Hypoxia-Induced Down-regulation of *BRCA1* Expression by E2Fs

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Abstract

Decreased *BRCA1* expression in the absence of genetic mutation is observed frequently in sporadic cancers of the breast and other sites, although little is known regarding the mechanisms by which the expression of this gene can be repressed. Here, we show that activating and repressive E2Fs simultaneously bind the *BRCA1* promoter at two adjacent E2F sites *in vivo*, and that hypoxia induces a dynamic redistribution of promoter occupancy by these factors resulting in the transcriptional repression of *BRCA1* expression. Functionally, we show that hypoxia is associated with impaired homologous recombination, whereas the nonhomologous end-joining (NHEJ) repair pathway is unaffected under these conditions. Repression of *BRCA1* expression by hypoxia represents an intriguing mechanism of functional *BRCA1* inactivation in the absence of genetic mutation. We propose that hypoxia-induced decreases in *BRCA1* expression and consequent suppression of homologous recombination may lead to genetic instability by shifting the balance between the high-fidelity homologous recombination pathway and the error-prone NHEJ pathway of DNA repair. Furthermore, these findings provide a novel link between E2Fs and the transcriptional response to hypoxia and provide insight into the mechanisms by which the tumor microenvironment can contribute to genetic instability in cancer. (Cancer Res 2005; 65(24): 11597-604)

Introduction

The BRCA1 protein plays a role in numerous cellular processes, including DNA repair, cell cycle checkpoint control, and transcription (1). Although germ line mutations in *BRCA1* account for a large proportion of inherited breast cancers, sporadic breast carcinomas rarely show mutations in the *BRCA1* gene (2, 3). As sporadic tumors account for >90% of the breast cancer burden, considerable research has been directed towards the study of other mechanisms by which the BRCA1 function is compromised in cases where somatic mutations are not observed. Indeed, decreased BRCA1 expression has been observed in many non-familial cancers of the breast and other sites, often directly correlating with increased tumor grade and poor prognosis (4–6).

We and others have proposed that the tumor microenvironment can contribute to genetic instability (7–15). Our laboratory has previously shown that hypoxia causes increased mutagenesis (8), functional impairment of nucleotide excision repair (9), and down-regulation of the mismatch repair gene, *MLH1* (16), and of the homologous recombination gene, *RAD51* (7). It has more recently been shown that hypoxia can repress the expression of another mismatch repair gene, *MSH2* (10). Collectively, these phenomena constitute a significant source of genetic instability induced by hypoxia, thus potentially accelerating the multistep process of tumor progression.

In parallel, emerging evidence indicates that members of the E2F family of transcription factors are involved in numerous pathways outside of their established roles as cell cycle regulators, including DNA damage response and apoptotic pathways (17–20). Here, we report that E2Fs mediate the repression of *BRCA1* gene expression in response to the key microenvironmental stress of hypoxia. Functionally, we show that homologous recombination is suppressed by hypoxia, whereas the nonhomologous end-joining (NHEJ) pathway is unaffected under these conditions. These data suggest that hypoxia-induced decreases in *BRCA1* expression and consequently diminished homologous recombination may lead to genetic instability by shifting the balance between the high-fidelity homologous recombination and the error-prone NHEJ pathways of double-strand break (DSB) repair.

Materials and Methods

Cells. MCF7, A549, and RKO cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown according to supplier instructions. 786-0 cell lines were a gift from Dr. W.G. Kaelin (Medical Oncology, Dana-Farber Cancer Institute, Boston, MA), and RKO-E7 cells were obtained from Dr. Kathleen Cho (Pathology, University of Michigan, Ann Arbor, MI).

Plasmids. The wild-type (WT) hypoxia-inducible factor-1α (HIF-1α) expression vector was obtained from G. Semenza (Oncology, Johns Hopkins University, Baltimore, MD). The HIF-1α mutant expression vectors and the 5X-HRE luciferase reporter construct were obtained from Dr. Zhong Yun (Therapeutic Radiology, Yale University School of Medicine, New Haven, CT; ref. 21). The E2F reporter vector (3XE2F) was from Panomics (Redwood City, CA). The Dp1 dominant-negative (Δ103-126) and p107 expression vectors were obtained from Dr. Ed Harlow (Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA). The p130 and E2F4 expression vectors were from Drs. Robert Weinberg (Biology, Massachusetts Institute of Technology, Boston, MA), and David Livingston (Dana-Farber Cancer Institute, Boston, MA), respectively. Transfections were done using the Fugene 6 reagent (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer's recommendations.

Hypoxia exposure. Hypoxic conditions were established as described previously (8).

Northern blot analysis. Total RNA was isolated using the TRIzol RNA isolation system (Life Technologies, Rockville, MD) followed by phenol-chloroform extraction. Northern blot analysis was done as described previously (16).

Quantitative real-time PCR analysis. Synthesis of cDNA from total RNA samples was done using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). To assess mRNA expression, cDNAs prepared from total RNA samples were used in PCR reactions containing Taqman Universal master mix and premixed Taqman probes and primers (Applied Biosystems, Foster City, CA). Fluorescence intensity was monitored in real time, and cycle thresholds (C_T) were calculated using the Mx3000p real-time PCR system (Stratagene, La Jolla, CA). Additional details are provided in the Supplementary Information.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assays were done essentially as described (22). Briefly, MCF7 and RKO cells were fixed in 1% formaldehyde followed by incubation in 0.125 mol/L glycine to stop cross-linking. Cells were then washed and recovered followed by lysis in SDS buffer. Lysates were sonicated yielding genomic DNA fragments with a bulk size of ∼200 to 1,000 bp followed by clarification by centrifugation. Supernatants were diluted and precleared with salmon sperm DNA/protein A-agarose (Upstate Biotech, Waltham, MA). Lysates were immunoprecipitated with 2 to 5 μg of the antibodies described below, with material from 5×10^6 cells used for each immunoprecipitation. Antibody-nucleoprotein complex mixtures were incubated overnight and recovered by incubation with 60 μL salmon sperm DNA/protein A-agarose for 1 hour at 4°C. Aliquots (100 μL) were reserved from the negative control (no antibody) samples before washes and processed in parallel with eluted samples and used as input DNA. Beads were washed five times (buffer recipes are shown in the Supplementary Information), and nucleoprotein complexes were eluted from protein A-agarose beads in IP elution buffer. Cross-links were reversed by addition of 4 μL of 5 mol/L NaCl and incubation at 65°C for 4.5 hours followed by a 1.5-hour digestion with RNase A and proteinase K at 50°C. DNA fragments were recovered using QIAquick PCR purification columns (Qiagen, Valencia, CA), samples were eluted in 50 μL and further diluted 1:5 in distilled water. Quantitative analysis of chromatin immunoprecipitation assays was then done as described in the Supplementary Information.

