

Hypoxia Provokes Base Excision Repair Changes and a Repair-Deficient, Mutator Phenotype in Colorectal Cancer Cells

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Abstract

Regions of acute and chronic hypoxia exist within solid tumors and can lead to increased rates of mutagenesis and/or altered DNA damage and repair protein expression. Base excision repair (BER) is responsible for resolving small, non-helix-distorting lesions from the genome that potentially cause mutations by mispairing or promoting DNA breaks during replication. Germline and somatic mutations in BER genes, such as MutY Homolog (*MUTYH/MYH*) and DNA-directed polymerase (*POLB*), are associated with increased risk of colorectal cancer. However, very little is known about the expression and function of BER proteins under hypoxic stress. Using conditions of chronic hypoxia, decreased expression of BER proteins was observed because of a mechanism involving suppressed BER protein synthesis in multiple colorectal cancer cell lines. Functional BER was impaired as determined by MYH- and 8-oxoguanine (OGG1)-specific glycosylase assays. A formamidopyrimidine-DNA glycosylase (Fpg) Comet assay revealed elevated residual DNA base damage in hypoxic cells 24 hours after H₂O₂ treatment as compared with normoxic controls. Similarly, high-performance liquid chromatography analysis demonstrated that 8-oxo-2'-deoxyguanosine lesions were elevated in hypoxic cells 3 and 24 hours after potassium bromate (KBrO₃) treatment when compared with aerobic cells. Correspondingly, decreased clonogenic survival was observed following exposure to the DNA base damaging agents H₂O₂ and MMS, but not to the microtubule interfering agent paclitaxel. Thus, a persistent downregulation of BER components by the microenvironment modifies and facilitates a mutator phenotype, driving genetic instability and cancer progression.

Implications: Aberrant BER is a contributing factor for the observed genetic instability in hypoxic tumor cells. *Mol Cancer Res*; 12(10); 1407–15. ©2014 AACR.

Introduction

Hypoxia is a common characteristic of solid tumors and is associated with decreased disease-free survival for many cancers, including colorectal cancer (1–9). Hypoxic tumor cells exist in colorectal cancer, and the level of hypoxia is prognostic for clinical outcome (10–19). Hypoxia may give rise to genetic instability by downregulating multiple DNA

repair pathways, including the DNA mismatch repair (MMR) and homologous recombination (HR) pathways (20–27). For example, Chan and colleagues demonstrated an alternate mechanism of translational inhibition of the HR proteins RAD51 and BRCA2 (23). Suppression of mRNA translation under hypoxia is controlled through at least two distinct pathways; first by PERK-mediated phosphorylation of eIF2 α which is required for the recruitment of aminoacylated tRNA, and second by disruption of the mRNA cap-binding complex, eIF4F (28). These changes can lead to altered sensitivity to chemotherapy and drive genetic instability.

Reactive oxygen species (ROS) generated either endogenously (during normal cellular physiology) or exogenously (by ionizing radiation or chemical carcinogens) can cause a wide range of DNA damage that must be properly repaired to prevent genetic instability. ROS leads to base lesion 8-oxo-2'-deoxyguanosine (8-oxo-dG), an oxidative derivative of guanosine (29, 30). DNA base excision repair (BER) corrects base lesions arising due to oxidative, alkylation, deamination, and depurination/depyrimidination damage (31–34). BER encompasses two general subcategories;

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short-patch BER (involving repair of a single nucleotide) and long-patch BER (producing a repair tract of at least two nucleotides). Repair of base lesions (e.g., 8-oxo-dG) is catalyzed by the BER proteins MYH, OGG1, MTH1, APE1, PCNA, POLD, FEN1, DNA POLB, and RPA (35, 36). In addition, the MMR pathway can provide additional protection by removing 8-oxo-dG mismatches from DNA (37–39).

Biallelic germline mutations in the *MYH* gene are associated with familial colorectal cancer (40, 41). To date, more than eighty mutations have been detected in this gene within MYH-associated polyposis (MAP) patients (42). Our laboratory has previously reported defective *in vitro* repair activity of mutant MYH proteins that are associated with familial colorectal cancer (43). The expression and function of the BER pathway under hypoxic conditions have not been previously studied. In the present work, we investigated the expression and function of BER proteins in human colorectal cancer cell lines under conditions of chronic hypoxic stress similar to conditions observed distant from the blood vasculature in tumors. Our findings indicate that both the expression and function of many BER proteins are downregulated by hypoxia with potential implications for genetic instability and malignant progression.

