Chronic Hypoxia Decreases Synthesis of Homologous Recombination Proteins to Offset Chemoresistance and Radioresistance

Norman Chan,[1,2] Marianne Koritzinsky,[3] Helen Zhao,[1,2] Ranjit Bindra,[4] Peter M. Glazer,[4] Simon Powell,[5] Abdellah Belmaaza,[6] Brad Wouters,[3] and Robert G. Bristow[1,2]

[1]Princess Margaret Hospital (University Health Network) and [2]Departments of Medical Biophysics and Radiation Oncology, University of Toronto, Toronto, Ontario, Canada; [3]Department of Radiation Oncology (Maastro), GROW Research Institute, Maastricht University, Maastricht, the Netherlands; [4]Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, Connecticut; [5]Department of Radiation Oncology, Washington University in St. Louis, St. Louis, Missouri; and [6]Department of Medicine and Microbiology, Centre de Recherche, Centre Hospialier de l'Université de Montreal-Hôpital Notre-Dame, Institut du Cancer de Montréal, Université de Montréal, Montréal, Quebec, Canada

Abstract

Hypoxic and/or anoxic tumor cells can have increased rates of mutagenesis and altered DNA repair protein expression. Yet very little is known regarding the functional consequences of any hypoxia-induced changes in the expression of proteins involved in DNA double-strand break repair. We have developed a unique hypoxic model system using H1299 cells expressing an integrated direct repeat green fluorescent protein (DR-GFP) homologous recombination (HR) reporter system to study HR under prolonged chronic hypoxia (up to 72 h under 0.2% O_2) without bias from altered proliferation, cell cycle checkpoint activation, or severe cell toxicity. We observed decreased expression of HR proteins due to a novel mechanism involving decreased HR protein synthesis. Error-free HR was suppressed 3-fold under 0.2% O_2 as measured by the DR-GFP reporter system. This decrease in functional HR resulted in increased sensitivity to the DNA cross-linking agents mitomycin C and cisplatin but not to the microtubule-interfering agent, paclitaxel. Chronically hypoxic H1299 cells that had decreased functional HR were relatively radiosensitive [oxygen enhancement ratio (OER), 1.37] when compared with acutely hypoxic or anoxic cells (OER, 1.96–2.61). Using CAPAN1 cells isogenic for *BRCA2* and siRNA to *RAD51*, we confirmed that the hypoxia-induced radiosensitivity was due to decreased HR capacity. Persistent down-regulation of HR function by the tumor microenvironment could result in low-fidelity DNA repair and have significant implications for response to therapy and genetic instability in human cancers. [Cancer Res 2008;68(2):605–14]

Introduction

Intratumoral hypoxia is an adverse clinical prognostic factor associated with decreased disease-free survival for cancers involving the prostate, cervix, breast, musculoskeletal, and head and neck (1–3). Chronic hypoxia and/or anoxia can develop in regions of solid tumors as a function of reduced O_2 diffusion with increasing distance from the vasculature. Acute hypoxia can arise due to transient restrictions in blood flow known to occur within the dynamic and tortuous tumor vasculature. Hypoxic tumor cells can be locally and systemically aggressive with a decreased sensitivity to apoptotic and other cell death signals, increased angiogenesis, increased proliferation, and an increased capacity for systemic metastasis (4–6). Experimental and spontaneous metastatic capacity can be increased when tumor cells are subjected to hypoxia and can be secondary to genetic instability (6–8). Anoxic cells can also be more chemoresistant and radioresistant than oxic cells. The latter can be expressed as the oxygen enhancement ratio (OER) that is defined as the ratio of doses required for the same biological effect (e.g., survival) in the absence or presence of oxygen. The OER for a wide range of cell lines and tissues is between 2.5 and 3.3 (9).

Acute anoxia and reoxygenation can lead to phosphorylation of the CHK2 and CHK1 kinases via the ataxia telangiectasia–mutated (ATM) and ataxia telangiectasia Rad3–related (ATR) kinases that enact G_1, S, and G_2 cell cycle checkpoints. These arrests can be used to repair the DNA damage caused by stalled or collapsed replication forks in S phase to reduce cell lethality (10–12), as residual or misrepaired DNA double-strand breaks (dsb) can lead to chromosomal deletion, translocations, and rearrangement and/or mitotic catastrophe (13).

In human cells, DNA-dsbs are primarily repaired through two different pathways: homologous recombination (HR) and nonhomologous end-joining (NHEJ; refs. 13, 14). HR is a template-guided, error-free pathway, predominantly operating in the S and G_2 phases of the cell cycle as it requires a repair template from a sister chromatid or chromosome. HR involves many proteins including RAD51, the RAD51 paralog complexes RAD51C-XRCC3 and RAD51B-C-D-XRCC2, and the p53, RPA, BRCA2, and BLM proteins (13, 15). In contrast, NHEJ is predominant in the G_1 and G_2 phases and is usually error prone (13, 16).

Inhibition of the DNA-dsb repair pathways has been linked to increased genetic instability and carcinogenesis. For example, DNA-PK_{cs}–deficient mice exhibit increased telomeric fusions (17), whereas overexpression of dominant-negative *RAD51* is associated with centrosome aberrations and increased tumorigenicity (18). Cells expressing a dominant-negative form of *RAD51* or hypomorphic alleles of *BRCA2* show impaired HR (19), and cells deficient in

Requests for reprints: Robert G. Bristow, Radiation Medicine Program, Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario, Canada M5G 2M9. Phone: 416-946-2936; Fax: 416-946-4596; E-mail: Rob.Bristow@rmp.uhn.on.ca.

doi:10.1158/0008-5472.CAN-07-5472

HR or NHEJ have a reduced capacity for DNA-dsb rejoining (20, 21). Therefore, maintaining error-free repair is imperative to preserve genomic stability (4).

Given that many human tumors contain hypoxic subpopulations, it is surprising that there are few data concerning DNA-dsb repair under conditions of acute versus chronic hypoxia. DNA repair in proliferating cells that have chronically adapted to low O_2 levels may be considerably different from cells that undergo acute anoxia/reoxygenation cycles that activate the ATM and ATR checkpoints. Therefore, numerous factors could alter overall DNA repair and cell fate within tumor subpopulations: the relative percentage of oxic or hypoxic or anoxic cells, the relative duration of hypoxic exposure and cell transit times (e.g., proportion of acute to chronic hypoxia), and differential protein function under a range of O_2 levels (e.g., differential biology between 0.0% and 5.0%; ref. 22). These factors need investigation as nonrepaired DNA breaks, as a function of the microenvironment, could activate oncogenes or inactivate tumor suppressor genes and drive a mutator phenotype and aggressive tumor cell phenotypes (4).

Recent studies from our laboratories and others have reported the effect of hypoxia on HR gene and protein expression (23–25). Gene expression studies in prostate cells conducted by Meng et al. (25) showed down-regulation of many HR mRNA species after treatment with chronic hypoxia (e.g., 72 h $\times$ 0.2% O_2). The expression of HR proteins was also decreased and was independent of p53 status, hypoxia-induced apoptosis, or altered cell cycle distribution. These studies supported earlier work in which an HIF-1α–independent down-regulation of RAD51 was also observed by Bindra et al. (23, 24) in a range of cancer cell lines after treatment with 48 h $\times$ 0.01% O_2 and in prostate and cervix cancer xenograft studies *in vivo* based on decreased RAD51 expression in areas that were avid for hypoxic biomarkers (i.e., EF5; ref. 24).