Chromatin immunoprecipitation assay antibodies and primers. Antibody details and primer sequences are provided in the Supplementary Information.

Transient and sequential chromatin immunoprecipitation assays. Transient and sequential chromatin immunoprecipitation assay procedures are described in detail in the Supplementary Information.

Western blot analysis. Western blot analysis was done as described previously (16), and specific antibody clones are described in the Supplementary Information.

In silico promoter analyses. 5′-Flanking regions (1-2 kb) of the *BRCA1* gene from *Homo sapiens, Pan troglodytes, Canis familiaris, Mus musculus*, and *Rattus norvegicus* were downloaded from the University of California Santa Cruz genome browser and aligned using the multiple sequence alignment program, ClustalW, which is especially suited for the analysis of functional noncoding sequences. In parallel, conserved motifs with a minimal length of eight nucleotides in the homologous sequences were identified using the phylogenetic analysis program, FootPrinter.[7] Several highly conserved binding sites were identified in the *NBR2/BRCA1* intergenic region using this approach, and a selection of these sites is shown in Fig. 2*A*.

Statistical analyses. Unpaired two-tailed *t* tests and other statistical analyses were done using the Microsoft Excel Plug-in, Analyze-it (Analyze-it, Ltd., Leeds, United Kingdom).

Luciferase reporter gene assays. Luciferase promoter assays were done as described previously (11), and additional information is provided in the Supplementary Information.

Fluorescence-activated cell sorting analysis. Fluorescence-activated cell sorting (FACS) analysis to isolate cells in different cell cycle stages was optimized and done as described previously (11).

Homologous recombination and nonhomologous end-joining assays. Measurement of the repair of an I-*Sce*I-generated DSB using the DR-GFP system has been described previously (23). Briefly, after genomic

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[7] http://bio.cs.washington.edu/software.html.

integration of the DR-GFP plasmid by stable transfection into MCF-7 cells, 50 μg of the I-*Sce*I expression vector pCBASce or an empty vector were transfected by electroporation using a BTX ECM 830 square wave electroporator (Holliston, MA). Cells were then exposed to 48 hours of normoxia or hypoxia followed by an additional 24 hours of incubation (i.e., recovery period) in normoxia. To measure homologous recombination events, percentages of green fluorescent protein (GFP)–positive cells were quantitated by flow cytometric analysis of trypsinized live cells. Specifically, homologous recombination events were calculated from plots of FL-1 (GFP) and FL-3 (autofluorescence) on the ordinate and abscissa, respectively, with a gate set to include 0.10% GFP$^+$ cells electroporated with an empty vector as background. Assessment of NHEJ activities were done as described in the Supplementary Information.

Results

Previous work by our group has shown that hypoxia induces mutagenesis and promotes genetic instability in mammalian cells (8, 11, 16). To further elucidate the mechanism for these effects, we examined patterns of DNA repair gene expression under hypoxia by transcriptome profiling (11). This analysis suggested that *BRCA1* gene expression may be decreased in response to hypoxic stress (data not shown). To follow up on this observation, we did Northern blot analyses, which revealed substantial decreases in *BRCA1* mRNA expression in MCF7 and A549 cells following exposure to hypoxia (0.01% O_2, 48 hours; Fig. 1*A*), and these decreases were also manifested at the protein level (Fig. 1*B*). Interestingly, the BRCA1 protein was also observed to migrate at a slower rate in samples from cells exposed to hypoxia compared with normoxia, which suggests post-translational modification (such as phosphorylation) under these conditions. Indeed, preliminary studies in our laboratory suggest that this protein is hyperphosphorylated in response to hypoxia (data not shown).

Decreases in *BRCA1* expression were also observed in numerous other human cell lines derived from a wide range of tissues using quantitative PCR and Northern blotting, including RKO, CaCo-2, PC3, DU145, and 5757 cells (data not shown). Additionally, exposure to the hypoxia-mimetic, desferrioxamine, induced decreases in *BRCA1* expression in MCF7 (Fig. 1*A*), A549, PC3, and DU145 cells (data not shown), and down-regulated *BRCA1* expression was also observed in response to moderate hypoxia (0.5-1% O_2; data not shown). Interestingly, decreased *BRCA1* levels were seen to persist for at least 48 hours following reoxygenation (Fig. S1*A*), at time points at which we have confirmed that these cells are actively proliferating in the S phase (11).

Analysis of the *BRCA1* promoter in the context of a luciferase reporter construct revealed that a 250-bp region from the proximal promoter mediates repression by hypoxia (∼10-fold; Fig. 1*C*). This region was identified by comparison of a series of nested *BRCA1* promoter luciferase constructs (data not shown). The activity of a hypoxia-inducible promoter (5X-HRE) increased ∼40-fold under the same conditions and served as a control to confirm physiologically relevant levels of hypoxia. The promoter of the *BRCA1* gene is bidirectional, as it is shared with the adjacent and oppositely transcribed gene, *NBR2* (24). Because these two genes are separated by ∼250 bp, it seemed likely that they might share similar transcription factor binding sites and that they may be coregulated (24, 25). We thus sought to determine whether *NBR2* gene expression was also repressed by hypoxia. Intriguingly, quantitative PCR analyses revealed that *NBR2* expression in MCF7 cells was unaffected by hypoxia (Fig. 1*D*); in the same samples, endogenous *BRCA1* expression was found to be reduced an average of 4-fold (Fig. 1*D*). In several other cell lines, we actually