Materials and Methods

Cell culture and hypoxic treatments

The RKO human colorectal cancer cell line was purchased from the American Type Culture Collection and grown in α MEM media supplemented with 10% FCS. The paired HCT116 human colorectal cancer cell lines (p53^{+/+}, p53^{-/-}; ref. 44) were a gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) and were grown in McCoy's 5A media supplemented with 10% FCS. All cell lines were authenticated using the STR AmpFISTR Identifier PCR Amplification Kit (Applied Biosystems, The Centre for Applied Genomics, The Hospital for Sick Children). Logarithmically growing cells were exposed to 0.2% O₂ with 5% CO₂ and balance N₂ using an Invivo2 400 Hypoxic Workstation (Ruskin). The chronic hypoxic gassing condition (72 hours with 0.2% O₂) was based on previous studies in which the translation of DNA repair genes was downregulated by hypoxia (23, 45). This gassing condition for RKO and HCT116 cells was optimal for (i) minimizing toxicity (e.g., 35%–50% cell kill based on clonogenic survival assays); (ii) no effect on S-phase fraction or cell proliferation; and (iii) maintaining the inhibitory effect of hypoxia on protein translation (see Supplementary Fig. S1).

Western blot analysis and siRNA treatments

Protein expression was determined by Western blot analysis based on a previously described protocol (23). Of note, 30 μ g whole-cell lysate was separated on 10% polyacrylamide gels and transferred onto nitrocellulose membrane. Membranes were incubated with the appropriate blocking buffer and primary/secondary antibodies. Imaging and densitometry were carried out using an Odyssey Infrared

Imaging System (LI-COR Bioscience). Hypoxic protein expression was compared with oxyc protein expression to generate quantitative data on relative protein expression under hypoxia versus oxia: [(hypoxic protein expression/hypoxic actin expression)]/[(oxic protein expression/oxic actin expression)]. Primary antibodies included: hypoxia-inducible factor-1 α (HIF1 α ; BD Transduction Laboratories); ACTIN (Sigma-Aldrich); RAD51, APE1, p53 (Santa Cruz Biotechnology); MYH, MSH2, DNA POLB, PCNA (Abcam); and OGG1, MTH1 (Novus Biologicals), and RPA (Oncogene).

MYH siRNA, *DNA POLB* siRNA, and the control oligonucleotide duplex were obtained from Invitrogen

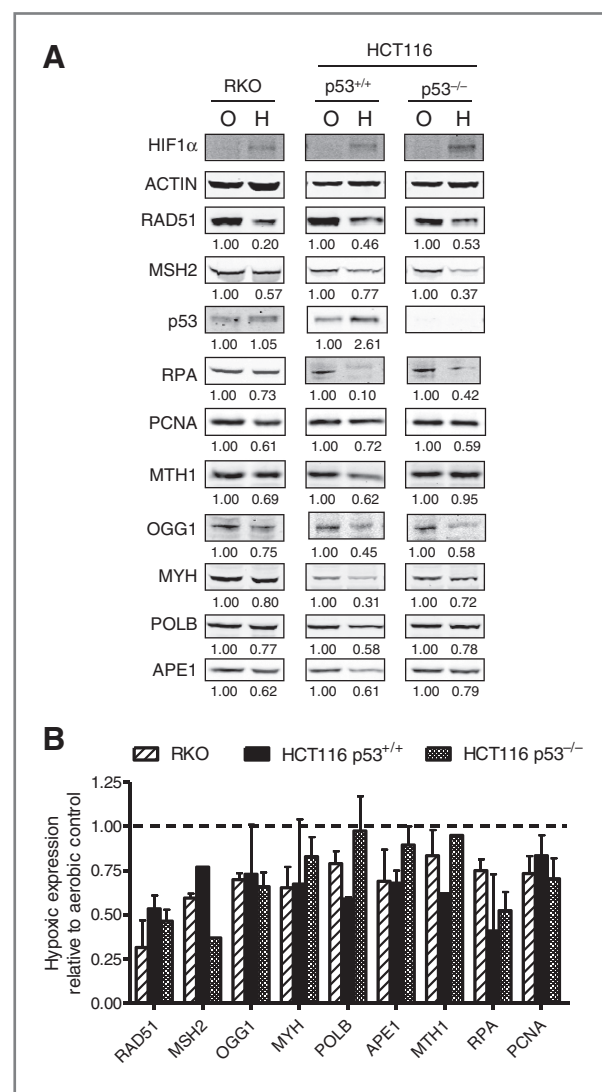


Figure 1. Hypoxia downregulates BER protein expression. A, the majority of the BER proteins are moderately downregulated under prolonged hypoxia (72 hours \times 0.2% O₂) in RKO and HCT116 cells expressing or lacking p53. B, quantification of hypoxia-mediated changes in BER protein expression. RAD51 (HR) and MSH2 (MMR) are repair proteins known to be suppressed by hypoxia and are shown as controls. HIF1 α is shown as a positive control for hypoxia. Mean \pm SEM.

and used with Lipofectamine 2000 transfection reagent (Invitrogen).

RNA isolation, reverse transcription, and qRT-PCR

Total RNA was isolated using the RNeasy isolation kit (Qiagen). Sample RNA or human reference RNA (Stratagene) was treated with DNase I (Roche Diagnostics). Reverse transcription was performed using the TaqMan Reverse Transcription Kit (Applied Biosystems).

