

Hypoxia-Induced Epigenetic Regulation and Silencing of the *BRCA1* Promoter[∇]

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Disruption of the *BRCA1* tumor suppressor can be caused not only by inherited mutations in familial cancers but also by *BRCA1* gene silencing in sporadic cancers. Hypoxia, a key feature of the tumor microenvironment, has been shown to downregulate *BRCA1* at the transcriptional level via repressive E2F4/p130 complexes. Here we showed that hypoxia also drives epigenetic modification of the *BRCA1* promoter, with decreased H3K4 methylation as a key repressive modification produced by the lysine-specific histone demethylase LSD1. We also observed increased H3K9 methylation coupled with decreased H3K9 acetylation. Similar modifications were seen in the *RAD51* promoter, which is also downregulated by hypoxia, whereas exactly opposite changes were seen in the promoter of the hypoxia-inducible gene *VEGF*. In cells containing the *BRCA1* promoter driving a selectable *HPRT* gene, long-term silencing of the promoter was observed following exposure to hypoxic stress. Clones with silenced *BRCA1* promoters were detected at frequencies of 2% or more following hypoxia, but at less than 6×10^{-5} without hypoxia. The silenced clones showed decreased H3K4 methylation and decreased H3K9 acetylation in the *BRCA1* promoters, consistent with the acute effects of hypoxic stress. Hypoxia-induced *BRCA1* promoter silencing persisted in subsequent normoxic conditions but could be reversed by treatment with a histone deacetylase (HDAC) inhibitor but not with a DNA methylation inhibitor. Interestingly, treatment of cells with inhibitors of poly(ADP-ribose) polymerase (PARP) can cause short-term repression of *BRCA1* expression, but such treatment does not produce H3K4 or H3K9 histone modification or *BRCA1* promoter silencing. These results suggest that hypoxia is a driving force for long-term silencing of *BRCA1*, thereby promoting genome instability and tumor progression.

Solid tumors constitute a unique tissue type, characterized by hypoxia, low pH, and nutrient deprivation. Previous work has shown that hypoxic stress is a source of genetic instability in tumors (3, 5, 7, 40, 58), causing increased point mutations (40), gene amplification (11, 58), and fragile-site induction (12). Our previous work revealed that *BRCA1* and *RAD51*, key genes in the homology-dependent repair (HDR) pathway, and *MLH1*, a key DNA mismatch (MMR) repair gene, are downregulated at the mRNA and protein levels in response to hypoxia via specific pathways of transcriptional regulation (3–5, 7). Moreover, *BRCA1* and *MLH1* have been found to be silenced in many sporadic cancers of multiple sites (8, 14, 16). The silencing of *BRCA1* and *MLH1* has been attributed primarily to promoter DNA hypermethylation at CpG sites (14). However, recent studies suggest that silenced promoters in cancer cells are also marked by characteristic histone modifications (9, 33, 48), and evidence is emerging that histone methylation may be a mediator of silencing that is independent of DNA methylation (26, 29, 30).

Posttranslational modification of histones is widely recognized as an important epigenetic mechanism in the organization of chromosomal domains and in gene regulation (31, 32, 36, 39). Methylation of lysine 4 and acetylation of lysine 9 of H3 have been associated with regions of active transcription,

whereas methylation of H3K9 and methylation of H3K27 are generally associated with gene repression (31, 32, 36, 39, 48). Hypoxia-induced histone modifications have recently been reported, and these can be found in both hypoxia-activated and hypoxia-repressed genes (20). The regulation of gene expression by hypoxia through covalent modification of histones is also supported by evidence that histone deacetylase (HDAC) activity plays a role in the activation of many hypoxia-inducible factor 1 (HIF-1)-responsive genes (22). In addition, certain histone demethylases and histone methyltransferases, including JMJD1A, JMJD2B, JARID1B, and G9a histone methyltransferase, have been identified as hypoxia- or HIF-1-regulated genes (2, 9, 27, 29, 53, 56).