Several recent studies have investigated the potential mechanism(s) for hypoxia-mediated changes in protein expression. A model of acute hypoxia-induced transcriptional repression has been proposed by Bindra et al. (23, 26) who showed that the hypoxic down-regulation of RAD51 and BRCA1 can be mediated by altered E2F transcriptional activation and repression. In other cell lines, the RNA and protein expression of HR genes is discordant after chronic hypoxic exposure (25), suggesting an additional level of genetic control. Indeed, a complementary model of hypoxia-induced translational repression has been proposed and could also explain decreased HR expression under hypoxia (27–29). In these studies, overall mRNA translation is severely, but reversibly, inhibited during hypoxia. The effect of hypoxia on the synthesis of DNA-dsb repair proteins has not been previously studied.

Despite the data above, hypoxia has not yet been directly linked to an HR-deficient cellular phenotype [e.g., sensitivity to mitomycin and other DNA cross-linking agents]. Herein, we report that exposure to chronic hypoxia (under conditions in which cell cycle checkpoints are not activated) induces down-regulation of the HR DNA-dsb repair pathway with the consequence of increased sensitivity to ionizing radiation and DNA cross-linking agents. We also show that this HR protein down-regulation is mediated, in part, by changes in the efficiency of HR mRNA translation. Our studies suggest that chronically hypoxic cells may be repair-deficient and may be a targetable pathway for novel chemoprevention and cancer treatment strategies.

Materials and Methods

Cell culture. The H1299 human lung carcinoma cell line was grown in RPMI 1640 supplemented with 10% FCS and 20 mmol/L HEPES. These cells express a direct repeat green fluorescent protein (DR-GFP) reporter system (19, 30). The CAPAN1 human pancreatic cancer cell lines (clones D2 and D2 B74) were grown in RPMI 1640 supplemented with 15% FCS, 10 mmol/L HEPES, 4.5 g/L dextrose, and 1 mmol/L pyruvic acid. These cells are isogenic for the expression and function of BRCA2 and are isogenic for functional HR as previously described (31).

Hypoxic treatments, cell proliferation, and cell cycle assays. T75 flasks containing logarithmically growing cells were exposed to a continuous flow of humidified 21% O_2 (normoxia), 0.2% O_2 (hypoxia), or 0% O_2 (anoxia) with 5% CO_2 and balanced N_2, using a custom designed glass manifold system based on a system described by Young et al. (32). An OxyLite oxygen probe (Oxford Optronix) verified that target O_2 levels were achieved within 6 h of gassing. Measurements of glucose and pH over periods of up to 72 h showed that these variables changed minimally under hypoxic gassing conditions (e.g., glucose range, 7.5–11.4 mmol/L; pH range, 7.29–7.40).

Relative cell proliferation was determined by daily counts of cells at 24, 48, and 72 h during gas treatment, and the data were plotted after normalizing against cell number at time 0. The cell cycle distribution of cells grown under the varying O_2 conditions was quantified using dual-variable flow cytometry (33). Briefly, gas-treated cells were incubated with 10 μmol/L bromodeoxyuridine (BrdUrd) for 30 min before collection without reoxygenation. Cells were then washed with $CaCl_2/MgCl_2$-free PBS and fixed in 70% ethanol. The DNA was denatured using 2 N HCl with 0.5% Triton X-100 and neutralized with 0.1 mol/L $Na_2B_4O_7$ (pH 8.5). Cells were stained with anti-BrdUrd FITC (BD Bioscience) and propidium iodide and analyzed using a FACSCalibre flow cytometer (BD Biosciences) and CellQuest software (BD Biosciences; ref. 33).

Western blot analysis and siRNA treatments. Protein expression was determined by Western blot analysis (25). Whole cell lysates were separated on 6% to 10% polyacrylamide gels and transferred onto nitrocellulose membrane. Membranes were incubated with the appropriate blocking buffer and primary/secondary antibodies. Imaging and densitometry was carried out using an Odyssey IR Imaging System (LI-COR Bioscience).

Primary antibodies included the following: HIF-1α (BD Transduction Laboratories); ACTIN (Sigma-Aldrich, Inc.); RAD51, BRCA1, and BRCA2 (Calbiochem); RAD51B, RAD51D, and RAD54 (Abcam, Inc.); and RAD51C, XRCC3, and RAD50 (Novus Biologicals, Inc.).

RAD51 siRNA duplex 1 and 2 and the negative oligo were obtained from Invitrogen and used at a concentration of 0.25 nmol/L for 24 h. Transfection of siRNA or negative oligo was achieved using Lipofectamine 2000 (Invitrogen).

RNA isolation, reverse transcription PCR, and real-time quantitative 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 PCR (RT-PCR) 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), *VEGF, GRP94, BRCA2, MDC1, RAD51, RAD51C*, and *RAD54*. Each PCR reaction was done in triplicate on an ABI real-time quantitative PCR 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 (29). Briefly, cell lysates were layered on a 10 mL continuous sucrose gradient. After centrifugation, the absorbance at 254 nm (derived primarily from rRNA) was measured continuously as a function of gradient depth. After background subtractions,

overall translation efficiency was estimated by calculating the fractional area under the curve corresponding to two or more ribosomes (polysomes). Data from three experiments were averaged and plotted with the SD of the data.

The translation efficiency of individual genes (*ACTIN, RAD51*, and *BRCA2*) was determined by assessing the distribution of the mRNA of the genes throughout the polysome profile. Fractions from sucrose gradients representing 2 to 3, 4 to 5, 6 to 7, and >7 polysomes were collected; mRNA was isolated and levels were determined by quantitative real-time PCR. The relative amount of mRNA in each of these fractions was determined after exposure to hypoxia.

HR assay. To evaluate HR repair of DNA-dsbs, H1299 cells containing the DR-GFP construct were transfected with pGFP (transfection efficiency control) or pCMV3xnlsI-SceI (functional endonuclease) or phCMV-1 I-SceI (negative control) under 21% or 0.2% O_2 conditions. Transient expression of I-SceI endonuclease generates a DNA-dsb at the integrated green fluorescent protein (GFP) gene sequences and stimulates HR. GFP signal was assayed at 3 days post-transfection on a FACSCalibre flow cytometer (BD Biosciences). For each experiment, 50,000 cells were scored per treatment group and the frequency of recombination events was calculated from the number of GFP-positive cells divided by the number of cells analyzed after correction for transfection efficiency. There were no differences in transfection efficiency or GFP expression under oxia or hypoxia (data not shown).

Clonogenic assays. Clonogenic assays were performed after gas and drug/IR treatments as previously described (34). IR was used to induce DNA-dsbs. Mitomycin and cisplatin were used to induce DNA interstrand cross-links. Paclitaxel is a microtubule-interfering agent that does not generally induce DNA damage as a mechanism for its toxicity. To determine clonogenic survival after IR treatment, H1299 and CAPAN1 cells were gassed for 6 h or 72 h and then exposed to 0 to 25 Gy IR using a ^{137}Cs irradiator (MDS Nordion) before, or after, reoxygenation. To determine clonogenic survival after mitomycin or cisplatin treatment, H1299 cells were gassed for 72 h and then reoxygenated and exposed to 1 h × 1 μg/mL mitomycin or 24 h × 0.5 μg/mL cisplatin. To determine clonogenic survival after paclitaxel treatment, cells were exposed to 0.01 μmol/L paclitaxel while being gassed for 72 h × 21% O_2 or 0.2% O_2.