Real-time PCR primers and fluorescent probes of target genes were obtained as Assays-On-Demand from Applied Biosystems. The following genes were assayed: 18S (endogenous control), *CAIX*, *MYH*, *OGG1*, *APE1*, and *DNA POLB*. Each PCR reaction was done in triplicate on an ABI 7900 RT-qPCR machine (Applied Biosystems) under the control of SDS software (Applied Biosystems). The fluorescence intensity threshold was set at 0.2 and the reaction cycle-threshold was obtained. Analysis was performed using the relative standard curve quantification method.

Polysomal fractionation and analysis

Polysomal fractionation and analysis were performed as described previously (46). Briefly, cell lysates were layered on a 10-mL continuous sucrose gradient. After centrifugation, the optical density at 254 nm (derived primarily from rRNA)

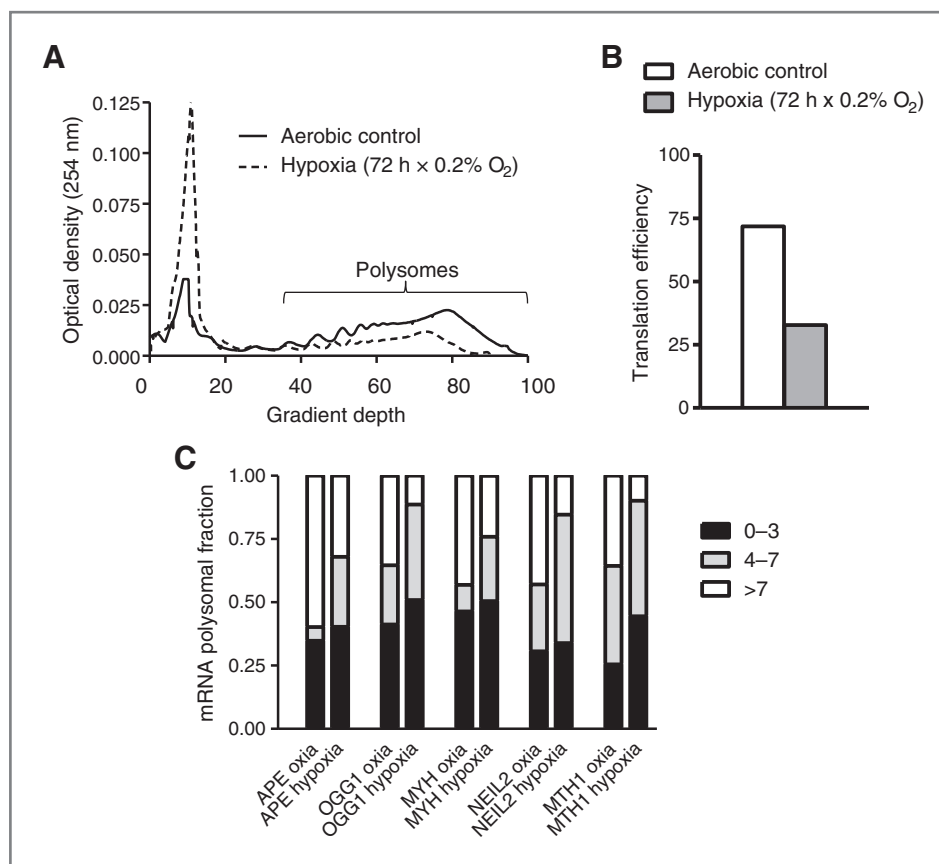
was measured continuously as a function of gradient depth. Following background subtractions, overall translation efficiency was estimated by calculating the fractional AUC corresponding to two or more ribosomes (polysomes).

Glycosylase activity assays

To assess MYH activity, a 5'-cy5-labeled 39-mer oligonucleotide containing adenine (A) at the 21-mer position from the 5'-end was hybridized with its complementary strand in a buffer containing 20 mmol/L Tris-HCl (pH 8), 10 mmol/L EDTA (pH 8), and 150 mmol/L NaCl to make a duplex DNA substrate where adenine is mispaired with 8-hydroxyguanine (GO) to create a A:GO mismatch (Supplementary Fig. S2A). To assess OGG1 activity, a 5'-³²P-labeled 49-mer oligonucleotide duplex containing a hydroxyguanine (GO) opposite cytosine was used (Supplementary Fig. S2B).

The glycosylase assay was performed as previously described (43). Briefly, 100 fmol labeled duplex was incubated with 100 μg whole-cell extract at 37°C for 1 hour in 10 μL buffer containing 50 mmol/L EDTA (pH 8), 500 μmol/L ZnCl₂, 250 mmol/L HEPES (pH 7), and 1.5% glycerol. The reaction was stopped by adding 10 μL denaturing PAGE gel loading buffer containing 10 mmol/L EDTA (pH 8), 98% formamide, 10 mg/mL blue dextran, and 200 mmol/L NaOH followed by heating at 90°C for 30 minutes.