In previous work, we found that hypoxia causes decreased expression of *BRCA1* and *RAD51* at the mRNA and protein levels, and we demonstrated that this repression is mediated, in part, by hypoxia-induced dephosphorylation and nuclear accumulation of p130, one of the retinoblastoma (Rb)-related pocket proteins, leading to the formation of repressive E2F4/p130 complexes and increased binding of these complexes to the *BRCA1* and *RAD51* promoters (3, 6). We also demonstrated that downregulation of these factors is linked to decreased DNA repair capacity, establishing a mechanism by which hypoxia can drive genetic instability in cancer cells (5, 7).

Since hypoxia occurs early in neoplastic growth and is a common feature of solid tumors, we asked in this work whether hypoxia might also be a driving force in the silencing of the *BRCA1* promoter. Recent evidence has shown that silenced *BRCA1* alleles found in sporadic cancers are associated not only with promoter DNA hypermethylation (14, 35, 50) but

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also with histone modifications in the promoter region (19). Hence, we hypothesized that hypoxia-induced downregulation of *BRCA1* might cause epigenetic histone modifications that mark the locus for potential silencing. We report here a series of chromatin immunoprecipitation (ChIP) studies revealing that hypoxia induces a set of repressive histone modifications at both the *BRCA1* and *RAD51* promoters, including H3K4 demethylation, H3K9 methylation, and H3K9 deacetylation. In contrast, exactly opposite changes were seen in the histone code at the promoter of the hypoxia-inducible *VEGF* gene. We showed that a key histone modification at the *BRCA1* and *RAD51* promoters in response to hypoxia, H3K4 demethylation, is mediated by the histone demethylase LSD1. We also showed that prolonged exposure of cells to hypoxia can promote the emergence of subclones in which the *BRCA1* promoter has undergone long-term silencing that persists even when cells are no longer in hypoxic conditions. In these silenced clones, the *BRCA1* promoter is characterized by H3K4 demethylation and H3K9 deacetylation. Such histone changes are mechanistically linked to the silencing because exposure of cells to an HDAC inhibitor after hypoxia reverses the *BRCA1* silencing. This work suggests that hypoxia drives not only genetic instability but also epigenetic alteration and tumor suppressor gene silencing in malignant cells.

MATERIALS AND METHODS

Cells. MCF-7, A549, RKO, and HCC 38 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown according to supplier instructions. MCF-7 PLU-1 knockdown cell lines, MCF-7 985, MCF-7 1000, and MCF-7 1100, with control MCF-7 SLR cells, were obtained from Qin Yan (Department of Pathology, Yale University) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1.5 $\mu\text{g}/\text{ml}$ of puromycin. RKO Neo and RKO E7 cells were obtained from Kathleen Cho (Department of Pathology, University of Michigan, Ann Arbor, MI) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 400 $\mu\text{g}/\text{ml}$ of G418. Dif-6-derived cells (described below) were cultured in DMEM supplemented with 5% FBS and 5% Serum Plus (SABC Bioscience). BSH8 cells were grown in 10 $\mu\text{g}/\text{ml}$ of azaserine (Sigma), 10 $\mu\text{g}/\text{ml}$ of hypoxanthine (Sigma), and 1.5 $\mu\text{g}/\text{ml}$ of puromycin (Invitrogen). BSH8 TGH22 and TGH31 cells were grown in 2.5 $\mu\text{g}/\text{ml}$ of 6-thioguanine (6-TG; Sigma) and 1.5 $\mu\text{g}/\text{ml}$ of puromycin. BSH8 2XTGH22 and 2XTGH31 cells were grown in 20 $\mu\text{g}/\text{ml}$ of 6-TG and 1.5 $\mu\text{g}/\text{ml}$ of puromycin.

Constructs. Lentivirus vectors for delivery of short hairpin RNAs (shRNAs) directed against JARID1A/RBP2-sh-1 and RBP2-sh-3 and control vector LLP were obtained from Marie Classon (Massachusetts General Hospital Cancer Center). Lentivirus shRNA vectors for LSD1 knockdown were obtained from Sigma-Aldrich (LSD1-1, catalogue number TRCN0000046068; LSD1-2, catalogue number TRCN0000046071; LSD1-3, catalogue number TRCN0000046072). The lentivirus shRNA vector for HIF-1 α knockdown was obtained from Zhong Yun (Department of Therapeutic Radiology, Yale University). The *BRCA1-HPRT* construct was made by cloning 1.3-kb full-length human hypoxanthine phosphoribosyltransferase (HPRT) cDNA (OriGene) downstream of the 218-bp human *BRCA1* promoter, which has been described previously (3).