Figure 1. Repression of *BRCA1* gene expression in hypoxia. *A,* Northern blot analysis of *BRCA1* mRNA expression in MCF-7 and A549 cells in response to normoxia (*N*) and hypoxia (*H*; 0.01% O₂, 48 hours; *left*) or to the hypoxia-mimetic, desferrioxamine (*DFX*; 250 μmol/L, 24 h; *right*). *VEGF* expression is shown to verify that physiologically relevant levels of hypoxia were present in the treated cells, and *18S* rRNA expression is presented to confirm equal sample loading. *B,* Western blot analysis of total amounts of BRCA1 protein in A549, RKO, and MCF7 cells in normoxia and hypoxia. MSH6 protein levels are unaffected by hypoxia (16) and served as a loading control. *C,* repression of *BRCA1* promoter activity in hypoxia. *Firefly* luciferase activity (normalized to total protein) representing *BRCA1* promoter activity in normoxia and hypoxia following transient transfection of RKO cells with pBRC-FF (*E*). The activities of a luciferase control vector driven by an SV40 promoter (*SV40*), a hypoxia-inducible promoter (*5X-HRE*), and a promoterless control plasmid (*pGL3*) as controls for specificity and physiologically relevant levels of hypoxia. *Columns,* means (*P*) from quadruplicate experiments; *bars,* SE. *D,* quantitative PCR analysis of endogenous *BRCA1*, *NBR2*, and *VEGF* mRNA expression in normoxic and hypoxic A549 cells, normalized to *18S* rRNA expression, and expressed as fold changes relative to normoxia (*H/N*) for each transcript. *Columns,* means from quadruplicate assays; *bars,* SE. *E,* schematic of the unidirectional and bidirectional *BRCA1* promoter constructs (pBRC-FF and pBRC-FF/RL, respectively) encompassing the *NBR2/BRCA1* promoter region used in the luciferase reporter gene assays. Nucleotide positions relative to the start of the first (untranslated) exon of the *BRCA1* gene. *Arrows,* ATG translation start codon of each luciferase gene. *F,* normalized *Renilla* and *firefly* luciferase activity representing *NBR2* and *BRCA1* promoter activity, respectively, in normoxia and hypoxia following transient transfection of MCF7 cells with pBRC-FF/RL, measured as in (*C*).
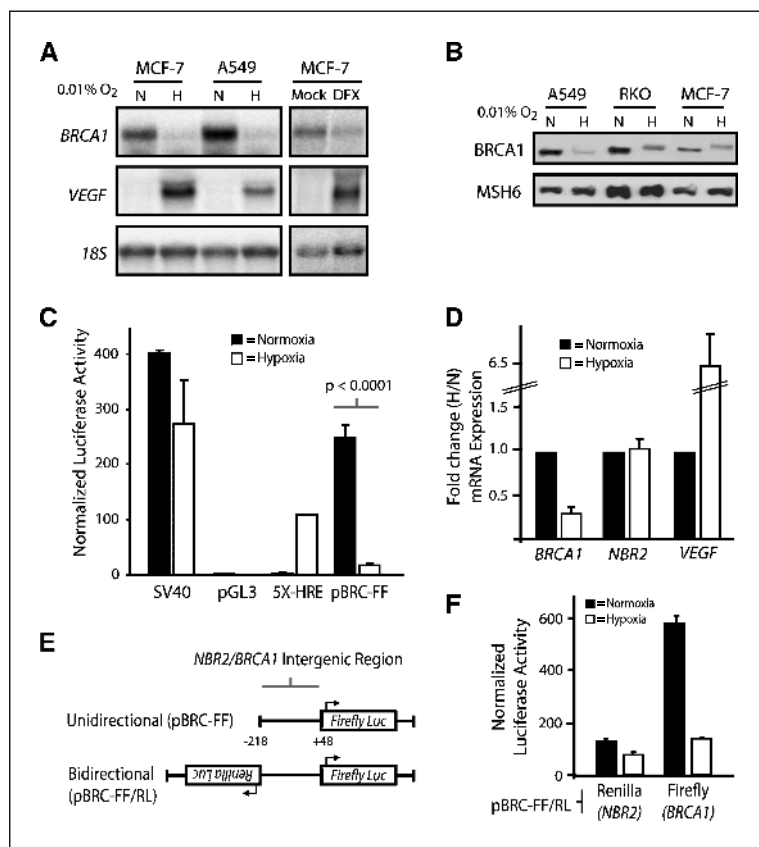
observed slight increases in *NBR2* mRNA expression after hypoxic exposure (Fig. 4*D*; data not shown).

To model the effect of hypoxia on *BRCA1* and *NBR2* promoter activity simultaneously, we constructed a bidirectional reporter construct containing the *NBR2/BRCA1* intergenic region driving the expression of *Renilla* and *firefly* luciferase genes in opposing orientations (schematic shown in Fig. 1*E*). Consistent with the mRNA expression patterns, *BRCA1* promoter activity was substantially repressed by hypoxia (4.2-fold), whereas *NBR2* promoter activity was minimally affected as measured using this bidirectional promoter construct in MCF7 cells (Fig. 1*F*). *NBR2* promoter activity was substantially lower than *BRCA1* promoter activity in normoxia, which is consistent with previous studies of *NBR2* gene expression (24, 25). Collectively, these data suggest that hypoxia can specifically down-regulate *BRCA1* expression via a mechanism involving unidirectional repression in the context of the bidirectional promoter.

Multispecies alignment of the proximal *BRCA1* promoter region revealed a 40-bp region of strong conservation (Fig. 2*A*), which includes a previously identified E2F consensus binding site (26) on the negative strand (relative to the *BRCA1* gene, designated E2FB). Further inspection of this region revealed a novel, conserved E2F site immediately adjacent to E2FB (designated E2FA). The E2F family of transcription factors can be subdivided into activating (E2F1-3a) and repressive (E2F3b-5; 6–8) proteins, and the transcriptional activities of these factors are dependent on their interactions with members of the Dp family and the repressive pocket proteins Rb, p130, and p107 (20, 27–29). Scattered reports have also implicated E2Fs in the regulation of *BRCA1* gene expression in selected mouse and human cell lines (22, 26, 30, 31). Coupled with the emerging role of E2F1 in DNA damage response pathways, we thus sought to determine whether E2Fs play a role

in the regulation of *BRCA1* expression by hypoxia. Mutation of either or both of these sites in the *BRCA1* promoter construct substantially attenuated repression of *BRCA1* promoter activity by hypoxia in MCF7 (Fig. 2*B*) and RKO cells (data not shown). In contrast, mutation of a conserved CCAAT box located upstream from the two E2F sites in the *BRCA1* promoter (shown in Fig. 2*A*) resulted in an ~35% decrease in promoter activity in normoxia in both cell lines (data not shown) but had no effect on the magnitude of repression by hypoxia (Fig. 2*B*). These data suggest that both the E2FA and E2FB sites specifically play a role in the regulation of *BRCA1* expression by hypoxia.

We next sought to determine whether the hypoxia-mediated repression of *BRCA1* could be attributed to cell cycle profile changes. However, decreases in *BRCA1* mRNA expression were observed both in G₁ phase– and S phase–specific cell populations from normoxic versus hypoxic A549 cells isolated by FACS as measured by quantitative PCR (Fig. S1*B*). These data suggest that the observed phenomenon is hypoxia specific rather than cell cycle phase dependent.