Figure 2. Global mRNA translation is moderately inhibited at 0.2% O₂ in RKO cells. A, RKO cells were treated with 72 hours × 0.2% O₂ and cell lysates were separated on sucrose gradients. The optical density (OD) at 254 nm is plotted as a function of gradient depth. OD decreases for hypoxic and anoxic cells at higher gradient depth, indicating decreased rRNA association with two or more ribosomes (polysomes—indicated in the dashed box). B, translation efficiency, quantified as the fraction of rRNA participating in polysomes, decreases under hypoxia. This equals the integration of the area under the "polysomes" part of the curve divided by the total area under the curve. C, the distribution of APE, OGG1, MYH, NEIL2, and MTH1 transcripts within the polysomes was determined. The translation efficiency for all BER genes is decreased as evidenced by a shift in transcripts toward reduced ribosome numbers.



Cleavage products were separated using a 14% denaturing PAGE gel running at 1400 V and 100 W for 1 hour.

Clonogenic assays

Clonogenic assays were performed to determine cell viability, following various gas and drug treatments as previously described (47). To determine clonogenic survival following MMS, H₂O₂, or paclitaxel treatment, RKO cells were gassed with 0.2% O₂ for 72 hours and then reoxygenated and exposed to varying drug doses for 1 hour before being replated for colony formation. All the drug treatment clonogenic survival data were normalized to the cell kill following hypoxic exposure, alone.

HPLC detection of 8-oxo-2'-deoxyguanosine

Oxidation of 2'-deoxyguanosine (dG) to 8-oxo-dG was quantified using an isocratic Series 200 HPLC system (PerkinElmer Instruments) equipped with a 5- μ m Exsil 80A-ODS C-18 column (5 cm \times 4.6 mm; Jones Chromatography), an electrochemical detector (Coulchem II),

a guard cell (model 5020), an analytical cell (model 5010; Coulchem; ESA), and an integrator (PerkinElmer NCI 900 Interface). Samples were filtered (0.22 μ m), injected into the HPLC-EC system, and eluted using a mobile phase consisting of 50 mmol/L KH₂PO₄ buffer, pH 5.5, methanol (95:5, v/v) at a flow rate of 0.8 mL/minute with a detector oxidation potential of +0.4 V (48). Chromatographs were analyzed using the TotalChrom chromatography software version 6.2.0 (PerkinElmer Instruments).

Fpg Comet assay detection of base damage

DNA base damage (including open ring forms of 7-methylguanine, 8-oxoguanine, 5-hydroxycytosine, 5-hydroxyuracil, aflatoxin-bound imidazole-ring-opened guanine, and imidazole ring opened N-2-aminofluorene-C8-guanine) was assessed using the Fpg FLARE Assay Kit (Trevigen) as per manufacturer's protocol. Briefly, 20 μ L of cells (1×10^6 cells/mL) in PBS were mixed with 200 μ L low melting agarose and 40 μ L of the mixed solution was spotted