Hypoxia. For hypoxia exposure, cells received a continuous flow of a humidified mixture of 95% N₂ and 5% CO₂ gas certified to <10 ppm O₂ for 48 h at 37°C, as previously described (40).

ChIP assays. ChIP assays were done essentially as described previously (3, 6). The primer sequences for the *BRCA1* and *RAD51* promoter regions have also been reported (3, 6). All the antibodies for the ChIP assays are available on request.

Assays for cells with *BRCA1* promoter silencing. Dif-6 cells were transfected by electroporation as described previously (47) with 10 μg of *BRCA1-HPRT* plasmid and 2 μg of a plasmid carrying puromycin resistance (Clontech). BSH8, a stable transfectant expressing functional HPRT, was established by selection first with medium containing 1.5 $\mu\text{g}/\text{ml}$ of puromycin and 1 week later with medium containing 10 $\mu\text{g}/\text{ml}$ of azaserine and 10 $\mu\text{g}/\text{ml}$ of hypoxanthine. The BSH8 clone was screened for physical linkage between puromycin resistance and

BRCA1-HPRT by demonstrating low-frequency loss of HPRT expression in the presence of puromycin and 2.5 $\mu\text{g}/\text{ml}$ of 6-TG but high-frequency loss when puromycin selection was omitted.

To test the impact of hypoxia on silencing of the *BRCA1* promoter in the BSH8 cells, the cells were plated in 100-mm dishes at 100,000 cells per dish and grown under 0.5% hypoxic conditions (or under normoxic conditions for the controls) for 2 weeks with passage twice per week. The cell population was then subject to selection for two additional weeks in the presence of 2.5 $\mu\text{g}/\text{ml}$ of 6-TG. This selection induced substantial cell death, and clones formed by surviving cells were counted for each condition tested. The TGH22 and TGH31 cell lines were isolated from the BSH8 cells as part of this process.

To measure *BRCA1-HPRT* reactivation by trichostatin A (TSA) or 5-aza-2'-deoxycytidine (5-Aza-dC), we incubated selected clones with silenced *BRCA1* promoters, BSH8 THG22, BSH 2XTGH22, and BSH8 TGH31, in medium containing 100 nM TSA (Wako, Richmond, VA) for 16 h to inhibit histone deacetylation or with medium containing 300 nM 5-aza-dC (Sigma) for 16 h to inhibit DNA methylation. After an additional 24 h, the cells were plated in 100-mm dishes (100,000 cells per dish) and the next day were exposed to medium containing 10 $\mu\text{g}/\text{ml}$ of azaserine and 10 $\mu\text{g}/\text{ml}$ of hypoxanthine to select for cells that had regained HPRT expression. Silencing or reactivation frequencies were calculated by dividing the number of clones growing under selection by the effective number of cells plated (as determined with the cloning efficiency plates).

To test the impact of TSA on initial *BRCA1* promoter silencing by hypoxia, we plated BSH8 cells in 100-mm dishes at 100,000 cells per dish and grew them under 0.5% oxygen hypoxic conditions for 2 weeks (as described above). The cells were treated with 100 nM TSA for 24 h when first removed from hypoxia. Then the cell population was selected in the presence of 2.5 $\mu\text{g}/\text{ml}$ of 6-TG for an additional 2 weeks under normoxic conditions.

To test for silencing of the *BRCA1* promoter by the poly(ADP-ribose) polymerase (PARP) inhibitor, 6(5H)-phenanthridinone (PHEN), we plated BSH8 cells in 100-mm dishes at 100,000 cells per dish and treated them with selected concentrations of PHEN for 2 weeks. Then the cells were selected in the presence of 2.5 $\mu\text{g}/\text{ml}$ of 6-TG for two additional weeks. Cells without selection were assayed as controls for cloning efficiency.

