediated G2 arrest [12,19,21,22,38,52]. When an arrested hypoxic cell becomes reoxygenated, it may either resume proliferation or undergo an irreversible loss of DNA replication ability and undergo cell death [38,53-55]. The length of the hypoxic stress may determine the ultimate

fate of a cancer cell [38]. Cell cycle changes however depend on the level of hypoxia. For example, oxygen levels such as 0.2% do not activate ATM or ATR and cell cycle checkpoint signaling [56]. Propagation of such a tumor cell with potentially altered DNA damage signaling and reoxygenation-induced DNA damage, can contribute to genetic instability and malignant progression [38].

HIF1α can also bind directly to minichromosome maintenance (MCM) proteins that are responsible for unwinding the DNA during replication [57]. Direct interaction between HIF1α and MCM7 results in increased prolyl hydroxylation-dependent HIF1α degradation, and an interaction with MCM3 results in HIF1α transactivation domain function inhibition [58]. HIF1α can block replication origin firing and DNA replication by binding to Cdc6, which is involved in recruiting MCM helicases to replication origins. HIF1α-Cdc6 interaction leads to enhanced MCM helicase loading and decreased recruitment of Cdc7 to replication origins, resulting inhibition of replication origin firing and overall DNA replication [57].

Hypoxia causes microsatellite and chromosomal instability

Studies have also documented an increased rate of spontaneous DNA mutations in cells exposed to hypoxia using reporter assays. This further supports the view of tumor microenvironment as a driving force of genomic instability (see Table 1) [59-62]. The concept of genetic instability covers a wide variety of genetic alterations from point mutations to chromosomal number. These changes are divided into two types: microsatellite instability (MSI) and chromosomal instability [63]. MSI is typically found in colorectal cancers and is caused by defective DNA mismatch repair (MMR) [64]. As hypoxia downregulates MMR, a model of tumor microenvironment-driven MSI has been proposed. This suggestion is supported by studies both in vitro and in vivo of colorectal cancer models [65-67]. High level of HIF1α associates with MSI in human colorectal carcinoma [68,69]. Further investigation in clinical settings will show whether the mechanistic laboratory findings of HIF-MMR-MSI can be generalized to other cancers in addition to colon carcinomas.

DNA double-strand break (DSB) repair is crucial for chromosomal integrity. Unrepaired DSBs can lead to formation of deletions, insertions, translocations and amplifications [83,84]. For example, cells deficient for BRCA1/2 develop spontaneous gross chromosomal aberrations [85-89]. Hypoxia is known to both inhibit DSB repair and to promote chromosomal instability in multiple ways [71,73,90]. Fragile sites are specific chromosomal regions prone to chromosomal breakage and rearrangements during replication stress and are induced under hypoxia [73,91]. This could be, in part, explained by hypoxia-mediated downregulation of DSB repair genes, as RNAi inhibition of DSB repair results in fragile site activation [92]. Additionally, ATM and ATR kinases maintain fragile site stability, and DSB biomarkers γ-H2AX and DNA-PKcs^{Thr2609} foci localize at fragile sites [92,93]. An unrepaired DSB can also lead to DNA amplification, which has been observed in hypoxic cells [70,71,75,79,94]. Additionally, the frequency of sister chromatid exchange (SCE), which is in part controlled by homologous recombination (HR) repair, may be increased in hypoxic primary human lymphocytes [81,95]. Human fibroblasts subjected to continual hypoxic conditions following exogenous DNA damage maintained increased chromosomal aberrations such as chromosome breaks, chromatid breaks, ring chromosomes, telomeric fusions, reciprocal translocations and double minutes [82]. Finally, hypoxia may also induce global deacetylation and methylation of histones, phosphorylation of H2AX and altered condensation states within the chromatin [90].

In order to prevent mitotic errors leading to genetic instability, the cell must properly align chromosomes during mitosis. The mitotic spindle is generated by the activity of centrosomes, which are composed of centrioles and pericentriolar material [96]. Defects in centrosomes and spindle formation lead to aneuploidy during the process of carcinogenesis and tumor progression [97,98]. Recently, a study has shown that hypoxia can modify centrosome function by altering the activity of prolyl-4-hydroxylases (PHDs) towards the protein Cep192 (a critical component of the centrosome) [99]. This allows for mediating signaling between oxygen tension and cell cycle control. Further studies are required to investigate whether these and other genes that are involved in mitosis and centrosome organization are altered in cancer cells within hypoxic sub-regions of solid tumors.