Results

Chronic hypoxia, but not anoxia, preserves H1299 cell proliferation and survival. Extremely low levels of oxygen (anoxia) can result in apoptosis and DNA fragmentation and decreased colony formation (35, 36). To study DNA-dsb repair without these confounding variables, we developed a tissue culture model using H1299 cells that would not be biased by cell toxicity, decreased proliferation, or altered cell cycle distribution under low O_2 conditions.

We initially determined the specificity of our gassing system to induce a differential phenotype when cells were gassed under hypoxia or anoxia. Our gassing system equally induced the hypoxic markers HIF-1α and *VEGF* under both 0.2% and 0% O_2 (Fig. 1*A*). However, the anoxia-specific marker *GRP94* was significantly induced only after anoxic gassing. The radiation clonogenic survival curves of H1299 cells yielded an OER of 2.61 under 0% O_2 gassing and a lower OER of 2.19 under 0.2% O_2 (Fig. 1*B*). Exposure to a chronic anoxic treatment alone (72 h × 0% O_2) resulted in a profound decrease in clonogenic survival, with a reduction in survival of >97% (Fig. 1*C*). In contrast, chronic hypoxic conditions (72 h × 0.2% O_2) only decreased survival on average by 18%. HR protein expression and function can be cell cycle specific and biased by altered cell cycle distribution after hypoxic gassing (37, 38). In our model, hypoxia (0.2% O_2) did not decrease cell proliferation or change the cell cycle distribution (Fig. 1*D*). We conclude that gassing for periods up to 72 h × 0.2% O_2 allows for the study of chronic hypoxia on DNA-dsb repair in H1299 cells without bias from cell toxicity, decreased proliferation, or altered cell cycle distribution.

Expression of DNA repair genes under chronic hypoxia. Using a variety of cancer cell lines, we have previously shown that RAD51 and other HR associated proteins can be differentially expressed in malignant versus normal cells (39) and can be down-regulated under hypoxia or anoxia. However, there is no general concordance between the effect on mRNA and protein under low O_2 gassing conditions, suggesting a role for translation in addition to transcription, in mediating low HR protein levels (24–26). In the current study, we compared the effect of prolonged hypoxia on HR gene transcription and mRNA translation in H1299 cells. Western blotting confirmed that the expression of the HR repair proteins RAD51, RAD51B/C, RAD54, and XRCC3 were all significantly down-regulated in this model (Fig. 2*A*) in which cell cycle distribution is unchanged (Fig. 1*D*). However, real-time PCR showed no significant changes in the transcript levels of the HR genes in H1299 cells (Fig. 2*B*).

To test the effect of hypoxia on the mRNA translation of HR genes, polysomal fractionation analysis was performed to determine the association of ribosomes with the mRNA of two key HR proteins, RAD51 and BRCA2. In this assay, actively translated mRNAs containing two or more ribosomes (polysomes) are separated from inefficiently translated mRNAs bound to zero or one ribosome (monosome) by sedimentation through a sucrose gradient. When recording the absorbance as a function of gradient depth (Fig. 2*B*), the fractional polysome area under the curve is a direct reflection of *de novo* protein synthesis. Exposure to anoxic conditions resulted in a rapid and substantial inhibition of mRNA translation that persisted for >72 h (Fig. 2*C*), indicating that at this concentration, the translation of the majority of gene transcripts is severely inhibited. Exposure of 0.2% O_2 resulted in a considerably smaller, but significant, decrease in overall translation efficiency. We also analyzed the mRNA translation of specific HR genes by determining their mRNA distribution within the polysome profiles. Under oxic conditions, the majority of the *RAD51* and *BRCA2* mRNA contain seven or more ribosomes, indicating highly efficient translation. However, after 48 h at 0.2% O_2, there is a severe drop in the number of ribosomes per transcript, indicating reduced translation of *RAD51* and *BRCA2* in hypoxic proliferating cells (Fig. 2*D*). This severe drop in translation efficiency of *RAD51* and *BRCA2* contrasts the modest inhibition of overall translation as well as a control gene (*ACTIN*) under these conditions. These data indicate that chronic hypoxia leads to a specific decrease in the synthesis of HR repair proteins by a novel mechanism of translational inhibition, even under conditions which cause only modest changes in the overall rates of mRNA translation and where cell proliferation is preserved.

HR is down-regulated by chronic hypoxia. The functional consequences of the decreased HR protein translation were determined using a stably integrated DR-GFP reporter construct that measures the frequency of HR in the context of chromatin-associated DNA repair in H1299 cells (Fig. 3*A*). The reporter construct contains a GFP gene, which is interrupted by a rare restriction site (I-SceI), and a downstream GFP fragment. Cells were transfected with a plasmid encoding for either a negative control, GFP (transfection efficiency control), or the endonuclease