Previous reports have suggested that *BRCA1* expression may potentially be affected by the transient overexpression of HIFs in selected cell lines (32, 33). To address this possibility, expression vectors encoding WT or proteolysis-resistant HIF-1α proteins were transiently transfected into HeLa and A549 cells, and total RNA was prepared from these cells after a 48-hour incubation in normoxia followed by Northern blot analysis. No effect of the HIF-1α expression vectors on *BRCA1* expression was seen (Fig. S2*A*), and similar results were obtained in RKO and MCF7 cells (data not shown). High levels of mutant HIF protein expression were confirmed in HeLa cells by Western blot analysis (Fig. S2*B*), and the cotransfection of these HIF-1α vectors with the 5X-HRE vector
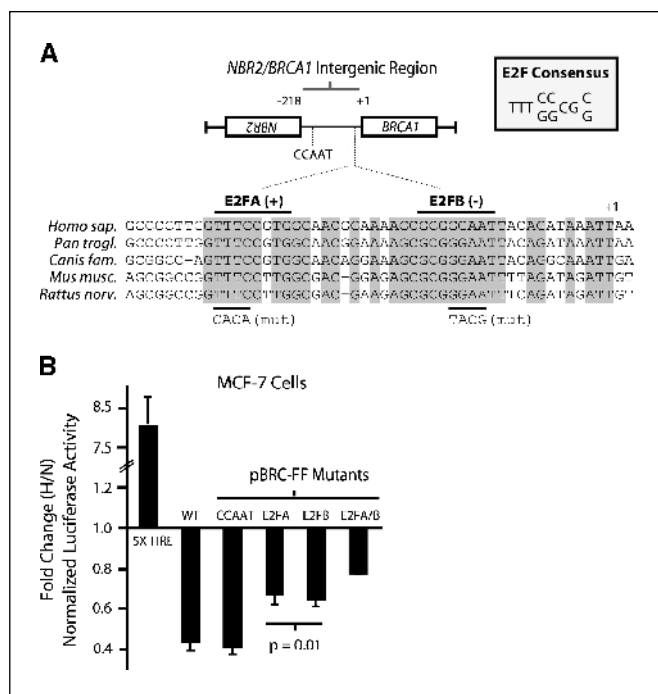
Figure 2. Repression of *BRCA1* expression by hypoxia is mediated by two E2F binding sites in the proximal *BRCA1* promoter. *A*, ClustalW multispecies alignment of the *NBR2/BRCA1* intergenic region using human (*Homo sap.*), chimpanzee (*Pan trogl.*), dog (*Canis fam.*), mouse (*Mus musc.*), and rat (*Rattus norv.*). A schematic of the *BRCA1* proximal promoter region with the approximate locations of the three highly conserved sites: CCAAT, E2FA, and E2FB. Nucleotide positions relative to the *BRCA1* gene as in Fig. 1*E. Top right,* E2F site consensus sequence (27). *Shaded areas,* 100% cross-species conservation; *top of alignment,* novel E2FA and previously identified E2FB (26) sites. *B,* effect of CCAAT, E2FA, and E2FB site mutation in the pBRC-FF construct on the repression of *BRCA1* promoter activity by hypoxia in MCF7 cells. E2FA/B contains both E2FA and E2FB site mutations. Fold change in normalized luciferase activity in hypoxia compared with normoxia (*H/N*) for each construct. *Columns,* means from four to eight pairs of N/H replicates; *bars,* SE. *P*s for the differences in H/N fold changes between WT and E2FA or E2FB.

described earlier resulted in a 50- to 200- fold increase in luciferase activity (Fig. S2*C*), whereas cotransfection of the HIF-1α vectors with *BRCA1* promoter luciferase constructs did not repress luciferase activity (data not shown). In addition, quantitative PCR analyses of mRNA expression in HeLa cells following transfection with the HIF-1α proline mutant vector further confirmed that overexpression of this protein can induce the expression of endogenous HIF-target genes, including *DEC1* (34), but does not seem to affect endogenous *BRCA1* expression (Fig. S2*D*). Finally, as an alternative approach to study the role of HIFs in the regulation of *BRCA1* expression, we assessed *BRCA1* expression by Northern blot analysis in VHL-mutant 786-0 cells either complemented with the VHL WT cDNA or with an empty vector. As the VHL protein targets multiple HIFs for ubiquitin-mediated proteolysis, VHL mutant cells overexpress HIF-2α (these cells do not express HIF-1α), and the expression of the VHL cDNA restores the normoxic regulation of HIF-2α (35). We found that *BRCA1* expression was not affected by VHL status or by HIF-2α expression (Fig. S2*E*), which suggests that HIF-2α does not play a critical role in the regulation of *BRCA1* expression. Taken together, these data suggest that the repression of *BRCA1* expression by hypoxia does not involve HIF-1α or HIF-2α proteins in the cell lines evaluated in the present study.

Based on the data presented above, we hypothesized that repression of *BRCA1* expression by hypoxia might be mediated by

either decreased binding of activating E2Fs or increased binding of repressive E2Fs at the *BRCA1* promoter in response to hypoxia. To test these hypotheses, we used the technique of quantitative chromatin immunoprecipitation to assess *BRCA1* promoter occupancy by activating and repressive E2Fs, as well as by associated pocket proteins, *in vivo*. Remarkably, hypoxia caused significant decreases in E2F1 occupancy and increases in occupancy by E2F4, p130, and p107 at the proximal *BRCA1* promoter in MCF7 cells (Fig. 3*A*). Relative promoter occupancies by each factor in normoxic and hypoxic cells are shown in detail in Fig. S3*D*. Significant increases in promoter occupancy by both E2F4 and p130 were also induced by hypoxia in other cell lines, including RKO (Fig. 3*B*). We detected minimal or no occupancy by other repressors such as E2F6, Rb, and HDAC1 at the *BRCA1* promoter under our conditions in these cell lines (Fig. 3*A* and Fig. S3*D*). *BRCA1* promoter occupancy by E2F4 and pocket proteins was detected to some degree even in normoxic cells (although these levels significantly increased in hypoxia; Fig. 3*A* and Fig. S3*D*), suggesting that these factors may play a role in the control of *BRCA1* expression even in normoxic cells. E2F binding was not detected by chromatin immunoprecipitation analysis at regions upstream or downstream from the proximal promoter region of *BRCA1* (Fig. S3*A* and *B*), indicating that the binding of these factors can be localized to the region containing the consensus sites described earlier. Importantly, total cellular levels of E2F1 were minimally affected by hypoxia, and slight decreases in E2F4 and E2F6 protein levels were observed in hypoxia in most of the cell lines examined (Fig. S1*C*). Hence, the observed alterations in promoter occupancy by these factors cannot be explained by changes in overall protein abundance.