Altogether, these studies support the concept that hypoxia can modify fragile sites, the repair of DNA damage, chromatin biology, and possibly mitosis in promoting genetic instability during tumor progression.

Hypoxia-mediated inhibition of DNA repair

The understanding of hypoxia in the context of signaling and DNA repair is increasing based on data using isogenic models that vary in specific DNA repair pathways. Below, we discuss the mechanisms of DNA repair downregulation in hypoxic cells in a pathway-specific manner (Figure 1).

DNA double-strand break repair

Ionizing radiation (IR) or radiomimetic drugs create DSBs, which are mainly repaired by HR or non-homologous end-joining (NHEJ) pathways in a cell cycle-dependent manner [100]. The proteins RAD51, BRCA1/2 and the

Table 1 Evidence linking hypoxia to tumor cell genetic instability

Author	% Oxygen	Cell system	Assays	Key findings
Rice et al. [70]	0%	AA8 (CHO)	Flow cytometry, gene copy analyses	- Anoxia induces S-phase overreplication and increases the frequency of dihydrofolate reductase gene amplification
Young et al. [71]	0% (<10 ppm)	KHT-C2-LP1 (M-fibrosarcoma), B16F10-A1 (M-melanoma)	Metastasis assay, flow cytometry	- Anoxia induces DNA overreplication and increases metastatic potential
Reynolds et al. [61]	0% (<10 ppm)	LN12 (M-fibroblasts)	Chromosome based λ shuttle vector, PCR, DNA sequence analysis	- Anoxia induces 3–4 fold increase in supF tRNA suppressor gene mutation (transversions and deletions) frequency
Rofstad et al. [72]	0% (<10 ppm and <100 ppm)	BEX-c (H-melanoma), SAX-c (H-melanoma)	Flow cytometry, Giemsa	- Anoxia followed by reoxygenation induces diplochromosomes and tetraploidization
Coquelle et al. [73]	0.02%	GMA32 (Chinese hamster fibroblasts)	Fluorescence in situ hybridization (FISH)	- Severe hypoxia induces fragile sites and generates homogeneously stained regions (HSRs)
Yuan et al. [74]	0% (<10 ppm)	3340 (M-fibroblast)	Host cell reactivation (HCR) assay, UV mutagenesis assay	- Anoxia induces 2-fold increase in supFG1 mutation frequency
Coquelle et al. [75]	0.02%	GMA32 (Chinese hamster fibroblasts), 112 (Chinese hamster fibroblasts)	Fluorescence in situ hybridization (FISH)	- Severe hypoxia activates fragile sites and generates double minutes and dicentric chromosomes
Mihaylova et al. [76]	0% <10 ppm	3340 (M-fibroblasts), HeLa (H-cervix adenocarcinoma), EMT6 (M-breast carcinoma)	β-galactosidase and supFG1 mutation assays	- Anoxia induces 2-fold increase in supFG1, cII and lacZ mutation frequency
Banath et al. [77]	i.p. pimonidazole	V79-VE (Chinese hamster fibroblasts), HCT116 (H-colon carcinoma), SCCVII (M-squamous cell carcinoma)	Flow cytometry, γ-H2AX foci, HPRT mutation assay, alkaline comet assay	- Hypoxia (cells distant to the blood vessels) followed by reoxygenation does not alter mutation frequency at HPRT locus, DNA strand break rejoining or resolution of γ-H2AX foci following ionizing radiation (IR)
Koshiji et al. [78]	1%	HCT116 (H-colon carcinoma), HEC59 (H-endometrial carcinoma)	β-galactosidase mutation assay, microsatellite analysis	- Hypoxia increases the frequency of microsatellite mutations
Papp-Szabo et al. [59]	0%	ME (R-mammary epithelial cells), MFib (R-mammary fibroblasts)	cII mutagenicity assay	- Anoxia increases the mutation frequency by 2-fold at cII locus without affecting colonogenic survival
Fischer et al. [79]	0%	TX3868 (H-glioblastoma)	Fluorescence in situ hybridization (FISH)	- Anoxia induces double minutes, fragile sites and anaphase-bridges and initiates gene amplification on chromosome 12q

Table 1 Evidence linking hypoxia to tumor cell genetic instability (Continued)