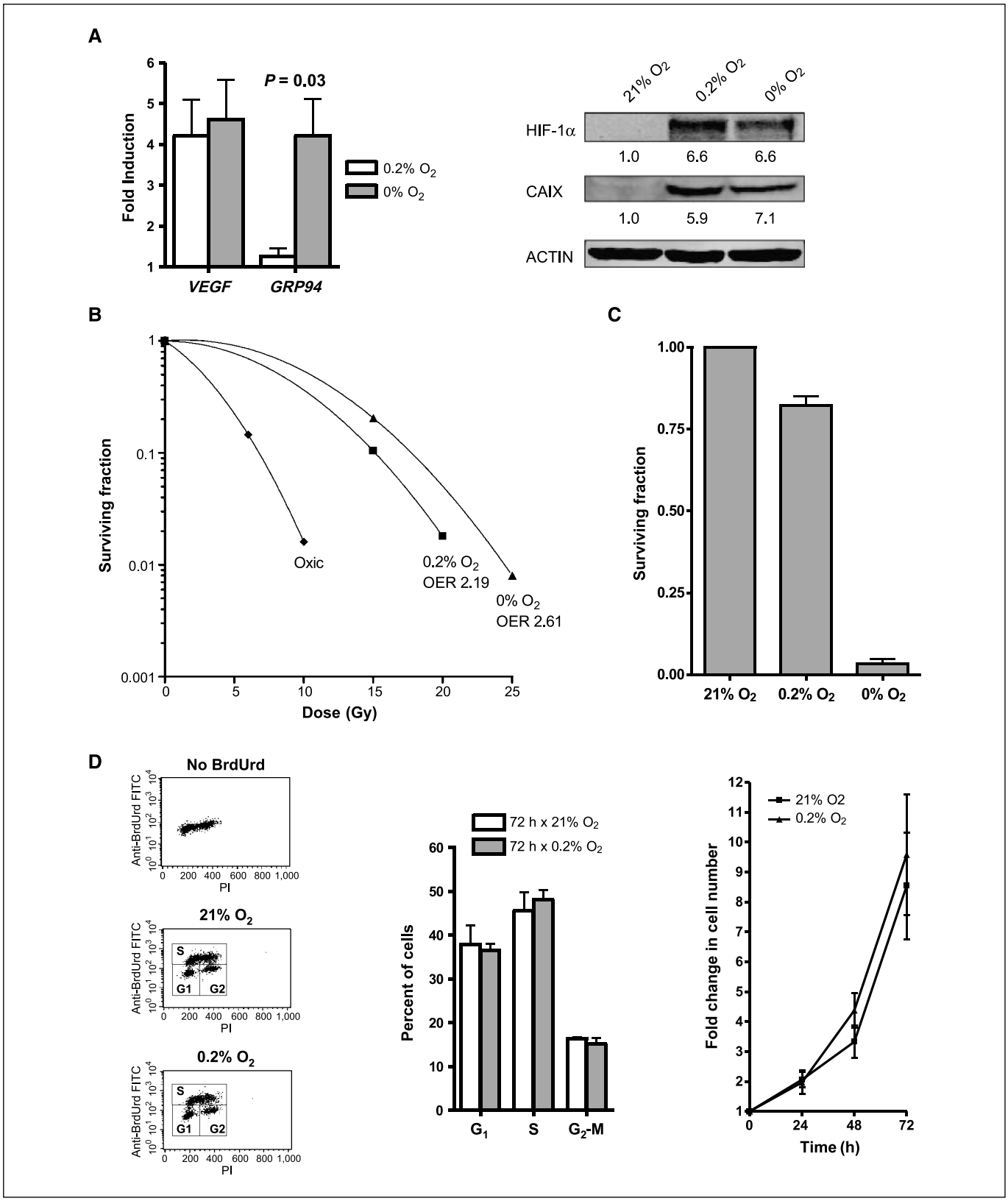

Figure 1. Differential phenotypes resulting from exposure to chronic hypoxia and anoxia on H1299 cells. *A,* induction of the hypoxic mRNA marker *VEGF* and the hypoxic protein markers HIF-1α and CAIX occurs after 72 h × 0.2% or 0% O_2. Induction of the anoxia-specific mRNA marker *GRP94* occurs only after 72 h × 0% O_2. *B,* representative clonogenic survival curves after IR exposure for cells irradiated under 21%, 0.2%, or 0% O_2 after a 6-h gas treatment. *C,* cell survival of H1299 cells after reoxygenation after 72 h × 0.2% or 0% O_2 is significantly decreased by 18% and 97%, respectively. *D,* representative flow cytometric profiles of H1299 cells after 72 h × 0.2% O_2. Anti-BrdUrd FITC signal is plotted on the *Y*-axis, with propidium iodide (*PI*) signal on the *X*-axis. The cell cycle profile is virtually unchanged under hypoxia. Cell proliferation is not significantly different after 24, 48, and 72 h of gassing at 21% or 0.2% O_2. *Columns,* mean; *bars,* SE.

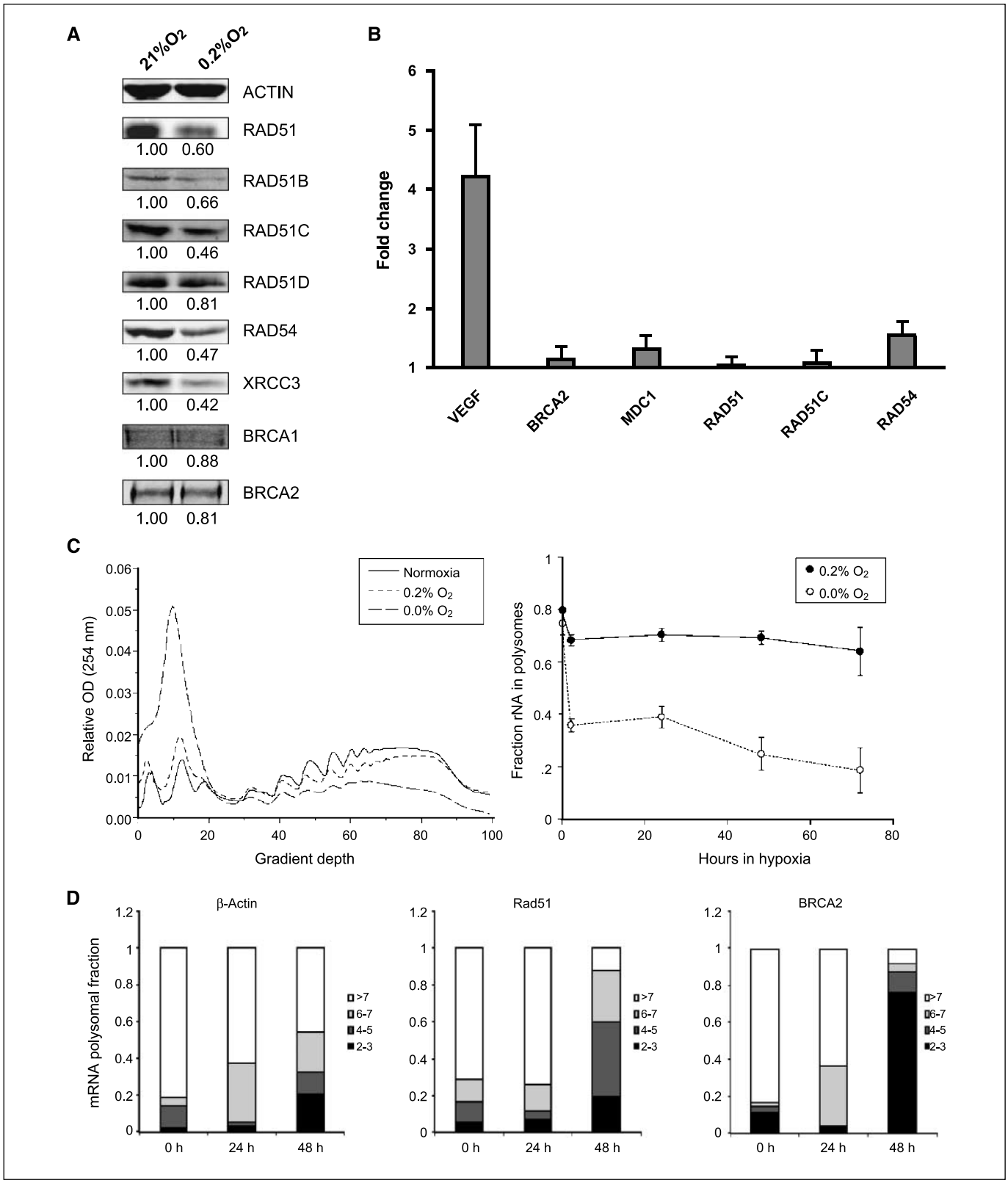

Figure 2. HR mRNA and protein expression changes induced by 72 h × 0.2% O_2 in H1299 cells. *A,* the majority of the HR proteins are down-regulated under prolonged hypoxia. *B,* real-time quantitative PCR analyses show no significant effects of prolonged hypoxia on the mRNA levels of the HR genes. Induction of *VEGF* is shown as a positive control. *C* to *D,* H1299 cells were treated up to 48 h × 0.2% O_2, and cell lysates were separated on sucrose gradients. *C,* the absorbance (*OD*) at 254 nm is plotted as a function of gradient depth. The overall translation efficiency, estimated as the relative amount of rRNA present in polysomes, decreases after 24 h of hypoxia and anoxia. *D,* the distribution of *ACTIN, RAD51*, and *BRCA2* transcripts within the polysomes was determined. The percentage of transcripts containing varying numbers of attached ribosomes after 0, 24, and 48 h of 0.2% O_2 is shown. At 48 h, the translation efficiency of both the *RAD51* and *BRCA2* genes is decreased as evidenced by a large shift in transcripts toward reduced ribosome numbers. *Points,* mean; *bars,* SE.

to generate a specific DNA-dsb. In this assay, functional GFP can only be restored if the DNA-dsb is repaired in an error-free manner using the downstream GFP fragment as a template for HR (19). The frequency of recombination as determined by flow cytometry was significantly reduced by a factor of 3 ($P = 0.017$) after 72 h × 0.2% O_2 gassing in proliferating cells (Fig. 3*A*).