Current models of E2F target gene regulation suggest that promoter occupancy by activating and repressive E2Fs may be mutually exclusive and influenced by either cell cycle phase or co-occupancy by other transcription factors (27, 29). However, our observation that both E2F1 and E2F4 bind the *BRCA1* promoter in normoxia, coupled with our delineation of the two adjacent E2F binding sites in the proximal promoter region, led us to hypothesize that these two factors might bind the *BRCA1* promoter *simultaneously*. To test this possibility, we used the technique of sequential chromatin immunoprecipitation (36). In sequential chromatin immunoprecipitation, a primary chromatin immunoprecipitation is first done with an antibody to a given transcription factor (e.g., E2F1), the immunoprecipitated protein-DNA complexes are washed and eluted and then subjected to a second immunoprecipitation with an antibody of a different specificity (e.g., E2F4) followed by quantitative PCR analysis with primers to the genomic region of interest (36). This methodology identifies genomic DNA fragments to which both the primary and secondary target proteins have been simultaneously cross-linked. Using this approach, we found evidence for co-occupancy by E2F1 and E2F4 at the *BRCA1* promoter in both RKO and MCF7 cells (Fig. 3*C* and *D*). Co-occupancy was detected both when E2F1 was used as the primary antibody and E2F4 as the secondary antibody and vice versa (Fig. 3*C*). These data provide strong evidence that E2F1 and E2F4 can bind the *BRCA1* promoter simultaneously *in vivo*, a phenomenon that has not been reported previously.

We next sought to further elucidate the importance of the E2F sites in the regulation of *BRCA1* promoter activity using a transient chromatin immunoprecipitation approach. In these assays, RKO and MCF7 cells were transiently transfected with WT or mutant *BRCA1* promoter luciferase constructs followed by a 24- to 48-hour

incubation (in normoxia). In addition, chromatin immunoprecipitation assays were then done as described earlier, except that quantitative PCR was done with primers specific to the promoter luciferase vectors rather than the endogenous promoter sequences (schematic shown in Fig. 4*A*). This technique has emerged recently as an alternative, *in vivo* approach to study the effects of mutating a particular DNA element on transcription factor binding (22). We detected substantial intracellular E2F4 binding to the WT *BRCA1* promoter construct in both RKO and MCF7 cells, whereas we found that mutation of either or both E2F sites led to a marked decrease in E2F4 binding to the respective mutant promoter constructs (Fig. 4*B*; quantified in Fig. 4*C*). Binding of E2F4 to the endogenous *BRCA1* promoter was examined for comparison, and binding to the 3′-end of the *DHFR* gene was used as a negative control in these experiments. Luciferase activity was also assayed from cells transfected with each promoter construct at the same time point(s) to correlate E2F4 binding (as measured by transient chromatin immunoprecipitation) with promoter activity. Intriguingly, we observed a consistent correlation of increased *BRCA1* promoter activity with decreased E2F4 binding among the WT and E2F mutant constructs in MCF7 cells (Fig. 4*C*). Collectively, these

data suggest that endogenous E2F4 can bind either E2F site within the *BRCA1* promoter *in vivo*, and that this binding is associated with the repression of *BRCA1* promoter activity.