Rodriguez-Jimenez et al. [80]	1%	C17.2 (M-multipotent neural precursor cells), M-primary neurospheres from CD31, BMMSC (H-mesenchymal stem cells), DPSC (H-mesenchymal stem cells)	Host cell reactivation (HCR) assay, microsatellite instability analysis	- Hypoxia increases mutation frequency of the β-galactosidase reporter gene and causes microsatellite instability
Keysar et al. [60]	<0.1%	$A_L(N)$ (CHO)	Complement cytotoxic assay, flow cytometry mutation assay	- Anoxia results in a significant induction of mutations especially large deletions in *CD59* gene
Lee et al. [81]	3%	Primary lymphocytes from healthy donors	Sister chromatid exchange (SCE) assay, microsatellite instability assay	- Hypoxia increases SCE but does not alter microsatellite instability
Pires et al. [38]	<0.02%	RKO (H-colon carcinoma), HCT116 (H-colon carcinoma), U2OS (H-osteosarcoma), IBR3 (H-fibroblast)	DNA fiber analysis, immunofluorescence	- Anoxia blocks DNA replication at the initiation and elongation stages and compromises DNA replication restart - Acute anoxia following reoxygenation (cycling hypoxia) does not affect DNA replication restart
Kumareswaran et al.* [82]	0.2%	GM05757 (H-fibroblasts)	Giemsa, Multicolor fluorescence in situ hybridization (M-FISH)	- Hypoxia increases the frequency of fragmented DNA, ring chromosomes, telomeric fusions, chromosomal translocations and marker chromosomes following exogenous DNA damage

CHO – Chinese hamster ovary cells; M – mouse; H – human; R – rat.
*only study investigating DNA repair under continual hypoxic conditions.

MRN complex (MRE11, RAD50, NBS1) together regulate HR during S and G2 phases of the cell cycle. Proteins such as KU70/80, DNA-PKcs and DNA-ligase IV function in NHEJ across all phases of the cell cycle [100].

The majority of HR proteins are repressed by chronic hypoxia [101]. This can occur through decreased transcription, translation, miRNA modulation and epigenetic silencing. The first mechanistic model suggests that HIF1α competes with and opposes MYC activity in hypoxic cells, inhibiting *Brca1* and *Nbs1* transcription [35,102-104]. Another model proposes that HR gene expression, including *Rad51* and *Brca1*, is repressed by the E2F-4/p130 complex independent of HIF [105-107]. The HIF-independent mechanism is supported by observations of downregulated RAD51 in isogenic HIF1α$^{-/-}$ mouse embryo fibroblasts (MEFs) under hypoxia, albeit by reduced efficiency [108]. Studies from our laboratory support a third model involving selective inhibition of protein synthesis. Hypoxia alters protein synthesis by pathways that modulate gene expression in both transcript-specific and a global manner; via unfolded protein response (UPR) and mammalian target of rapamycin (mTOR) signaling [109]. Our findings indicate that in chronically hypoxic proliferating cells, RAD51 and BRCA2 are downregulated due to selective inhibition of mRNA translation [56]. Yet another layer to hypoxia-regulated HR expression involves altered chromatin modification and *Brca1* promoter silencing in severe hypoxia [110]. Finally, miRNA may play a role in HR suppression and can affect *Rad52* gene expression [111].

The impact of hypoxia and DNA repair on malignant progression is demonstrated in studies indicating that repressed HR is linked with cancer initiating cell formation [112]. Breast tumor-initiating cells overexpress polycomb protein EZH2, which is further induced by HIF1α under hypoxia [112,113]. EZH2 inhibits *Rad51* transcription in hypoxic CD44^{+}CD24$^{-/low}$ cells, which is associated with increased genomic abnormality [112]. This EZH2-RAD51 signaling (via RAF1 amplification) promotes mammosphere formation and malignant progression [112].

The function of NHEJ in hypoxia-driven genetic instability and radiation response is more controversial. Inhibited expression of *DNA-PKcs, Ku70, Ku80* and *DNA-ligase IV* has been observed under hypoxia [101,114]. NHEJ factors are downregulated in hypoxic wild-type MEFs and in normoxic HIF1α$^{-/-}$ MEFs [115]. In cervical tumors, KU70/KU80 expression correlates with oxygen pressure and is inhibited with increasing distance to blood vessels [116]. We observed an increase in residual DSBs in G0/G1 synchronized human fibrobalsts under hypoxic conditions following exogenous DNA damage (Figures 2 and 3) [82]. On the other hand, induction of *Ku70* may occur under hypoxia in some cell lines [114]. KU70 could indeed contribute to hypoxic tumor cell resistance to radiation, as expression of a dominant negative form of KU70 sensitizes hypoxic glioma and colorectal cells to radiation [117]. Other reports have proposed redundancy or increased NHEJ under hypoxia [118-120]. An outstanding question in the field is whether the MRN complex, ATM and DNA-PKcs kinases differentially sense DSBs under oxia vs hypoxia (Figure 1). Varying model systems and tumor microenvironment conditions might explain the differing observations, and further investigation will clarify the role of hypoxia in NHEJ control.