Western blotting. After the designated treatments, cells were lysed in RIPA buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Igepal CA-630, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Clontech). The primary antibodies used for Western blotting were as follows: monoclonal anti-*BRCA1* (D-9; Santa Cruz Biotechnology), β -actin (C4; Santa Cruz Biotechnology), anti-JARID1B/PLU-1 (ab50958; Abcam), anti-Rad51 (3C10; Upstate), anti-JARID1A/RBP2 (catalogue number 3867; Cell Signaling), and anti-LSD1 (catalogue number 4218; Cell Signaling).

Quantitative real-time PCR analysis. For quantitative PCR analysis of *BRCA1* and *RAD51* mRNA expression, total RNA was prepared using an Absolutely RNA miniprep kit (Agilent Technologies). Two micrograms of RNA was used to synthesize cDNA using a High Capacity cDNA reverse transcription kit (Applied Biosystems). The resulting cDNA was used in PCRs containing TaqMan universal PCR master mix (Applied Biosystems), premixed TaqMan probes and primers for *BRCA1* and *RAD51*, and 18S (Applied Biosystems). The Mx3000P real-time PCR system (Stratagene) was used to monitor fluorescence intensity in real time to allow quantitative comparisons, as described previously (3, 6).

For quantitative PCR analysis of *BRCA1* promoter-driven *HPRT* mRNA expression, total RNA was isolated from cell culture with an RNeasy minikit (Qiagen) according to the manufacturer's instructions. Total RNA samples were converted to cDNA using a QuantiTect reverse transcription kit (Qiagen) with removal of genomic DNA contamination. One hundred nanograms of cDNA was used as input in a subsequent quantitative-PCR analysis for either *HPRT* (Applied Biosystems) or *GAPDH* (Applied Biosystems) with iQ supermix (Bio-Rad) and a Bio-Rad iCycler instrument. *HPRT* results were normalized in relation to *GAPDH* mRNA levels.

RESULTS

Hypoxia decreases H3K4 methylation at the *BRCA1* and *RAD51* promoters. To test the hypothesis that downregulation of *BRCA1* and *RAD51* during hypoxia is linked to histone modifications, we used the technique of quantitative chromatin immunoprecipitation (qChIP) to probe histone changes at *BRCA1* and *RAD51* promoters during hypoxia. We first examined histone H3K4 methylation levels at the promoters because the methylation status of this residue is a key histone

marker that is associated with increased transcriptional activity. MCF-7 breast cancer cells were exposed or not exposed to hypoxia at 0.01% oxygen for 48 h, and chromatin was prepared for analysis. ChIP analysis was performed using either an antibody that simultaneously recognizes the mono-, di-, and trimethylated forms of H3K4 (H3K4me1,2,3) or antibodies that individually recognize either H3K4me1, H3K4me2, or H3K4me3. Hypoxia was found to cause substantial decreases in the levels of H3K4 me1,2,3 at the *BRCA1* (Fig. 1A) and *RAD51* (Fig. 1B) promoters. Similarly, the levels of H3K4me2 and H3K4me3 were also decreased at these promoters under hypoxic conditions (Fig. 1A and B). Representative agarose gel images corresponding to panels A and B are available on request. H3K4me1 levels were low preexposure and remained low under hypoxic conditions (data not shown). Decreased H3K4 methylation levels were also induced by hypoxia in other cell lines tested, including RKO colon cancer cells (data available on request). In contrast, at the promoter of the hypoxia-induced *VEGF* gene, levels of all forms of H3K4 methylation, H3K4me1,2,3, H3K4me2, and H3K4me3, were increased by hypoxia in both MCF-7 (Fig. 1C; also data available on request) and RKO (data available on request) cells. We also conducted a time course experiment in which we placed MCF-7 cells under hypoxia and then collected the cells 12, 24, 48, and 72 h later for analysis by ChIP of H3K4 methylation at the *BRCA1* and *RAD51* promoters (Fig. 1D and E). In both cases, promoter H3K4 demethylation occurred by 12 h and persisted through 72 h, although there was a slight uptick in H3K4 methylation at the 72-h time point. Collectively, these results demonstrate that hypoxic stress causes decreased H3K4 methylation levels at the *BRCA1* and *RAD51* promoters.