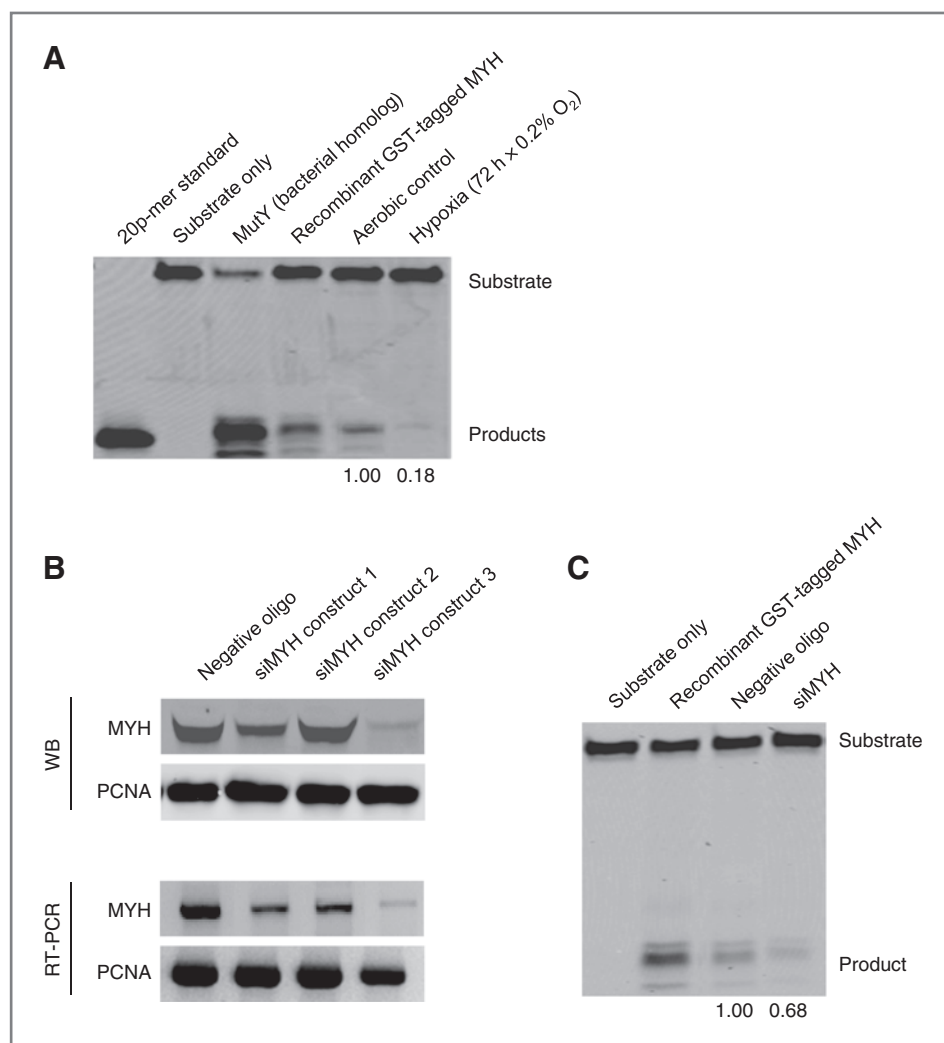
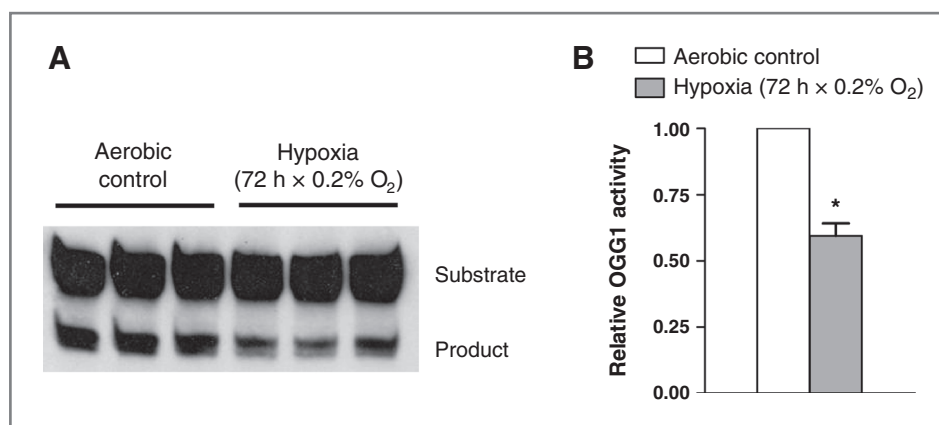


Figure 3. Enzymatic MYH glycosylase activity is decreased in hypoxic cells. A, MYH glycosylase activity was assessed by incubating 100 fmol 5'-Cy5-labeled duplex substrate with 100 μ g whole-cell extract from aerobic or hypoxic (72 hours \times 0.2% O₂) RKO cells at 37°C for 30 minutes and analyzed by denaturing PAGE to detect the cleavage products. Substrate alone is shown as a negative control and bacterial MutY and recombinant GST-tagged human MYH are shown as positive controls. B, as a control, siMYH (40 nmol/L \times 48 hours) was used to decrease MYH protein and mRNA expression leading to (C) decrease glycosylase activity. For the glycosylase assay, a mixture of the three siRNAs was used to knockdown MYH.

Figure 4. Enzymatic OGG1 glycosylase activity is decreased in hypoxic cells. A, OGG1 glycosylase activity was assessed by incubating 100 fmol duplex substrate with 100 μ g whole-cell extract from aerobic or hypoxic (72 hours \times 0.2% O₂) RKO cells at 37°C for 30 minutes and analyzed by denaturing PAGE to detect the cleavage products. B, OGG1 activity quantified as the percent incision of the substrate and expressed relative to the aerobic control. Mean \pm SEM. The asterisk denotes a significant difference with $P < 0.05$ on a Student *t* test.



onto agarose coated microscope slides. Slides were incubated in lysis buffer overnight at 4°C and treated with 5 \times diluted formamidopyrimidine-DNA glycosylase (Fpg) solution for 30 minutes. Slides were electrophoresed (30 V, 4°C, 45 minutes) in alkaline electrophoresis buffer (pH > 13) and dried with 70% ethanol. Comets were stained with ethidium bromide and imaged with an Axioskop fluorescent microscope (Carl Zeiss) and Komet analysis software (Andor Technologies). Plotted data represent the tail moment of Fpg-treated cells (measures single strand break (SSB), double-strand break (DSB), and base damage) subtracted by the tail moment of Fpg untreated cells (measures SSBs and DSBs) to give a measurement of base damage alone.