Mismatch repair

MMR repairs DNA base substitutions and misalignments, which occur during DNA replication [122]. Mammalian MMR uses proteins such as MutSα (MSH2 + MSH6), MutSβ (MSH2 + MSH3), and MutLα (MLH1 + PMS2) [122].

The involvement of MMR in the hypoxic response is fairly well characterized. The hypoxia-driven genetic instability in colorectal cancers is consistent with inhibited *Mlh1* transcription in low oxygen [76]. Mechanistically, MMR inhibition under hypoxia involves at least MYC and DEC transcription factors. Interplay of HIF1α and MYC has been suggested to regulate MMR expression; MYC-dependent regulation of MSH2 and MSH6 in oxic cells may be replaced by HIF1α under hypoxia [35,78,104]. In addition, knockdown of HIF1α reverses hypoxia-driven inhibition of MMR expression [78,123]. Repression of MMR gene expression by decreased MYC and increased MAX, MAD and MNT association on *Mlh1* and *Msh2* promoters have been observed in hypoxic cells [107]. MYC, MAD and MNT (as part of the "max network" containing basic helix-loop-helix zipper (bHLHZ) motifs) form heterodimers with MAX resulting in sequence-specific DNA binding [124]. These DNA-bound heterodimers can then alter chromatin structure to modulate transcription [124]. Additionally, hypoxia-induced transcription repressors DEC1 and DEC2 contribute to *Mlh1* inhibition [125]. Hypoxic MMR regulation is also influenced by the state of chromatin acetylation [66,76,80,125].

Nucleotide excision repair and Fanconi anemia pathway

Chemicals covalently bound to DNA forming bulky adducts, as well as chemical-caused DNA crosslinks and UV-induced DNA lesions, are repaired by nucleotide excision repair (NER). NER in mammals uses two pathways: global genome repair (GGR) and transcription-coupled repair (TCR) [126]. GGR involves multiple sequential steps including sensing of the lesion (XPC-HR23B-Centrin 2 complex), opening of a denaturation bubble (TFIIH, XPA-RPA complex), incision of damaged strand (XPG, XPF-ERCC1 complex), displacement of lesion-containing oligonucleotides and gap filling (DNA
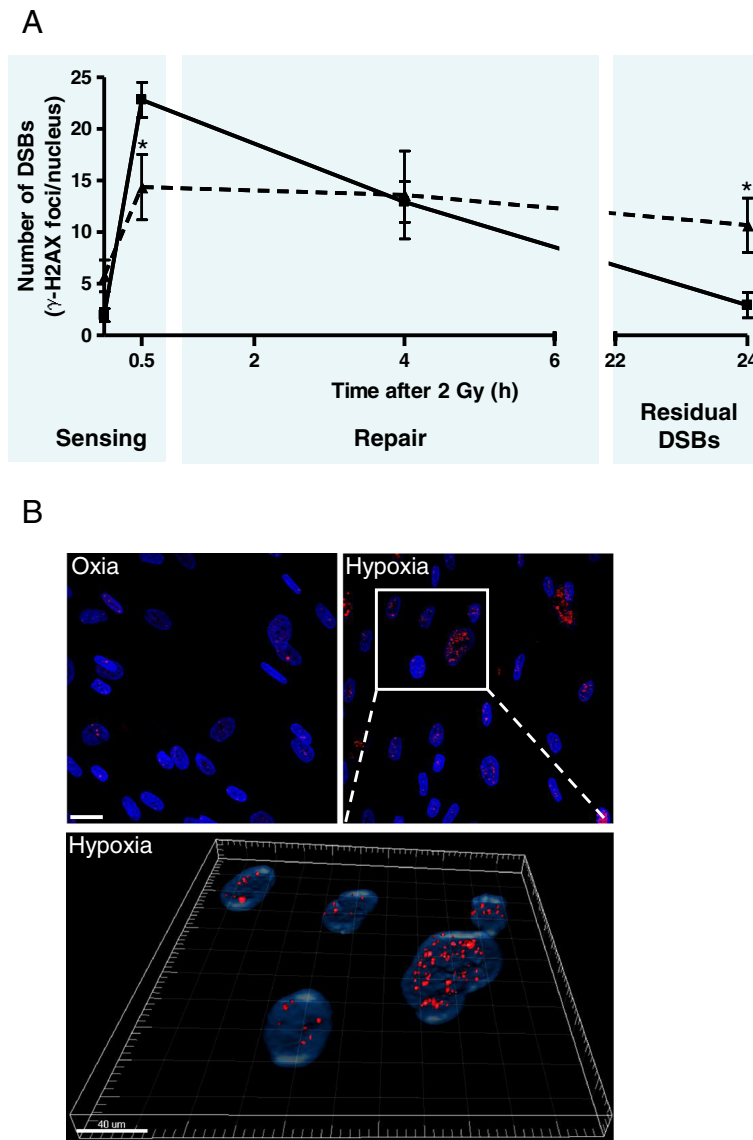
Figure 2 Decreased repair of DNA double strand breaks (DNA-DSBs) under continual hypoxia. A, Despite a decrease in the initial number of induced and sensed DSBs measured by γ-H2AX foci at 30 minutes following 2 Gy, hypoxic (0.2% O_2) G0/G1 synchronized human fibroblasts have an increased number of residual γ-H2AX foci at 24 hours. The asterisk represents a significant difference (*P < 0.05) between oxic control (solid) and hypoxic treatment (dashed). Plot is adapted from data published in Kumareswaran et al. [82]. **B**, Two dimensional (top panels) and three dimensional (bottom panel) confocal images of G0/G1 fibroblasts with increased number of residual γ-H2AX foci under continual hypoxia at 24 hours following 2 Gy of irradiation. Scale bar = 10 μm.