The lysine-specific demethylase LSD1, but not JARID1B/PLU-1 or JARID1A/RBP2, is required for hypoxia-induced H3K4 demethylation at the *BRCA1* and *RAD51* promoters. The identification of specific histone demethylases has changed the understanding of histone methylation, which had been considered a static modification but is now understood to be dynamically regulated (10, 25). Several H3K4-specific demethylases have been identified, including LSD1 (42), JARID1A/RBP2 (24), and JARID1B/PLU-1 (55). We sought to determine whether one or more of the above H3K4 demethylases might be involved in hypoxia-decreased H3K4 methylation levels at the *BRCA1* and *RAD51* promoters.

We first examined the changes in protein levels of LSD1, RBP2, and PLU-1 in response to hypoxia treatment. We found that there was no change in the levels of LSD1, whereas RBP levels were decreased and PLU-1 levels were increased in MCF-7, RKO, and A549 cells (data available on request). Hypoxia-induced upregulation of *PLU-1* had also been seen in another study (27).

Based on its upregulation in response to hypoxia, PLU-1 therefore seemed to be a good candidate for regulation of *BRCA1* and *RAD51* in hypoxia. To test the role of *PLU-1* in hypoxia-decreased *BRCA1* and *RAD51* expression, we used stable shRNA-mediated knockdown of *PLU-1* in MCF-7 cell lines. Three cell line subclones with shRNA knockdown of PLU-1 were established (MCF-7 985, MCF-7 1000, and MCF-7 1100) along with vector control cells (MCF-7 SLR). Reduction in the baseline expression of PLU-1 in normoxia and attenuation of PLU-1 induction in hypoxia were seen in all

three shRNA-expressing lines compared to those of the vector control cells (data available on request). Using these engineered MCF-7-derived cell lines, we found that knockdown of PLU-1 had no effect on hypoxia-mediated suppression of *BRCA1* and *RAD51* protein levels (data available on request). Nonetheless, we also examined *BRCA1* and *RAD51* mRNA levels in response to hypoxia in MCF-7 SLR and MCF-7 1000 cells, the latter chosen because they showed the most robust knockdown of *PLU-1*. We found that knockdown of PLU-1 in the MCF-7 1000 cell line yielded increased expression of *BRCA1* mRNA under normoxic conditions relative to that of the vector control cells (data available on request), consistent with a prior report (55). However, hypoxia-induced downregulation of *BRCA1* mRNA was still seen in the MCF-7 1000 cell line in spite of the PLU-1 knockdown (data available on request). In like fashion, *RAD51* mRNA expression levels were downregulated in hypoxia in both the MCF-7 SLR and MCF-7 1000 cells (data available on request). Turning to chromatin analysis, we found that there was no significant difference in hypoxia-induced H3K4 demethylation at the *BRCA1* and *RAD51* promoters between the vector control cells and the MCF-7 1000 cells with PLU-1 knockdown (data available on request), further ruling out a role for PLU-1 in this process. Similar negative results were also seen in cells with shRNA-mediated knockdown of *JARID1A/RBP2* (data available on request).

Turning to LSD1, we established three MCF-7-derived cell lines with stable shRNA-mediated knockdown of LSD1 (Fig. 2A). As a control, a cell line was established with shRNA-targeting green fluorescent protein (GFP), which had no effect on LSD1 levels (Fig. 2A). We chose one LSD1 knockdown line, MCF-7 shLSD1-1, for further analysis. These cells were exposed or not exposed to hypoxia for 48 h and analyzed for H3K4 methylation status in comparison to that of the shGFP-expressing control cells. We found that knockdown of LSD1 (Fig. 2A) clearly prevented the hypoxia-induced demethylation of H3K4 at both the *BRCA1* and *RAD51* promoters (Fig. 2B and C; quantified in Fig. 2E). However, at the *VEGF* promoter, H3K4 methylation was still seen to increase in response to hypoxia, consistent with hypoxia-induced *VEGF* gene activation, which is not affected by LSD1 knockdown (Fig. 2D; quantified in Fig. 2E).