E2F transcription factors bind to DNA as a heterodimer with Dp proteins, and dominant-negative Dp mutants have been developed which can specifically disrupt E2F transcriptional activity (37). Exogenous overexpression of one such mutant Dp protein, Dp1(Δ103-126), was found to substantially reduce WT *BRCA1* promoter activity in normoxia, and this reduction was either completely or partially abolished in the E2FA and E2FB *BRCA1* promoter mutants, respectively (Fig. S1*D*). Exogenous overexpression of E2F4 protein along with p130, on the other hand, was found to substantially repress WT *BRCA1* promoter activity. Consistent with our above analysis of the promoter, this repression was either completely or partially abolished by mutation of the E2FA and E2FB sites, respectively (data not shown). These data suggest that activating E2F complexes mediate baseline induction of *BRCA1* promoter activity in normoxia, because this activation is disrupted by forced expression of a dominant-negative Dp1 protein. Second, this basal activity can be repressed by forced overexpression of E2F4 and p130. In addition, the effects of dominant-negative Dp1
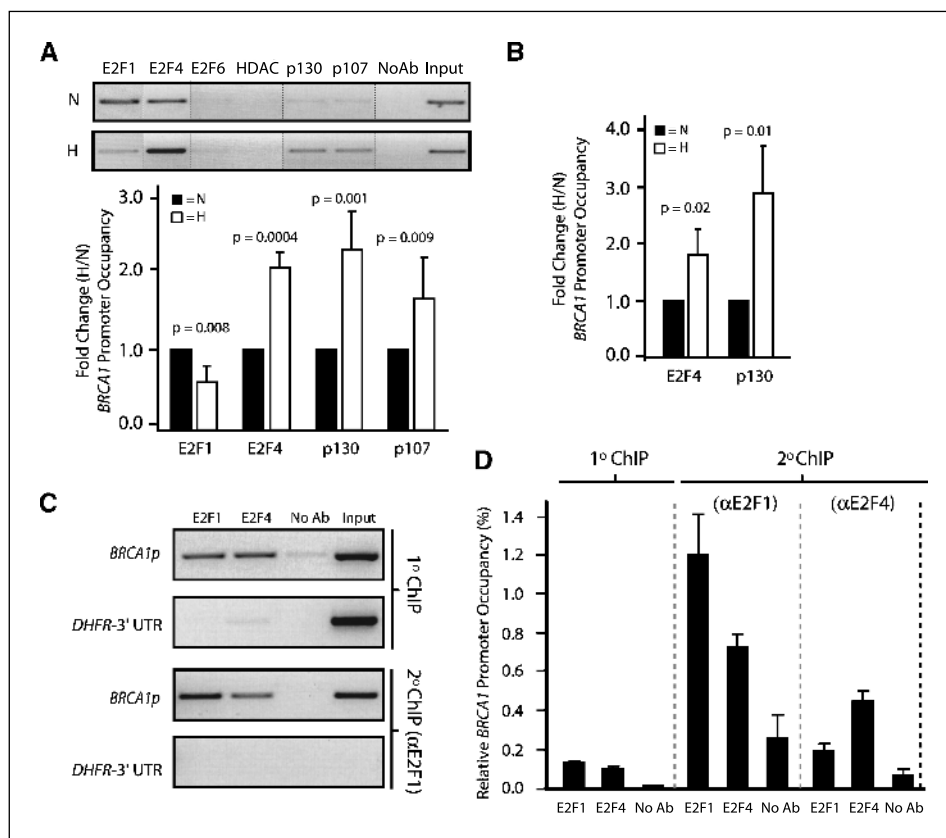
Figure 3. *In vivo* analysis of *BRCA1* promoter occupancy by E2Fs and pocket proteins in normoxic and hypoxic cells. *A,* chromatin immunoprecipitation (*ChIP*) assays were done in MCF7 cells following a 48-hour exposure to normoxia (*N*) or hypoxia (*H*) with antibodies to the indicated proteins to determine *BRCA1* proximal promoter occupancy by these factors. *Top,* representative agarose gel containing *BRCA1* amplification products obtained by PCR with several chromatin immunoprecipitation assay samples. *Dashed lines,* separate lanes from the same agarose gel. *BRCA1* promoter occupancy is expressed as the fold change relative to that observed in normoxia (*H/N*). *Bottom,* quantification based on six independent chromatin immunoprecipitation assays. *Columns,* means from a total of six pairs of H/N replicates; *bars,* SE. *P*s were calculated based on the difference in promoter occupancy in normoxia and hypoxia for each transcription factor. *B,* the analyses discussed in (*A*) were extended to RKO cells with similar results. *Columns,* means from four pairs of H/N replicates; *bars,* SE. *C,* sequential chromatin immunoprecipitation analysis was done to determine co-occupancy at the *BRCA1* promoter by E2F1 and E2F4 in RKO cells. Chromatin immunoprecipitation analysis was first done as described in (*A*) with antibodies specific to either E2F1 or E2F4 (*1°ChIP*), and separate aliquots from these immunoprecipitates were then each subjected to a second immunoprecipitation (*2°ChIP*) with either E2F1 or E2F4 antibodies. As a negative control, PCR was done with primers specific to the 3′-UTR of the *DHFR* gene, a region that does not bind E2Fs (50). *D,* quantitative PCR analysis of sequential chromatin immunoprecipitation assays. Relative *BRCA1* promoter occupancy (%) is shown as calculated in Fig. S2*C*. The input signal is set as 100% (not depicted in graph) for each set of assays (*1* and *2ChIPs*). *Columns,* means from duplicate sequential chromatin immunoprecipitation assays; *bars,* SE.
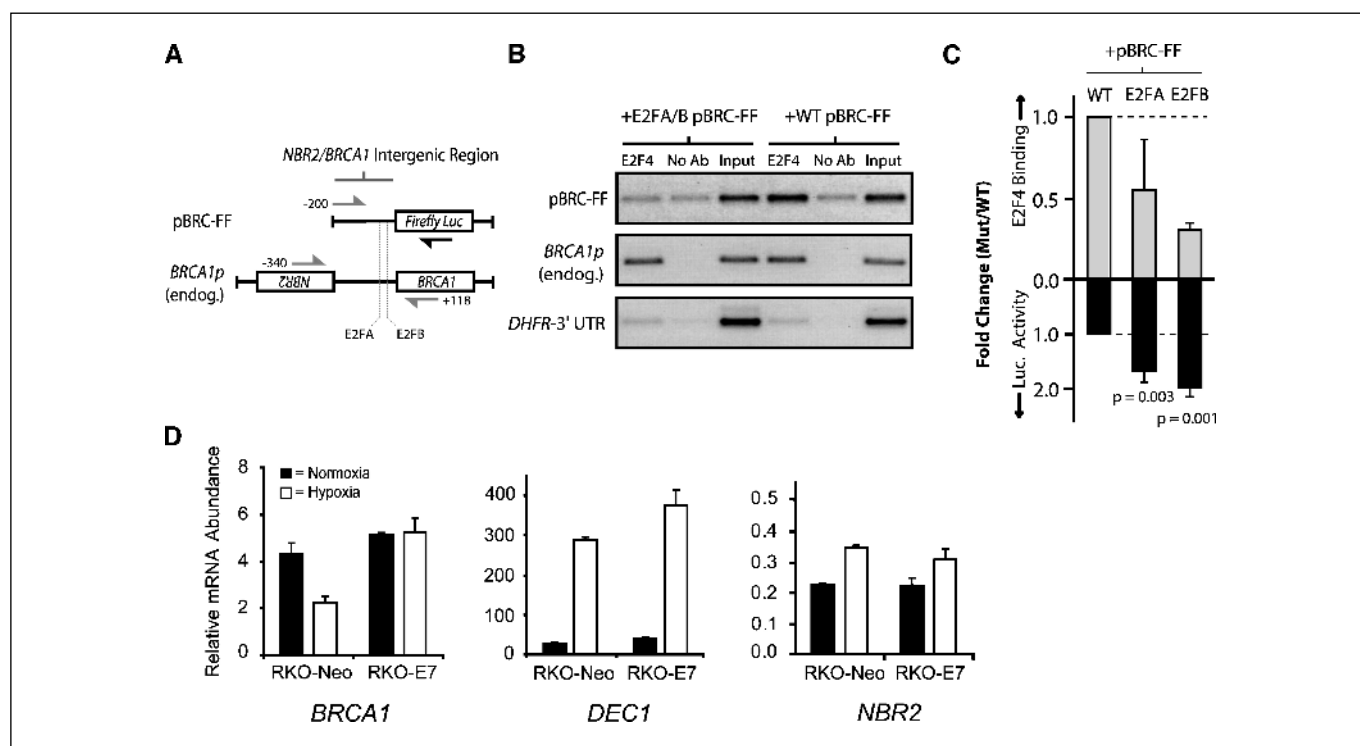
Figure 4. Elucidation of the role of E2F4 binding in the regulation of *BRCA1* gene expression. *A,* E2F4 binding to the proximal *BRCA1* promoter. The schematic depicts the locations of primers used to amplify either the promoter region contained within the pBRC-FF constructs or the endogenous *BRCA1* proximal promoter (*BRCA1p*) in the transient chromatin immunoprecipitation assays shown in (*B*) and (*C*), and in the endogenous chromatin immunoprecipitation assays shown in Fig. 3*A-E*. Nucleotide positions as in Fig. 1*E*, and the approximate locations of the E2F sites are shown for reference. *B,* transient chromatin immunoprecipitation analysis of intracellular E2F4 binding to transiently transfected pBRC-FF WT or E2FA/B mutant promoter constructs in RKO cells. Chromatin immunoprecipitation assays were done essentially as in Fig. 3*A* using primers specific to either pBRC-FF or the endogenous *BRCA1* promoter as depicted in (*A*). Binding to the 3′-UTR of the *DHFR* gene served as negative control as in Fig. 3*C*. *C,* correlation between endogenous E2F4 binding to pBRC-FF constructs (WT, E2FA, and E2FB; as calculated by quantitative PCR analysis) and luciferase activity associated with each promoter construct from plates assayed in parallel with the transient chromatin immunoprecipitation assays in MCF7 cells. Both E2F4 binding and luciferase activity associated with the mutant constructs are shown as the fold change relative to that obtained with WT pBRC-FF (*Mut/WT*). *Dashed lines,* no change are shown for reference. *Columns,* mean chromatin immunoprecipitation and luciferase values from duplicate transient chromatin immunoprecipitation assays and six individual replicates, respectively; *bars,* SE. *P*s were calculated relative to WT pBRC-FF luciferase activity. *D,* effect of HPV E7 expression on *BRCA1* repression by hypoxia. Quantitative PCR analysis of *BRCA1*, *DEC1*, and *NBR2* mRNA expression in RKO-E7 and RKO-Neo cells following exposure to normoxia or hypoxia (48 hours, 0.01% O_2), normalized to *18S* rRNA expression. *Columns,* means from duplicate experiments with two separate RKO-E7 and RKO-Neo clones; *bars,* SE.