Pol δ and ε) and ligation (ligase III, ligase I) [126]. On the other hand, TCR requires CSA, CSB and XAB2 to sense the lesion and proceeds to GGR for the next sequential steps [126]. Both decreased and increased ability of cells to repair UV-damaged DNA in conditions of hypoxia and low pH have been reported [74,120]. Indication for NER in the hypoxic response comes from findings of XPC and XPD as direct HIF1α targets, and inhibition of HIF1α perturbs the removal of UVB-induced 6–4 photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs) [127]. Also, HIF1α associates

with the gene promoter of CSB/ERCC6, which functions in recruiting NER repair proteins to the damaged DNA, and is induced by hypoxia. CSB mutant cells fail to activate HIF-dependent hypoxic response [128]. Finally, RAD23B protein is repressed under hypoxia and by miRNA-373 [111]. Further investigation is needed to establish the role of hypoxia in NER.

Fanconi anemia (FA) is a hereditary disorder with predisposition to cancer [129]. The FA pathway includes 14 FANC genes, which function in ubiquitination-phosphorylation pathways and participate in repairing
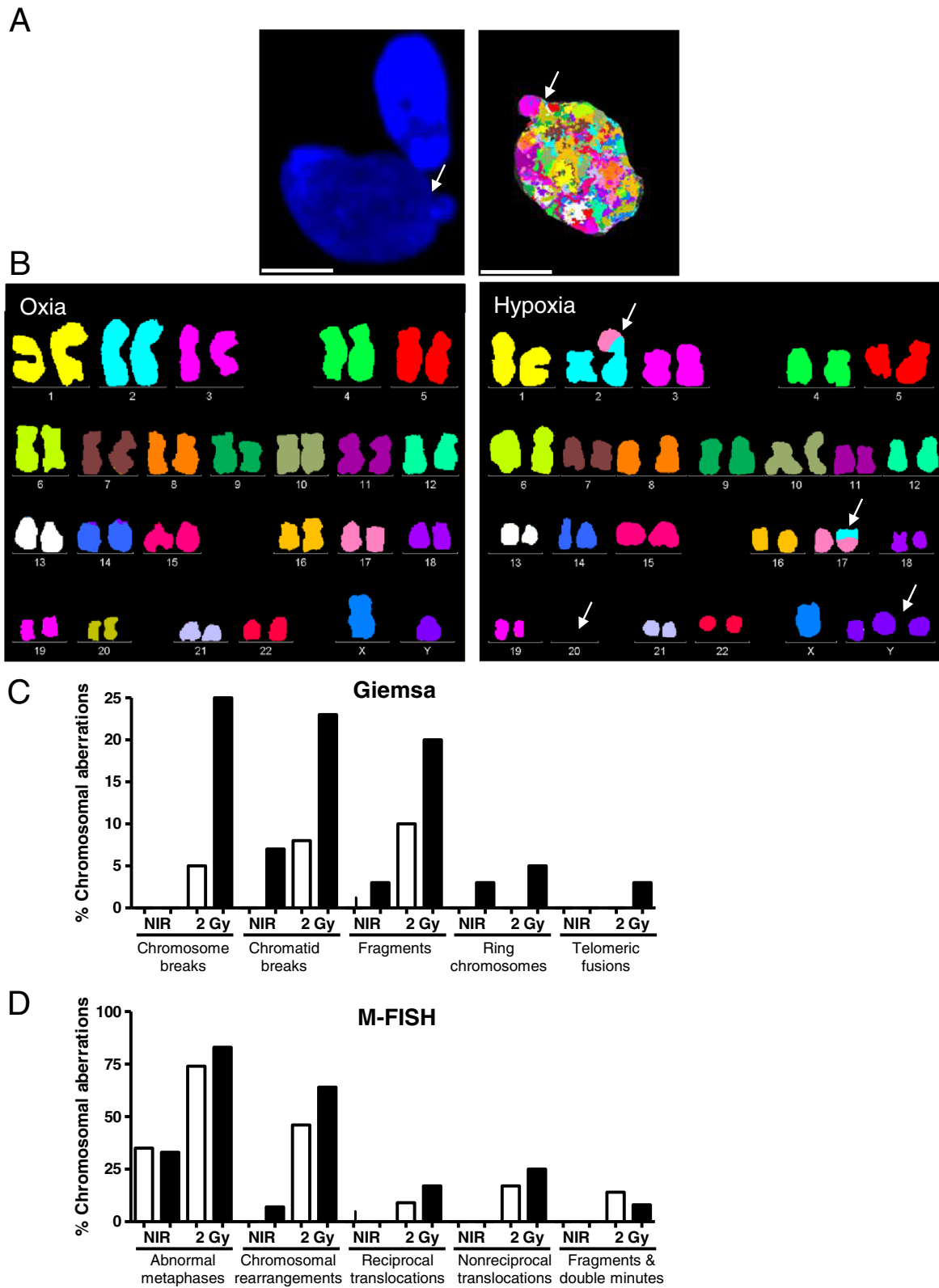
Figure 3 (See legend on next page.)