Interestingly, LSD1 knockdown also blocked the reduction in H3K9 acetylation that otherwise occurs at the *BRCA1* and *RAD51* promoters in response to hypoxia (Fig. 2B and C; quantified in Fig. 2F), consistent with cross talk between the H3K4 and H3K9 modifications. LSD1 knockdown again had no effect at the *VEGF* promoter with respect to the dynamics of H3K9 acetylation.

We next examined the changes in *BRCA1* and *RAD51* mRNA levels in response to hypoxia with or without LSD1 knockdown, using the same set of cell lines as above (MCF-7 shGFP and MCF-7 shLSD1). After the cells were exposed or not exposed to hypoxia for 48 h, mRNA levels for *BRCA1* and *RAD51* were evaluated by quantitative real-time reverse transcription (RT)-PCR (Fig. 2G). LSD1 knockdown blocked about half of the reduction in mRNA levels for these factors that otherwise occurs in response to hypoxia, again consistent with a key role for LSD1 and H3K4 promoter methylation status in the regulation of *BRCA1* and *RAD51* in hypoxia.

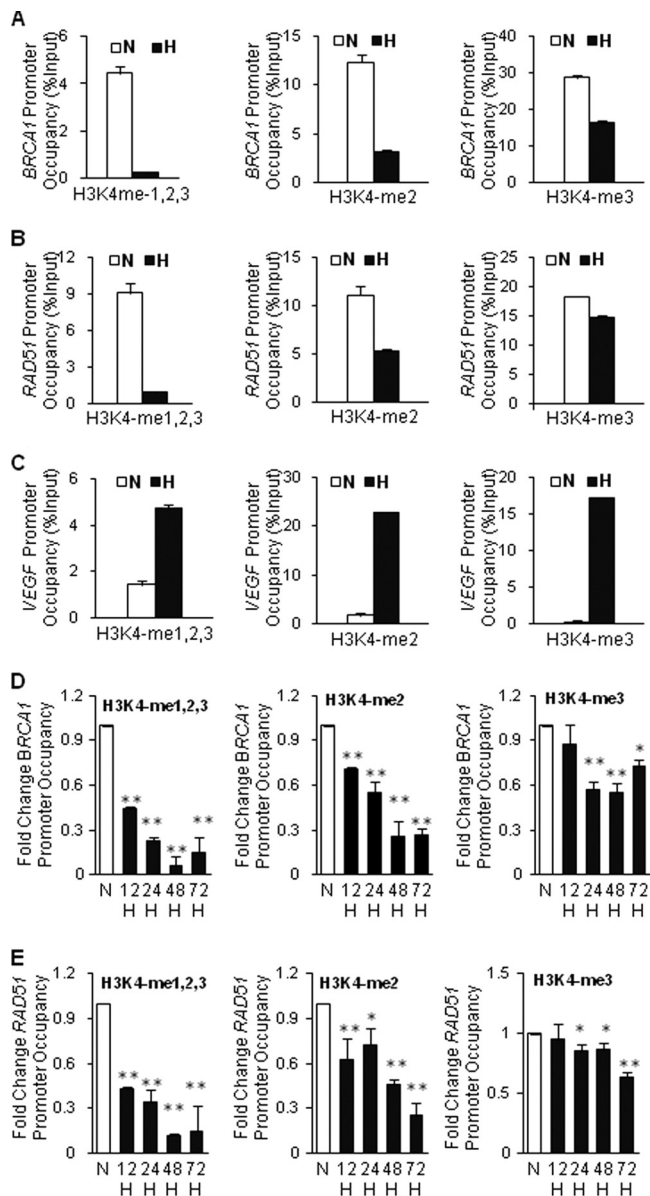


FIG. 1. Decreased H3K4 methylation levels at the *BRCA1* and *RAD51* promoters in response to hypoxia in MCF-7 cells. ChIP assays were performed on MCF-7 cells following exposure to normoxia (N; white bars) or to hypoxia at 0.01% oxygen (H; black bars) for various times, as indicated. Cells were then collected for ChIP analyses using antibodies to the indicated H3K4 methylation forms to determine H3K4 methylation levels at the *BRCA1*, *RAD51*, and *VEGF* promoters. (A) ChIP analysis of H3K4 methylation levels at the *BRCA1* promoter after 48 h of normoxia or hypoxia as quantified by real-time PCR. Specific antibodies that either simultaneously recognize the mono-, di-, and trimethylation forms of H3K4 (H3K4me1,2,3) or that individually recognize either the dimethylated form of H3K4 (H3K4me2) or the trimethylated form of H3K4 (H3K4me3) were used. Relative promoter occupancies (% input) are shown with error bars based on standard errors (SEs) calculated from at least three replicates. The input signal is set as 100% (not depicted in graphs) for each set of assays. (B) ChIP analysis of H3K4 methylation levels at the *RAD51* promoter after 48 h of N or H as quantified by real-time PCR. Relative promoter occupancies (% input) by the indicated H3K4 methylated forms are shown with error bars based on SEs calculated from at least three replicates. (C) ChIP analysis of H3K4 methylation levels at the *VEGF* promoter after 48 h of N or H as quantified by real-time PCR. Relative promoter occupancies (% input) by the indi-

Together, these results identify LSD1 as the key histone demethylase that mediates epigenetic regulation of *BRCA1* and *RAD51* in response to hypoxia.