To further test for a functional cellular defect in HR, cells were exposed to hypoxia and then treated with mitomycin, cisplatin, or paclitaxel. Mitomycin is a bioreductive alkylating agent that is selectively toxic to HR-deficient cells by causing DNA interstrand cross-links whose repair is HR dependent (40, 41). Cisplatin is a commonly used platinum-based chemotherapeutic that also causes interstrand crosslinks. In keeping with the observed effects of hypoxia on the DR-GFP HR assay, cells pretreated with chronic hypoxia showed significantly increased sensitivity to both mitomycin ($P = 0.05$) and cisplatin ($P = 0.006$; Fig. 3*B*). As a control, paclitaxel, a chemotherapeutic agent that interferes with microtubules and does not cause DNA damage, had similar toxicity under hypoxic and oxic conditions. We conclude that HR is functionally down-regulated by chronic hypoxia. These results are supported by down-regulation of RAD51 via siRNA, leading to increased sensitivity to mitomycin as well as decreased HR as measured by the DR-GFP assay (Fig. 3*C*).

Cellular radioresistance is decreased after chronic hypoxia. Oxygen enhances the biological effects of radiation by interacting with and "fixing" DNA damage after the indirect effect of IR in the vicinity of DNA (9). Acute anoxia generally decreases IR-induced DNA-dsbs by 2- to 3-fold, reflecting the OER for this O_2

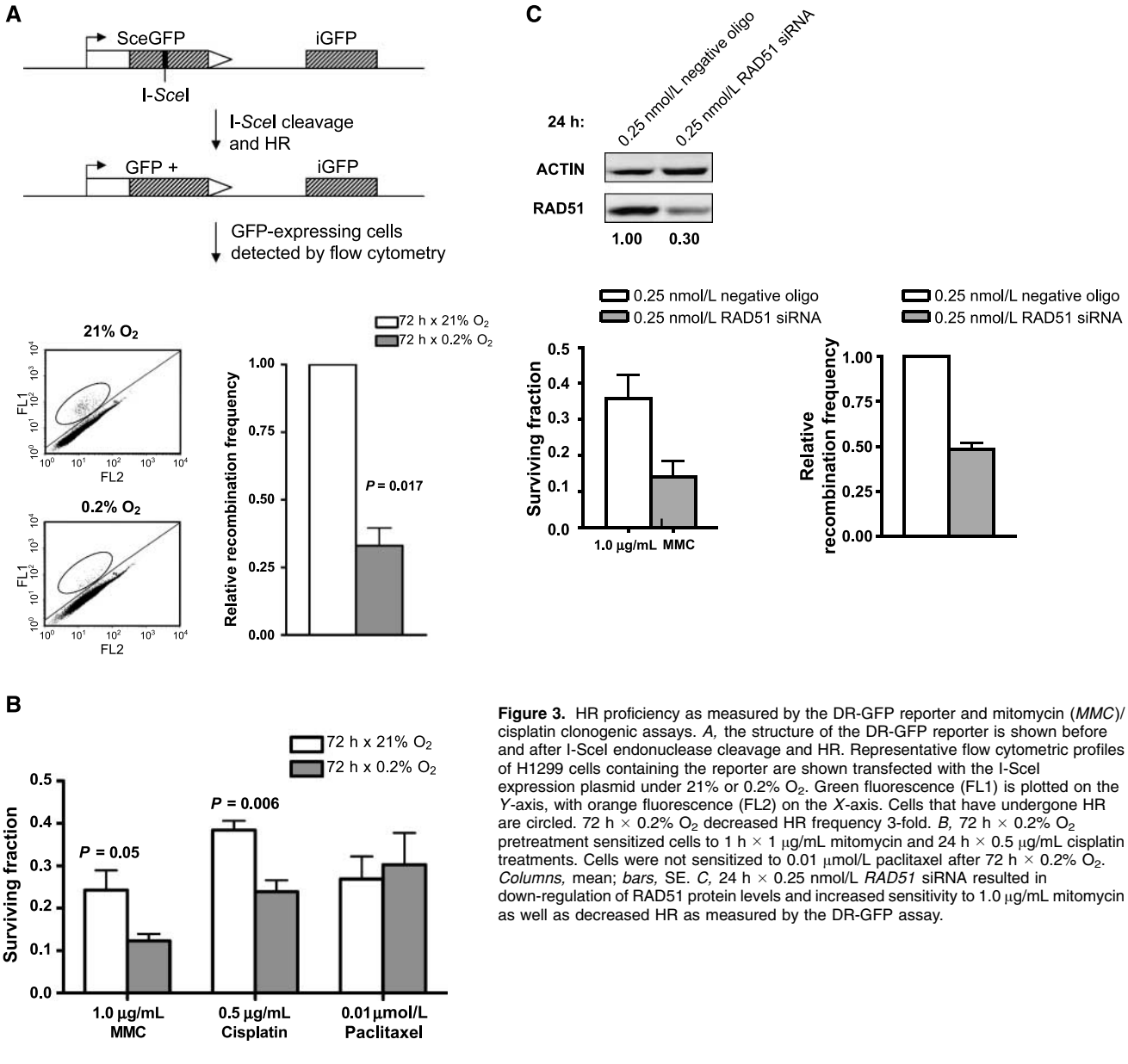

Figure 3. HR proficiency as measured by the DR-GFP reporter and mitomycin (*MMC*)/cisplatin clonogenic assays. *A,* the structure of the DR-GFP reporter is shown before and after I-SceI endonuclease cleavage and HR. Representative flow cytometric profiles of H1299 cells containing the reporter are shown transfected with the I-SceI expression plasmid under 21% or 0.2% O_2. Green fluorescence (FL1) is plotted on the *Y*-axis, with orange fluorescence (FL2) on the *X*-axis. Cells that have undergone HR are circled. 72 h × 0.2% O_2 decreased HR frequency 3-fold. *B,* 72 h × 0.2% O_2 pretreatment sensitized cells to 1 h × 1 μg/mL mitomycin and 24 h × 0.5 μg/mL cisplatin treatments. Cells were not sensitized to 0.01 μmol/L paclitaxel after 72 h × 0.2% O_2. *Columns,* mean; *bars,* SE. *C,* 24 h × 0.25 nmol/L *RAD51* siRNA resulted in down-regulation of RAD51 protein levels and increased sensitivity to 1.0 μg/mL mitomycin as well as decreased HR as measured by the DR-GFP assay.