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Figure 3 Hypoxia induces chromosomal aberrations following exogenous damage. A, Chromatin bridges or anaphase bridges in fibroblasts maintained under continual hypoxic (0.2% O_2) conditions following 2 Gy of irradiation. These bridges can break into fragments and give rise to micronuclei [121]. The type, the number, and the fate of chromosome bridges under hypoxia is not known and requires further investigation. Representative DAPI stained and M-FISH images of fibroblasts are shown. Scale bar = 10 μm. **B**, M-FISH karyotype of fibroblasts maintained under oxic (21% O_2) conditions following 2 Gy of irradiation or hypoxic (0.2% O_2) conditions following 2 Gy of irradiation. Shown are reciprocal translocation between chromosomes 2 and 17, loss of chromosome 20 and two extra copies of chromosome Y in hypoxic cells following 2 Gy of irradiation. **C**, Percentages of chromosomal aberrations in oxic and hypoxic fibroblasts as measured by Giemsa staining analysis. NIR = non-irradiated; white columns = oxia (21% O_2); black columns = hypoxia (0.2% O_2). **D**, Percentages of chromosomal aberrations in oxic and hypoxic fibroblasts as measured by M-FISH analysis. NIR = non-irradiated; white columns = oxia (21% O_2); black columns = hypoxia (0.2% O_2). Plots are based on quantitative assessment of data published in Kumareswaran et al. [82].

DNA interstrand crosslinks created by agents such as (mitomycin C) MMC or cisplatin [129]. Little is known regarding the role of FANC in the hypoxic response. However, FANCC and FANCD2 cells exhibit increased IR sensitivity under hypoxia compared to wild-type cells [118,130]. UBE2T is an E2 conjugating enzyme that operates in the FA pathway to mono-ubiquitinate FANCD2 and FANCI. UBE2T expression is inhibited under hypoxia by a mechanism involving decreased promoter activity, independent of HIF1α, HIF1β or HIF2α. Consistent with the FA phenotype, both anoxic and UBE2T knockdown cells are hypersensitive to MMC-induced DNA crosslinks [131].