Hypoxia increases H3K9 methylation and decreases H3K9 acetylation at the *BRCA1* and *RAD51* promoters. H3K9 methylation is a critical epigenetic mark for gene repression and silencing (13, 17). It has been reported that hypoxia induces H3K9 methylation at different gene promoters, which is correlated with hypoxic repression and silencing of those genes (9, 20). For this reason, we examined the effect of hypoxia on H3K9 methylation levels at the *BRCA1* and *RAD51* promoters. There were significant increases in H3K9 me3 levels at both the *BRCA1* and *RAD51* promoters in MCF-7 cells in response to hypoxia (Fig. 3A). Similar results were found in RKO cells (Fig. 3B). Conversely, there was no increase in H3K9me3 levels at the *VEGF* promoter in either cell line (Fig. 3A and B). We also examined the kinetics of H3K9 methylation at the *BRCA1* and *RAD51* promoters over time in response to hypoxic stress (Fig. 3C). We observed increased methylation beginning at about 24 h and peaking around 48 h, a slightly slower response than we observed for demethylation of H3K4 (Fig. 1D and E), suggesting that H3K4 demethylation is an earlier modification that may be upstream of H3K9 methylation at these promoters under hypoxic stress.

We next tested for the putative role of specific H3K9 methyltransferases, including G9a, SUV39-h, and SETDB1 (28), by looking for interactions with either the *BRCA1* or *RAD51* promoters in response to hypoxia by ChIP. We could not detect any of these factors in association with either promoter (data not shown), and so the factor mediating the increased H3K9 methylation remains to be determined.

H3K9 acetylation (H3K9ac) is a marker of transcriptional activation. Following up the initial observations presented in Fig. 2F, we went on to confirm in multiple experiments that hypoxia produces slight decreases in H3K9 acetylation levels at both the *BRCA1* and *RAD51* promoters (Fig. 3D), whereas it substantially increases H3K9 acetylation at the *VEGF* promoter in MCF-7 (Fig. 3D). Similar results were obtained in RKO cells (data not shown).

H3K27 methylation is another mark of transcriptional repression, and so we analyzed the *BRCA1* and *RAD51* promot-

ated H3K4 methylated forms are shown with error bars based on SEs calculated from at least three replicates. (C) ChIP analysis of H3K4 methylation levels at the *VEGF* promoter after 48 h of N or H as quantified by real-time PCR. Relative promoter occupancies (% input) by the indicated H3K4 methylation forms are shown with error bars based on SEs calculated from at least three replicates. Representative agarose gel images corresponding to panels A, B, and C are shown in data available on request. (D) Time course assay of H3K4 methylation at the *BRCA1* promoter in MCF-7 cells placed under hypoxia and collected at the indicated times for analysis by ChIP and quantification by real-time PCR. Promoter occupancy levels are expressed as the fold change relative to those for normoxia, based on three independent ChIP assays, with error bars based on SEs. Significant differences were identified as $P < 0.05$ (indicated by *) or $P < 0.01$ (indicated by **) compared to normoxic levels. (E) Time course assay of H3K4 methylation at the *RAD51* promoter in MCF-7 cells placed under hypoxia and collected at the indicated times for analysis by ChIP and quantification by real-time PCR, as for panel D.