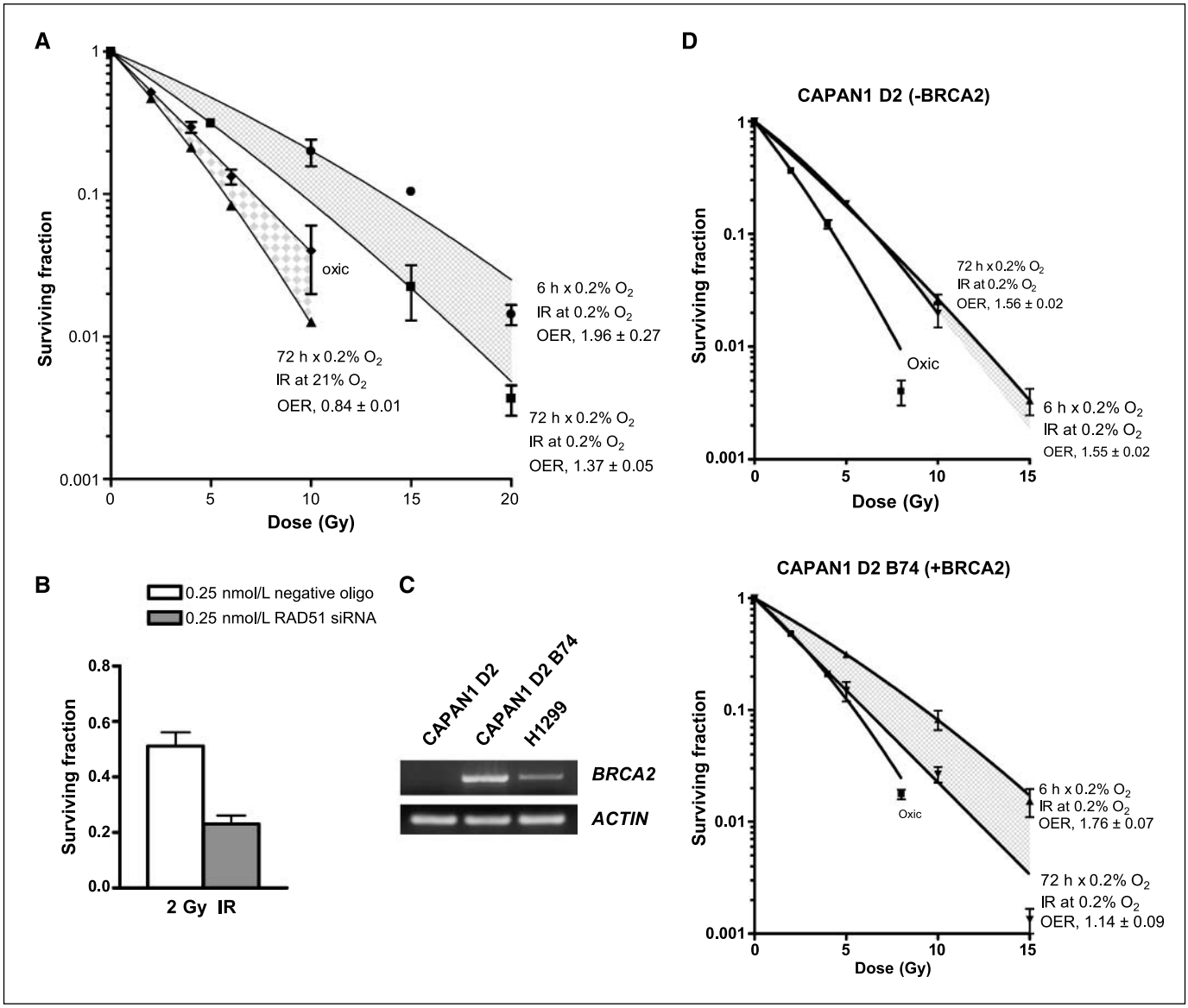

Figure 4. The effect of prolonged hypoxia on cellular radiosensitivity in H1299 and isogenic BRCA2 cells. The clonogenic survival was determined for H1299 and CAPAN1 cells treated for 6 to 72 h × 21% or 0.2% O_2 and irradiated 0 to 20 Gy with or without cellular reoxygenation. *A,* acutely hypoxic H1299 cells were 2-fold more radioresistant than oxic cells (OER, 1.96 ± 0.27). In contrast, chronically hypoxic H1299 cells were only slightly more radioresistant than oxic cells (OER, 1.37 ± 0.05). Chronically hypoxic cells that were reoxygenated before irradiation were more radiosensitive than oxic cells (OER, 0.84 ± 0.01). *B,* 24 h × 0.25 nmol/L *RAD51* siRNA resulted in increased sensitivity to 2 Gy IR. *C,* RT-PCR detection of *BRCA2* expression in the H1299 and BRCA2-complimented cell line D2 B74 and its parental line CAPAN1 D2. *D,* BRCA2-deficient CAPAN1 cells had a similar radiosensitivity after either 6 (OER, 1.56 ± 0.02) or 72 h (OER, 1.55 ± 0.02) gassing with 0.2% O_2. BRCA2-complemented cells were almost 2-fold more radioresistant than oxic cells after acute gassing (6 h) with 0.2% O_2 (OER, 1.76 ± 0.07). After 72 h, prolonged hypoxia the radiosenstivity was reduced similar to the oxic control in BRCA2-complemented cells (OER, 1.14 ± 0.09). *Points,* mean; *bars,* SE.

level. As expected, H1299 cells gassed with an acute hypoxic treatment showed a 2-fold increase in radioresistance (OER, 1.96 ± 0.27; Fig. 4*A*). This is lower than the OER for acute anoxia (Fig. 1*C*). Cells treated with chronic hypoxia (72 h × 0.2% O_2) had a reduced radioresistance (OER, 1.37 ± 0.05). Furthermore, cells treated 72 h × 0.2% O_2 and then reoxygenated before irradiation were more radiosensitive than the oxic control (OER, 0.84 ± 0.01). Knocking down RAD51 with siRNA to levels similar to the levels seen under chronic hypoxia also sensitizes H1299 cells to IR (Fig. 4*B*). We conclude that chronic hypoxic conditions, which lead to an HR-deficiency, results in reduced hypoxic radioresistance.

To further prove that the effect on radiosensitivity was HR dependent, we determined the effect of chronic hypoxia on the hypoxic radiosensitivity of paired CAPAN1 pancreatic adenocarcinoma cell lines that are isogenic for BRCA2 (D2 and D2 B74; Fig. 4*C*). In support of our H1299 gassing data, BRCA2-deficient cells show no difference in radiosensitivity between cells treated with either acute (OER, 1.56 ± 0.02) or chronic hypoxia (OER, 1.55 ± 0.02; Fig. 4*D*). Complementing this cell line with functional BRCA2

increased the baseline radioresistance (OER, 1.76 ± 0.07). However, their hypoxia-mediated radioresistance was decreased after chronic hypoxia in a manner similar to that observed for the H1299 cell line (OER, 1.14 ± 0.09). These data support our hypothesis that the inherent radioresistance of acutely hypoxic cells is abrogated under chronic hypoxia and that this is dependent on functional HR.

Discussion

The experiments outlined in this paper were conducted under conditions of chronic prolonged hypoxia that prevented alterations in cell cycle distribution or anoxia-induced cell cycle checkpoints: all factors that could potentially bias HR expression and function. This is the first report of chronic hypoxia-induced suppression of HR DNA-dsb repair proteins at the translational level leading to functional impairment of the HR pathway. Furthermore, we report for the first time that hypoxia-mediated inhibition of HR gene expression results in a functional HR impairment with increased sensitivity to IR, mitomycin, and cisplatin and confirming an HR-deficient phenotype.

Many studies have assessed the expression and function of DNA repair genes solely under anoxic (0% O_2) rather than hypoxic conditions (11). Anoxia can induce an ATM- and ATR-mediated G_1 and intra-S (DNA replicative) arrest and a further G_2 phase arrest upon reoxygenation (42–44). This could lead to altered S and G_2 fractions that may bias HR protein expression and function, given that HR is cell cycle dependent (37). However, our data support a cell cycle–independent decrease in HR function. Although HR might require to restart collapsed replication forks in S phase, the residual HR capacity after 0.2% gassing may just be sufficient to allow normal cellular proliferation.

In this report, we studied the biology of chronic and prolonged hypoxia, which can reflect longer cell transit times across intratumoral oxygen partial pressure gradients in certain slowly growing tumors (e.g., prostate and other cancers). This biology may be very different than conditions of cyclic acute anoxia that can produce reactive oxygen species during subsequent reoxygenation. Indeed, the effects on HR protein expression and function are not observed until at least 24 h of hypoxic gassing (25). Therefore, prolonged hypoxic conditions in adapted tumor cells with decreased DNA repair and absent checkpoint control could lead to the accumulation of DNA damage, genetic instability, and mutator and/or resistant phenotypes (45). Studies using innovative models that allow for long-term hypoxic incubation (e.g., 3–4 weeks) with subsequent characterization of cell phenotype and genotype will be required to directly test this hypothesis.