Results

Expression of BER genes under chronic hypoxia

In the current study, we compared the effect of prolonged hypoxia on the expression of the BER genes in RKO cells. Western blotting confirmed that the expression of many BER repair proteins, including OGG1, MYH, DNA POLB, APE1, RPA, and PCNA, was significantly downregulated in RKO cells after exposure to 72 hours \times 0.2% O₂ (Fig. 1). A similar hypoxic exposure to paired HCT116 cells, expressing or lacking p53, confirmed downregulation of many BER proteins and suggested this was a p53-independent effect. However, real-time PCR showed no significant changes in the mRNA levels of some of these BER genes in RKO cells (Supplementary Fig. S1).

To test the effect of hypoxia on mRNA translation in RKO cells, polysomal fractionation analysis was performed to determine the association of mRNAs with ribosomes. In this assay, actively translated mRNAs associated with two or more ribosomes (polysomes) are separated from inefficiently translated mRNAs bound to one (monosome) or no ribosomes by sedimentation through a sucrose gradient. When recording the optical density as a function of gradient depth (Fig. 2A), the fractional polysome AUC is a direct reflection of *de novo* protein synthesis. Exposure to hypoxic conditions resulted in a substantial inhibition of mRNA translation (Fig. 2B), indicating that under these conditions, the translation

of the majority of gene transcripts is severely inhibited. We also analyzed the mRNA translation of specific BER genes, by determining their mRNA distribution

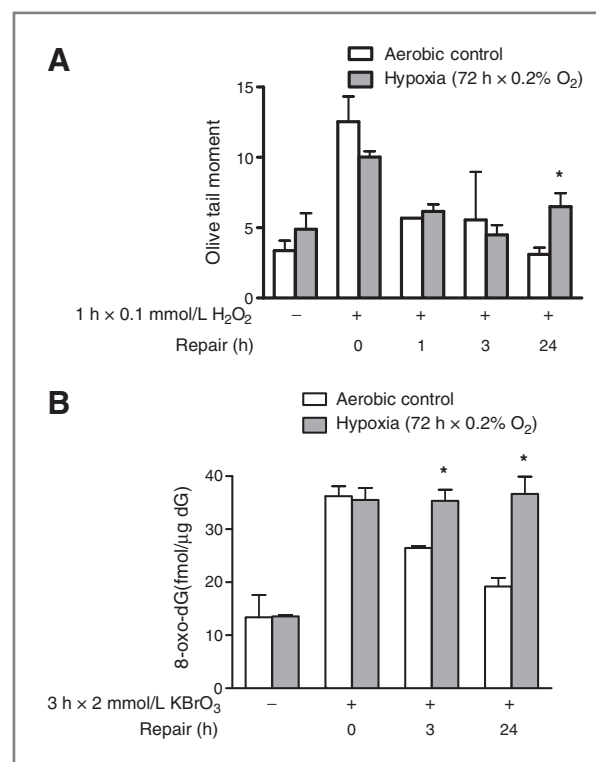


Figure 5. Hypoxic RKO cells have increased residual base damage following H₂O₂ and KBrO₃ treatments. A, using the Fpg comet assay hypoxic RKO cells (72 hours \times 0.2% O₂) displayed increased residual base damage 24 hours following H₂O₂ treatment (1 hour \times 0.1 mmol/L). Olive tail moment (OTM) is a measure of DNA damage calculated by multiplying the percentage of DNA in the tail by the distance between the head and center of gravity of DNA in the tail. OTMs displayed are the OTM of Fpg-treated comets subtracted by the OTM of Fpg untreated comets to give a measure of base damage only. B, using HPLC detection, hypoxic RKO cells (72 hours \times 0.2% O₂) displayed increased residual 8-oxo-dG base lesions 3 and 24 hours following KBrO₃ treatment (3 hours \times 2 mmol/L). Mean \pm SEM. The asterisk denotes a significant difference with $P < 0.05$ on a Student *t* test.

within the polysome profiles (Fig. 2C). After 72 hours at 0.2% O₂, there is a decrease in the number of ribosomes per transcript, especially in transcripts containing >7 ribosomes, indicating decreased translation of these mRNAs.

Functional BER is downregulated by chronic hypoxia

The functional consequences of the decreased BER protein expression were determined using a MYH-specific glycosylase assay. Whole-cell extracts from aerobic and hypoxic cells were assayed for MYH glycosylase activity on a 39-mer synthetic duplex DNA substrate containing an A:GO (adenine:hydroxyguanine) mismatch. Functional MYH will excise A opposite to GO, resulting in two cleavage products that are separated on a denaturing PAGE gel (Supplementary Fig. S1A). The slower migrating product is a 20-mer aldehyde generated by cleavage of the 3' phosphodiester bond (β -elimination) by NaOH treatment. Similarly, the faster migrating product is a 20-mer product generated by cleavage of both phosphodiester bonds (β,δ -elimination; ref. 49). Separation through a denaturing PAGE gel will cleave the hydrogen bonds holding the double-stranded oligonucleotide together and subsequently only the Cy5-labeled 20-mer products will be detectable. Similar to the bacterial homologue MutY and recombinant MYH, whole-cell extract prepared from aerobic RKO cells (72 hours \times 21% O₂) generated two cleavage products as expected, whereas whole-cell extract from hypoxic cells (72 hours \times 0.2% O₂) also produced the same products but to a much lesser extent (Fig. 3A). These findings were validated using siRNA to knock down MYH. Depletion of both mRNA and protein was achieved (Fig. 3B) and resulted in

decreased glycosylase activity (Fig. 3C). A similar assay to test OGG1-specific glycosylase activity (Supplementary Fig. S1B) also revealed a comparable decrease in functional BER activity under hypoxia (Fig. 4).