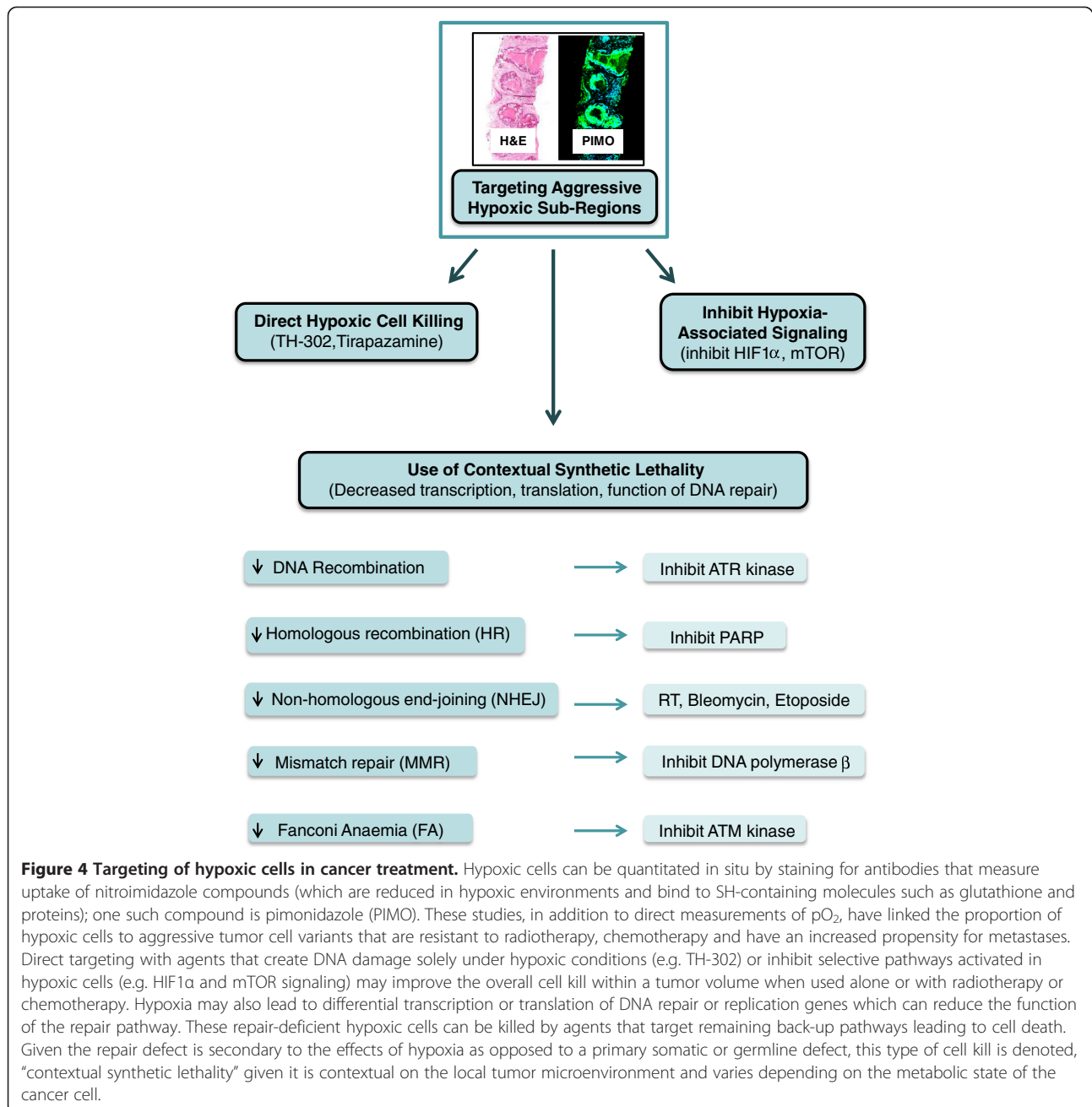## Therapeutic targeting of hypoxic tumor cells

The success of anti-cancer therapies is currently challenged by increased local and systemic resistance of tumor cells residing in the hypoxic microenvironment. However, the hypoxic phenotype can also provide an opportunity to specifically target cells in the tumor microenvironment and improve the therapeutic index (e.g. kill more cancer cells than normal cells) (see Figure 4). The development of therapeutic agents that are selectively activated upon exposure to low oxygen is of great interest [32]. For example, tirapazamine and apaziquone, both bioreductive prodrugs that induce DNA damage, have been tested in Phase III clinical trials [32]. A newer compound, TH-302, is a 2-nitroimidazole triggered hypoxia-activated prodrug of the cytotoxin bromo-isophosphoramide mustard (Br-IPM), which causes DNA damage under hypoxic/anoxic conditions [132]. The antitumor activity of TH-302 has been shown to be dose-dependent and decreased the hypoxic fraction in xenografts of varying histology. TH-302 also induces DNA damage (as measured by γ-H2AX) in hypoxic regions in vivo and can further kill cells through a time-dependent "bystander effect". This compound is currently in Phase II-III clinical trials in combination with chemotherapy.