expression and of E2F4/p130 overexpression are attenuated in promoter constructs with either E2FA or E2FB site mutations, indicating that both E2F sites are required to mediate regulation by the respective E2Fs. Coupled with the *in vivo* analyses of *BRCA1* promoter occupancy, these findings suggest that repression of *BRCA1* expression by hypoxia is mediated by a dynamic shift from activating E2F1 to repressive E2F4 complexes at the *BRCA1* promoter.

To further study the role of repressive E2F complexes in the repression of *BRCA1* by hypoxia, we examined *BRCA1* mRNA expression following exposure to normoxia or hypoxia in RKO cell lines stably transfected with an HPV E7 cDNA (RKO-E7) or an empty vector (RKO-Neo). The HPV E7 oncoprotein inactivates pocket protein function both by disrupting the formation of E2F/pocket protein complexes and also by targeting pocket proteins for degradation (38). Importantly, E2F4 is a weak repressor in the absence of pocket protein binding; thus, E7 can be used to effectively inactivate the repressive functions of this factor (27). As shown in Fig. 4*D*, whereas hypoxia induced substantial decreases in *BRCA1* mRNA expression levels in RKO-Neo cells, no such decreases were observed in RKO-E7 cells. In contrast, *DEC1* expression was significantly induced by hypoxia in both cell lines, and *NBR2* expression was slightly induced in both cases. Hence, these genes were not affected by E7 expression. Collectively, these

data suggest that the repression of *BRCA1* expression by hypoxia is specifically mediated by E2F4 and pocket proteins.

We next sought to determine whether hypoxia-mediated decreases in BRCA1 expression were associated with functional changes in homologous recombination repair using an intrachromosomally based DSB repair assay (23). In this system, a DSB is generated by expressing the I-*Sce*I endonuclease, whose 18-bp recognition site has been integrated into the *GFP* gene such that it disrupts the gene. Repair of the cleaved I-*Sce*I site by homologous recombination gives rise to a functional *GFP* gene when the template used for repair is a truncated *GFP* fragment located downstream in the plasmid, and homologous recombination activity is measured by flow cytometric analysis of the number of GFP+ cells following *Sce*I expression. Homologous recombination activity was found to be substantially reduced following *Sce*I expression in hypoxic compared with normoxic MCF7 cells containing the chromosomally integrated repair substrate (0.53% versus 4.3%, respectively; Fig. 5*A*), and these differences persisted for up to 96 hours after hypoxia (data not shown).

In contrast, we did not detect any effect of hypoxia on the NHEJ pathway of DSB repair. First, the expression patterns of several key NHEJ proteins were not altered by hypoxia (Fig. 5*B*). To assess NHEJ activity in normoxia versus hypoxia, we used a luciferase

plasmid religation assay. In this system, restriction endonuclease digestion of pGL3 with *Hin*dIII or *Nco*I results in cleavage at the linker region between the promoter and luciferase coding sequence or at the ATG start codon, respectively. End-joining activity resulting in religation of the linearized plasmid is detected as luciferase activity following transient transfection into cells. In this manner, the assay permits the study of both overall and precise end-joining activity when *Hin*dIII and *Nco*I, respectively, are used to linearize the vector. The validation and use of this protocol to assess NHEJ activity has been described previously (39). RKO cells were transiently transfected with either linearized or uncut vector 6 hours before normoxic or hypoxic exposure (48 hours) immediately followed by measurement of luciferase activity. Intriguingly, no statistically significant differences in NHEJ activity were observed between normoxia and hypoxia (Fig. 5*C*). Collectively, these data suggest that homologous recombination activity is suppressed, whereas the NHEJ pathway may remain undiminished in hypoxic cells.

Discussion

We have shown here that hypoxia induces the down-regulation of *BRCA1* expression, a critical tumor suppressor gene involved in numerous cellular pathways, including DNA repair, cell cycle checkpoint control, and transcriptional regulation (1). This down-regulation occurs via a dynamic redistribution of E2Fs and pocket proteins at the *BRCA1* promoter and is associated with a functional decrease in homologous recombination activity in hypoxic cells.

It has been proposed that the BRCA1 protein functions as a caretaker of genomic integrity through its role in repairing DNA DSBs, instead of by directly inhibiting cell growth (1, 40). Specifically, recent studies have suggested that *BRCA1* functions as a tumor suppressor primarily through its role in promoting high-fidelity homologous recombination while simultaneously suppressing the error-prone NHEJ pathway (1, 41). Based on our findings that hypoxia suppresses homologous recombination activity, although NHEJ seems unaffected under these conditions, we propose a novel mechanism of hypoxia-induced genetic instability involving inappropriate shunting of DSBs from the homologous recombination repair pathway to the NHEJ pathway under these conditions.