Hypoxia can alter the balance of activating and repressive E2F transcription factors, thereby affecting the expression of BRCA1 and RAD51 protein in a HIF-1α–independent manner (23, 26). However, in our H1299 model, the mRNA levels of the HR repair genes was unaffected after 72 h × 0.2% O_2. This suggested an additional mode of regulation of HR proteins under hypoxia. Polysomal fractionation analysis of *RAD51* and *BRCA2* transcripts revealed that in H1299 cells, the expression of these genes is regulated at the translational level. Regulation of mRNA translation can dramatically alter individual gene expression and possibly also explain reduced HR protein levels under chronic hypoxic conditions (46). Importantly, this effect was specific to HR repair genes under conditions of hypoxia, which resulted in only a modest reduction in the overall rate of cellular mRNA translation. A similar treatment had little effect on the levels of NHEJ proteins (data not shown), supporting specificity for the HR pathway under these study conditions.

Translational control is increasingly recognized as a mechanism for altering specific gene expression and is highly influenced by sequences within the 5′ and 3′ untranslated regions. Under hypoxia, this suppression is controlled through at least two distinct pathways: first, by phosporylated extracellular signal-related kinase–mediated phosphorylation of eIF2α, which is required for the recruitment of aminoacylated tRNA and second, by disruption of the mRNA cap–binding complex, eIF4F (47). Both pathways result in the inhibition of mRNA translation but lead to important differences in the translation of specific genes. Further studies using cells with defined defects in each of these pathways will be required to elucidate the exact mechanisms involved in translational control of HR protein expression under chronic hypoxia. However, it seems that both altered transcription and translation are important in determining decreased DNA repair protein expression, and the relative contributions of each mechanism may depend on cell type, oxygen concentration, and experimental design.

The relative radioresistance acquired by anoxic cells, as manifested in the OER, has recently been shown to be associated with HR proficiency (48). Irradiation of Chinese hamster cell lines with mutations in HR genes (e.g., *XRCC2, XRCC3, BRCA2*, and *RAD51C*) under oxic (20% O_2) and anoxic (<0.1% O_2) conditions revealed a decreased OER (1.5–2.0) when compared with the OER for HR-proficient wild-type cells (2.6–3.0). No effect on OER was noted for NHEJ-deficient cells. In support of this recent study, we observed that the hypoxic radioresistance observed after acute hypoxia (6 h × 0.2% O_2; OER, 1.96 ± 0.27) in HR-proficient cells was mostly lost after chronic hypoxia (72 h × 0.2% O_2; OER, 1.37 ± 0.05). This is consistent with the effects on radiosensitivity due to impairment of HR proficiency (21). This HR effect on radiosensitivity may in part be due to an altered number and repair of DNA-dsbs, DNA-single strand breaks, DNA-DNA interstrand cross-links, or DNA protein cross-links and requires further study (48).

Our observations open up the possibility for novel therapeutic strategies that target HR and chronic hypoxia. Fractionated radiotherapy is a successful cancer treatment modality that maintains the therapeutic ratio (e.g., increased killing in tumor cells relative to normal tissues) by differential cellular repair between malignant and normal tissues (49). Traditionally, acutely hypoxic cells have been documented as being extremely radioresistant due to the biophysics of oxygen free radical attack and fixation of DNA lesions. The process of reoxygenation during radiotherapy is thought to offset tumor radioresistance to improve the likelihood of local control and may alter the proportion of both acute and chronic hypoxia. Based on our data, we suggest that the relative radiosensitivity of chronically hypoxic cells may be an additional factor in determining radiotherapeutic local control.

Novel molecular-based therapies could take advantage of DNA repair deficiencies to selectively kill hypoxic cells that would otherwise acquire a mutator phenotype. The therapeutic ratio may be favored in this approach, given that few normal tissues are hypoxic. Furthermore, malignant tissues may have decreased repair capability and discordance between cell cycle checkpoint

control and DNA repair, which could make them more susceptible to CHK2-CHK1 targeting and agents that sensitize one repair pathway over another (49). This concept could also include the use of chemotherapeutic agents such as etoposide, or PARP inhibitors, that can preferentially sensitize HR-deficient cells (11, 13).

A prerequisite for the use of novel therapies based on these preclinical studies is the ability to predict the fraction of acute and chronic hypoxic cells in solid tumors and their relative oxygen concentration. One strategy could involve using a serial injection of two different hypoxic markers, such as pimonidazole and EF5, in combination with markers of proliferation (e.g., BuDr and IuDr) and blood vessels as described by Ljungkvist et al. (50). Intratumoral regions that are matched for the two hypoxic markers are chronically hypoxic, and those mismatched for staining are acutely hypoxic. Such studies are under way in our laboratory, whereby the relative repair of DNA-dsbs can be tracked as a function of acute and chronic hypoxia and correlated to tumor radioresponse and chemoresponse. This concept will be clinically feasible only through the development of innovative noninvasive imaging techniques that can track acute and chronic hypoxia during treatment to allow effective intervention with novel antihypoxia therapies.

Acknowledgments

Received 9/18/2007; revised 10/31/2007; accepted 10/31/2007.