Translational control is an important contributor to the hypoxic adaptation and gene expression alongside with HIF-dependent pathways [109]. Therefore, targeting mTOR and UPR could provide another opportunity to enhance selective tumor cell kill [32,133,134]. Clinically relevant agents that influence mTOR or UPR signaling include for example imatinib, nelfinavir and sunitinib, which can improve tumor oxygenation and inhibit angiogenesis [109,135].

Synthetic lethality is a phenomenon that arises when mutations in two or more genes result in cell death, while a cell with a mutation in either gene alone is viable [136]. Over the recent years, this has started to attract attention as a way to attack the Achilles' heel of a cancer cell. For example, inhibition of poly(ADP-ribose) polymerase (PARP), which normally functions in single-strand break (SSB) and base-excision repair (BER), is synthetically lethal with BRCA-deficient tumors [137]. In addition to targeting cancerous mutations, synthetic lethality based on tumor microenvironment has emerged, where the extrinsic differences of tumor cells are used to widen the therapeutic index [136]. In this "contextual" synthetic lethality, the hypoxic phenotype with defective DNA repair can be exploited, together with inhibiting a backup DNA repair pathway, to specifically kill hypoxic cells. Therapies would therefore preferentially kill tumor cells with reduced DNA repair capacity, and spare normal tissue with physiological oxygenation state and functional DNA repair. Indeed, hypoxic HR-defective cells are sensitive to PARP inhibition [108,138]. PARP inhibition induces DNA damage in proliferating cells and kills hypoxic cells specifically in S phase [108]. Synthetic lethality in the HR pathway has also been documented between RAD52 and BRCA2, as well as between splicing factor proline and glutamate-rich (SFPQ)/PSF and RAD51D [139,140]. Additionally, PTEN null astrocytes were found to be sensitive to PARP inhibition due to lower expression of *Rad51B-D* [141]. However, recent data from our laboratory failed to observe a correlation between PTEN status and RAD51 function [142].

In MMR, inhibition of POLB in MSH2-deficient; and inhibition of POLG in MLH1-deficient cells, produces a synthetic lethal phenotype [143]. An siRNA screen identified inhibited PTEN-induced putative kinase 1 (PINK1) as lethal in cells deficient in MLH1, MSH2 and MSH6

Figure 4 Targeting of hypoxic cells in cancer treatment. Hypoxic cells can be quantitated in situ by staining for antibodies that measure uptake of nitroimidazole compounds (which are reduced in hypoxic environments and bind to SH-containing molecules such as glutathione and proteins); one such compound is pimonidazole (PIMO). These studies, in addition to direct measurements of pO$_2$, have linked the proportion of hypoxic cells to aggressive tumor cell variants that are resistant to radiotherapy, chemotherapy and have an increased propensity for metastases. Direct targeting with agents that create DNA damage solely under hypoxic conditions (e.g. TH-302) or inhibit selective pathways activated in hypoxic cells (e.g. HIF1α and mTOR signaling) may improve the overall cell kill within a tumor volume when used alone or with radiotherapy or chemotherapy. Hypoxia may also lead to differential transcription or translation of DNA repair or replication genes which can reduce the function of the repair pathway. These repair-deficient hypoxic cells can be killed by agents that target remaining back-up pathways leading to cell death. Given the repair defect is secondary to the effects of hypoxia as opposed to a primary somatic or germline defect, this type of cell kill is denoted, "contextual synthetic lethality" given it is contextual on the local tumor microenvironment and varies depending on the metabolic state of the cancer cell.

[144]. Given that most HR factors and MMR are down-regulated under hypoxia, determining whether these synthetic lethal interactions could be exploited to target hypoxic tumor cells, would be of great interest. Future investigations will show if these observations could have an impact on radiation- and clinical oncology.

Conclusions

A number of molecular mechanisms have been proposed to explain hypoxic inhibition of HR and MMR-mediated DNA repair based on biochemical and cell biology endpoints. Molecular pathways may play differing roles depending on tissue type, microenvironment conditions and proliferation status; or alternatively, each might have a relative contribution for a global DNA repair-deficient phenotype. Dissecting these pathways could help designing anti-cancer treatments that inhibit DNA repair and sensitize tumor cells to radio- and chemotherapies. Also, a better understanding of therapies targeting the proliferating hypoxic cell subpopulations could increase selective killing of resistant tumor cells. Clinical trials using these approaches will require careful assessment

of the tumor microenvironment using imaging or other techniques in order to incorporate hypoxia assessment as a part of a standard of care. This approach will serve well to be one step closer to individualized cancer medicine and improved patient outcome.

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