In the regulation of *BRCA1* expression, the data indicate that transcriptional control by E2Fs is mediated by two adjacent, conserved E2F sites in the proximal *BRCA1* promoter region. Although recent studies have shown that different E2F family members can bind to distinct promoter elements independently, our results show that E2F1 and E2F4 can bind the *BRCA1* promoter simultaneously *in vivo*. Furthermore, our findings suggest that hypoxic stress can induce a shift favoring a higher proportion of occupancy at the two E2F sites in the *BRCA1* promoter by repressive E2F4 complexes versus activating E2Fs. Taken together, these data thus expand our understanding of how members of the E2F family of transcription factors can regulate gene expression, as they suggest that multiple E2Fs can exert combinatorial regulation of gene expression in response to cell stress.

As discussed earlier, we have found that E2F1 binding to the *BRCA1* promoter is decreased in hypoxia without a change in total E2F1 protein levels. Recent studies suggest that E2F1 promoter occupancy is regulated by post-translational modifications, including acetylation and phosphorylation, in response to various genotoxic stresses (17–19, 42, 43). It is thus possible that decreased occupancy of the *BRCA1* promoter by E2F1 in hypoxia is caused by post-translational modification(s) of E2F1 under these conditions. Future studies directed at studying whether such modifications occur under these

conditions likely will provide further insight into the mechanism(s) by which hypoxia affects E2F1 target gene specificity.

Emerging evidence indicates that E2F4 may play an oncogenic rather than a tumor suppressor role in cells (28, 44, 45), and recent studies have implicated a role for this factor in both genotoxic stress response and in apoptotic pathways (46, 47). Our discovery that dynamic changes in promoter occupancy by E2F1 and E2F4 play a role in the cell cycle–independent repression of *BRCA1* by hypoxia supports the concept of an expanded role for E2Fs in the regulation of DNA repair beyond their well-established roles in cell cycle progression and the maintenance of quiescence, and it provides the basis for future studies to elucidate the distinct roles of E2Fs in the transcriptome response to hypoxia (48).
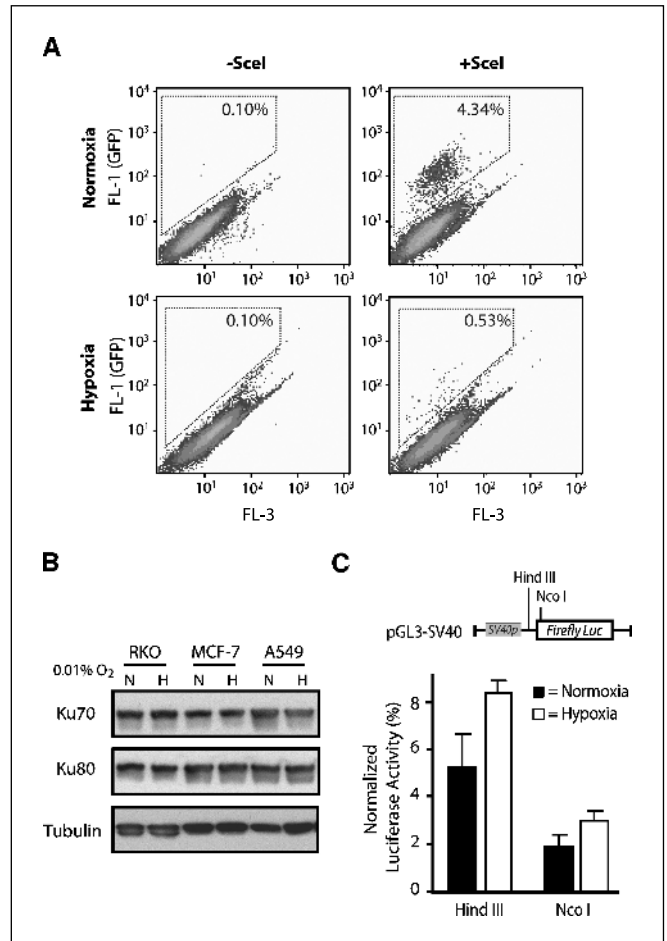
Figure 5. Analysis of homologous recombination and NHEJ pathways of DSB repair in hypoxia. *A,* impaired homologous recombination repair following hypoxic exposure, as detected using the DR-GFP recombination substrate. MCF7 cells containing the DR-GFP substrate were transiently transfected either with an I-*Sce*I expression vector (+*Sce*I) to induce a site-specific DSB, or with an empty vector (−*Sce*I) as a control. Cells were then exposed to 48 hours of normoxia or hypoxia followed by an additional 24 hours of incubation in normoxia (i.e., recovery period) before flow cytometric analysis of duplicate samples. Percentages of GFP⁺ cells based on duplicate experiments. *B,* Western blotting was done to analyze total amounts of the NHEJ proteins Ku70 and Ku80 in RKO, MCF7, and A549 cells in normoxia and hypoxia. Tubulin protein levels served as a loading control as in Fig. 3*C*. *C,* functional changes in NHEJ in hypoxia were assessed using a plasmid end-joining assay. RKO cells were transiently transfected with either linearized or uncut vector 6 hours before normoxic or hypoxic exposure (48 hours) immediately followed by measurement of luciferase activity. Luciferase activity in the cleaved samples was expressed as a percentage of the activity obtained following transfection with undigested plasmid (each first normalized to total protein). *Columns,* means from three independent assays each done in triplicate; *bars,* SE.

Intriguingly, we have found substantial homology between the proximal promoter of *BRCA1* and another homologous recombination–associated gene, *RAD51*, which encompasses the E2F sites shown in Fig. 2*A*. As we have previously shown that this gene is also repressed by hypoxia (11), it is thus possible that key genes within the homologous recombination pathway may be coregulated by E2Fs in response to hypoxic stress. In addition, further examination of target gene promoter occupancy by E2Fs in the context of other cell stresses such as ionizing radiation, acidosis, or following exposure to DNA damaging agents, likely will provide further insight into the emerging role of both activating and repressive E2Fs in stress response pathways.

Repression of *BRCA1* expression, in particular, by hypoxia represents an intriguing mechanism of functional *BRCA1* inactivation in the absence of genetic mutation. As hypoxia is a common feature of solid tumors, it is tempting to speculate that hypoxia-mediated repression of *BRCA1* expression could partially explain the observations of decreased BRCA1 expression in many sporadic cancers. In addition, in light of recent publications reporting that *BRCA1*-deficient cells are hypersensitive to poly(ADP-ribose) polymerase inhibitors (49), our findings suggest that such inhibitors may be particularly effective against hypoxic cancer cells in which *BRCA1* expression is downregulated.

Acknowledgments

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