Grant support: The Terry Fox Foundation-NCIC Hypoxia PMH Program Grant (15004) and a NCIC operating grant (17154) to R.G. Bristow; the NIH (grants R01ES005775 and P01CA129186) to P.M. Glazer; the Dutch Science Organization (ZonMW-NOW Top grant 912-03-047 to B. Wouters and ZonMW-VENI Grant 016.056.015 to M. Koritzinsky), the Dutch Cancer Society (Koningin Wilhelmina Fonds grant UM 2003-2821 to B. Wouters), and the EU sixth framework program (Euroxy program to B. Wouters). R.G. Bristow is a Canadian Cancer Society Research Scientist.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Kaanders JH, Wijffels KI, Marres HA, et al. Pimonidazole binding and tumor vascularity predict for treatment outcome in head and neck cancer. Cancer Res 2002;62:7066–74.
2. Nordsmark M, Bentzen SM, Rudat V, et al. Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. Radiother Oncol 2005;77:18–24.
3. Vaupel P. Tumor microenvironmental physiology and its implications for radiation oncology. Semin Radiat Oncol 2004;14:198–206.
4. Chan N, Milosevic M, Bristow RG. Tumor hypoxia, DNA repair and prostate cancer progression: new targets and new therapies. Future Oncol 2007;3:329–41.
5. Charlesworth PJ, Harris AL. Mechanisms of disease: angiogenesis in urologic malignancies. Nat Clin Pract 2006;3:157–69.
6. Chaudary N, Hill RP. Hypoxia and metastasis. Clin Cancer Res 2007;13:1947–9.
7. Cairns RA, Hill RP. Acute hypoxia enhances spontaneous lymph node metastasis in an orthotopic murine model of human cervical carcinoma. Cancer Res 2004; 64:2054–61.
8. Rofstad EK, Galappathi K, Mathiesen B, Ruud EB. Fluctuating and diffusion-limited hypoxia in hypoxia-induced metastasis. Clin Cancer Res 2007; 13:1971–8.
9. Hill R, Bristow R. The Scientific Basis of Clinical Radiotherapy. In: Tannock H, Harrington L, Bristow R, editors. The Basic Science of Oncology. 4th ed. New York: McGraw-Hill Ltd; 2005. p. 289–321.
10. Bindra RS, Glazer PM. Genetic instability and the tumor microenvironment: towards the concept of microenvironment-induced mutagenesis. Mutat Res 2005;569:75–85.
11. Bristow RG, Ozcelik H, Jalali F, Chan N, Vesprini D. Homologous recombination and prostate cancer: a model for novel DNA repair targets and therapies. Radiother Oncol 2007;83:220–30.
12. Freiberg RA, Krieg AJ, Giaccia AJ, Hammond EM. Checking in on hypoxia/reoxygenation. Cell Cycle 2006; 5:1304–7.
13. Helleday T, Lo J, van Gent DC, Engelward BP. DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair (Amst) 2007; 6:923–35.
14. Rothkamm K, Kruger I, Thompson LH, Lobrich M. Pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol 2003; 23:5706–15.
15. Rodrigue A, Lafrance M, Gauthier MC, et al. Interplay between human DNA repair proteins at a unique double-strand break *in vivo*. EMBO J 2006;25: 222–31.
16. Weterings E, van Gent DC. The mechanism of non-homologous end-joining: a synopsis of synapsis. DNA Repair (Amst) 2004;3:1425–35.
17. Collis SJ, DeWeese TL, Jeggo PA, Parker AR. The life and death of DNA-PK. Oncogene 2005;24: 949–61.
18. Bertrand P, Lambert S, Joubert C, Lopez BS. Overexpression of mammalian Rad51 does not stimulate tumorigenesis while a dominant-negative Rad51 affects centrosome fragmentation, ploidy and stimulates tumorigenesis, in p53-defective CHO cells. Oncogene 2003; 22:7587–92.
19. Stark JM, Pierce AJ, Oh J, Pastink A, Jasin M. Genetic steps of mammalian homologous repair with distinct mutagenic consequences. Mol Cell Biol 2004; 24:9305–16.
20. Ouyang H, Nussenzweig A, Kurimasa A, et al. Ku70 is required for DNA repair but not for T cell antigen receptor gene recombination *In vivo*. J Exp Med 1997; 186:921–9.
21. Powell SN, Kachnic LA. Roles of BRCA1 and BRCA2 in homologous recombination, DNA replication fidelity and the cellular response to ionizing radiation. Oncogene 2003;22:5784–91.
22. Wilson GD. Cell kinetics. Clin Oncol (R Coll Radiol) 2007;19:370–84.
23. Bindra RS, Glazer PM. Repression of RAD51 gene expression by E2F4/p130 complexes in hypoxia. Oncogene 2007;26:2048–57.
24. Bindra RS, Schaffer PJ, Meng A, et al. Down-regulation of Rad51 and decreased homologous recombination in hypoxic cancer cells. Mol Cell Biol 2004;24: 8504–18.
25. Meng AX, Jalali F, Cuddihy A, et al. Hypoxia down-regulates DNA double strand break repair gene expression in prostate cancer cells. Radiother Oncol 2005;76: 168–76.
26. Bindra RS, Gibson SL, Meng A, et al. Hypoxia-induced down-regulation of BRCA1 expression by E2Fs. Cancer Res 2005;65:11597–604.
27. Wouters BG, van den Beucken T, Magagnin MG, Koritzinsky M, Fels D, Koumenis C. Control of the hypoxic response through regulation of mRNA translation. Semin Cell Dev Biol 2005;16:487–501.
28. Koritzinsky M, Magagnin MG, van den Beucken T, et al. Gene expression during acute and prolonged hypoxia is regulated by distinct mechanisms of translational control. EMBO J 2006;25: 1114–25.
29. Koritzinsky M, Seigneuric R, Magagnin MG, van den Beucken T, Lambin P, Wouters BG. The hypoxic proteome is influenced by gene-specific changes in mRNA translation. Radiother Oncol 2005;76:177–86.
30. Romanova LY, Willers H, Blagosklonny MV, Powell SN. The interaction of p53 with replication protein A mediates suppression of homologous recombination. Oncogene 2004;23:9025–33.
31. Abaji C, Cousineau I, Belmaaza A. BRCA2 regulates homologous recombination in response to DNA damage: implications for genome stability and carcinogenesis. Cancer Res 2005;65:4117–25.
32. Young SD, Marshall RS, Hill RP. Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci U S A 1988;85: 9533–7.
33. Bristow RG, Hu Q, Jang A, et al. Radioresistant MTp53-expressing rat embryo cell transformants exhibit increased DNA-dsb rejoining during exposure to ionizing radiation. Oncogene 1998;16:1789–802.
34. Bromfield GP, Meng A, Warde P, Bristow RG. Cell death in irradiated prostate epithelial cells: role of apoptotic and clonogenic cell kill. Prostate Cancer Prostatic Dis 2003;6:73–85.
35. Papandreou I, Krishna C, Kaper F, Cai D, Giaccia AJ, Denko NC. Anoxia is necessary for tumor cell toxicity caused by a low-oxygen environment. Cancer Res 2005; 65:3171–8.
36. Johnson AB, Barton MC. Hypoxia-induced and stress-specific changes in chromatin structure and function. Mutat Res 2007;618:149–62.
37. Aylon Y, Liefshitz B, Kupiec M. The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle. EMBO J 2004;23: 4868–75.
38. Essers J, Hendriks RW, Wesoly J, et al. Analysis of mouse Rad54 expression and its implications for homologous recombination. DNA Repair (Amst) 2002; 1:779–93.
39. Fan R, Kumaravel TS, Jalali F, Marrano P, Squire JA, Bristow RG. Defective DNA strand break repair after DNA damage in prostate cancer cells: implications for genetic instability and prostate cancer progression. Cancer Res 2004;64:8526–33.
40. Dronkert ML, Kanaar R. Repair of DNA interstrand cross-links. Mutat Res 2001;486:217–47.
41. McHugh PJ, Spanswick VJ, Hartley JA. Repair of DNA

interstrand crosslinks: molecular mechanisms and clinical relevance. Lancet Oncol 2001;2:483–90.
42. Hammond EM, Dorie MJ, Giaccia AJ. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. J Biol Chem 2003;278:12207–13.
43. Freiberg RA, Hammond EM, Dorie MJ, Welford SM, Giaccia AJ. DNA damage during reoxygenation elicits a Chk2-dependent checkpoint response. Mol Cell Biol 2006;26:1598–609.
44. Gibson SL, Bindra RS, Glazer PM. Hypoxia-induced phosphorylation of Chk2 in an ataxia telangiectasia mutated-dependent manner. Cancer Res 2005;65:10734–41.
45. Subarsky P, Hill RP. The hypoxic tumour microenvironment and metastatic progression. Clin Exp Metastasis 2003;20:237–50.
46. Harding HP, Novoa I, Zhang Y, et al. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell 2000;6:1099–108.
47. Wouters BG, van den Beucken T, Magagnin MG, Lambin P, Koumenis C. Targeting hypoxia tolerance in cancer. Drug Resist Updat 2004;7:25–40.
48. Sprong D, Janssen HL, Vens C, Begg AC. Resistance of hypoxic cells to ionizing radiation is influenced by homologous recombination status. Int J Radiat Oncol Biol Phys 2006;64:562–72.
49. Choudhury A, Cuddihy A, Bristow RG. Radiation and new molecular agents part I: targeting ATM-ATR checkpoints, DNA repair, and the proteasome. Semin Radiat Oncol 2006;16:51–8.
50. Ljungkvist AS, Bussink J, Kaanders JH, van der Kogel AJ. Dynamics of tumor hypoxia measured with bioreductive hypoxic cell markers. Radiat Res 2007;167:127–45.