Hypoxic cells accumulate residual base damage

Decreased BER protein expression and functional repair in hypoxic cells suggested that hypoxic cells might accumulate residual base damage following base damaging treatments. Indeed, hypoxic RKO cells display elevated residual base damage 24 hours following H₂O₂ exposure compared with aerobic cells as assessed by the Fpg comet assay (Fig. 5A). The Fpg comet assay is a variation of an alkaline comet assay that specifically measures base damage (including open ring forms of 7-methylguanine, 8-oxoguanine, 5-hydroxycytosine, 5-hydrouracil, aflatoxin-bound imidazole ring-opened guanine, and imidazole ring opened N-2-amino-fluorene-C8-guanine). Similarly, hypoxic RKO cells display elevated residual 8-oxo-dG base lesions 3 and 24 hours following KBrO₃ exposure compared with aerobic cells as assessed by high-performance liquid chromatography (HPLC; Fig. 5B). These results support the hypothesis that hypoxic cells have decreased functional BER consistent with a decrease in BER protein expression.

Cell survival is compromised in BER-deficient hypoxic cells

To further test for a functional cellular defect in BER, cells were exposed to hypoxia, treated with H₂O₂, methyl methanesulfonate (MMS) or paclitaxel, and then assessed for clonogenic survival. H₂O₂ causes oxidative DNA damage and MMS is an alkylating agent. Both of these agents cause

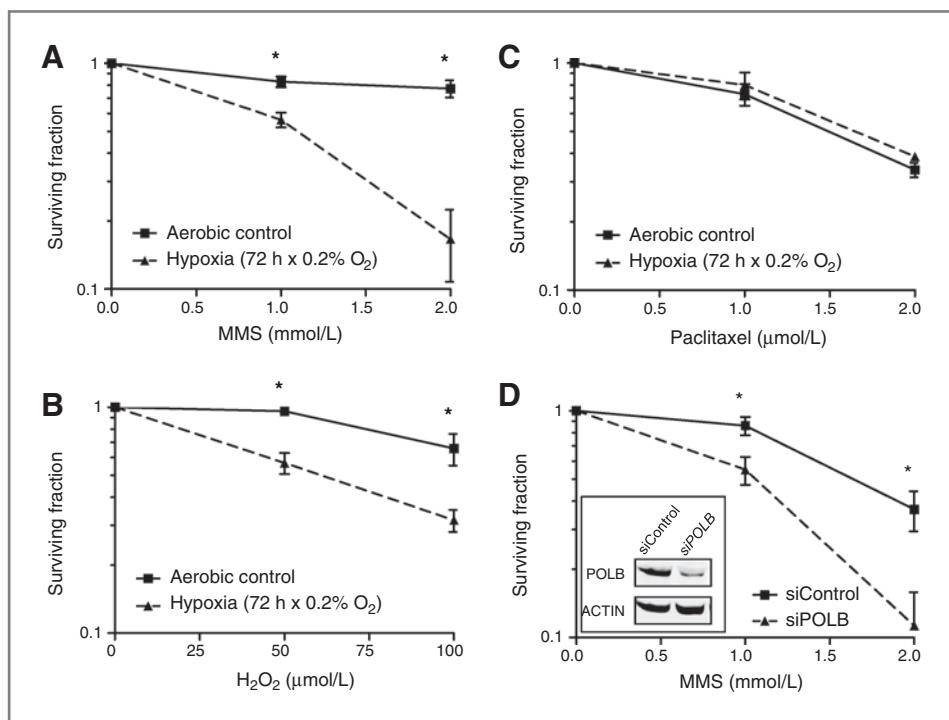


Figure 6. Hypoxic RKO cells are more sensitive to MMS and H₂O₂. BER proficiency, as measured clonogenic survival following (A) MMS and (B) H₂O₂ treatment, is downregulated by hypoxia in RKO cells. Of note, 72 hours \times 0.2% O₂ pretreatment sensitized cells to 1-hour MMS and H₂O₂ treatments. C, hypoxic cells were not sensitized to a 1-hour paclitaxel treatment. D, of note, 48 hours \times 1 nmol/L DNA POLB siRNA resulted in downregulation of DNA POLB protein levels and (B) increased sensitivity to a 1-hour MMS treatment as measured by clonogenic assays. Mean \pm SEM. The asterisk denotes a significant difference with $P < 0.05$ on a Student t test.

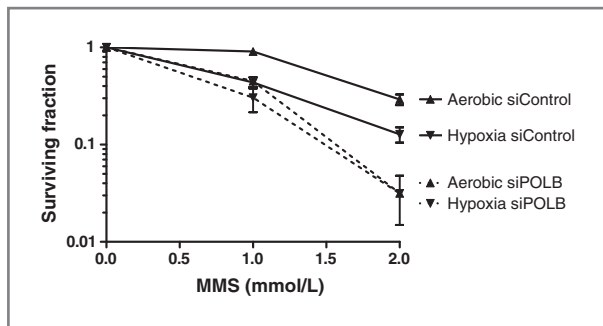


Figure 7. Increased sensitivity to MMS under hypoxia due to a BER defect. Of note, 72 hours \times 0.2% O_2 or 72 hours \times 1 nmol/L DNA POLB siRNA resulted in increased sensitivity to a 1-hour MMS treatment as measured by clonogenic assays. However, hypoxia does not further sensitize RKO cells in which DNA POLB is already knocked down. Mean \pm SEM.

damage that is selectively toxic to BER-deficient cells (50). In keeping with the observed toxic effects of hypoxia on the glycosylase assay, cells pretreated with chronic hypoxia showed significantly increased sensitivity to both MMS (Fig. 6A) and H_2O_2 (Fig. 6B). As a control, paclitaxel, a chemotherapeutic agent that interferes with microtubules and does not cause DNA damage, had similar toxicity under hypoxic and aerobic conditions (Fig. 6C). We conclude that BER is functionally downregulated by chronic hypoxia. These results are supported by siRNA knockdown of DNA POLB leading to increased sensitivity to MMS (Fig. 6D). In addition, combining hypoxia with siRNA knockdown of DNA POLB does not further sensitize RKO cells to MMS compared with siRNA alone (Fig. 7).

Discussion

In the current study, we provide the first report of hypoxia-mediated suppression of BER protein expression and function in colorectal cancer. The decreased protein expression seems to be the result of decreased protein synthesis as overall mRNA translation is inhibited while the levels of the BER mRNA transcripts are unchanged. Hypoxia-mediated changes in mRNA translation have previously been reported and can dramatically alter individual gene expression (51). Under hypoxia, this suppression is controlled through at least two distinct pathways; first by PERK-mediated phosphorylation of eIF2 α , which is required for the recruitment of aminoacylated tRNA, and second by disruption of the mRNA cap-binding complex, eIF4F (28).

Functionally, BER is suppressed as assessed by MYH- and OGG1-specific glycosylase assays and increased sensitivity to the DNA base damaging agents H_2O_2 and MMS. In addition, hypoxic cells have increased residual base damage (including 8-oxo-dG) following H_2O_2 and $KBrO_3$ treatments. Components of the BER pathway (OGG1, MTH1, and MYH) and MMR pathway (MSH2, MSH6, and MLH1) work together to protect the genome against this mutagenic base lesion (38, 39). MMR has also been shown to be impaired under hypoxic conditions (21, 52). The

residual 8-oxo-dG accumulated under hypoxic conditions is therefore probably a result of impaired BER and MMR pathways. It has previously been documented that hypoxia-pretreated cells have increased radiosensitivity and at the time this was attributed to a hypoxia-mediated defect in HR (23). However, in light of these findings, decreased BER may also play a role. Indeed hypoxia has been shown to inhibit multiple DNA repair pathways, including MMR (21), HR (23) and now BER, all of which are involved in colorectal cancer risk and tumor progression.

The effect of hypoxia on suppression of BER protein levels is not as great as the effect previously shown on HR and MMR proteins. This may be due to relatively longer half-lives of the BER proteins. However, even a minor reduction in BER protein levels may have important implications for carcinogenesis and tumor progression in the colon. Mutation of the *MYH* gene has been associated with familial colorectal cancer with more than 80 mutations detected within MAP patients (40, 41, 53). Previously our laboratory has characterized the functional activity of mutant MYH proteins known to be associated with increased clinical risk of familial colorectal cancer (43). It was determined that several missense and frameshift mutations were dysfunctional for DNA-binding and glycosylase activity potentially leading to carcinogenesis. Furthermore, hypoxia has been documented in colorectal tissues ranging from normal mucosa to benign adenoma to carcinomas (54–57). It is conceivable that in colorectal cells, with or without a BER mutation, a hypoxic microenvironment could lead to further reduction in functional protein expression driving genetic instability, carcinogenesis, and tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Chan, M. Ali, G.P. McCallum, R. Kumareswaran, P.G. Wells, S. Gallinger

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Chan, M. Ali, G.P. McCallum, R. Kumareswaran, M. Koritzinsky, B.G. Wouters, P.G. Wells, R.G. Bristow

Writing, review, and/or revision of the manuscript: N. Chan, M. Ali, R. Kumareswaran, B.G. Wouters, P.G. Wells

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Chan, R. Kumareswaran, P.G. Wells, S. Gallinger, R.G. Bristow

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