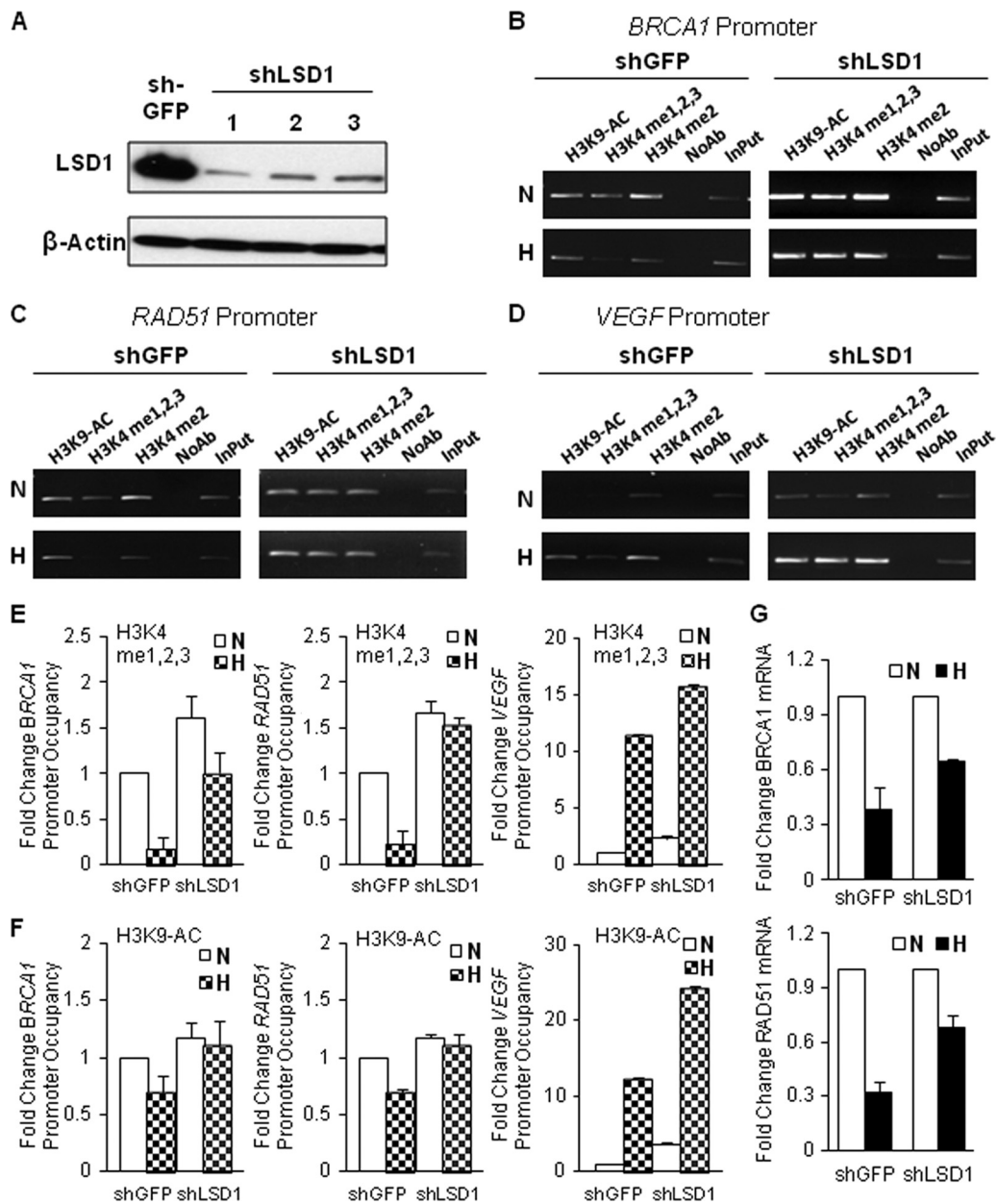


FIG. 2. The histone demethylase LSD1 mediates hypoxia-induced H3K4 demethylation at the *BRCA1* and *RAD51* promoters. MCF-7 cells were transduced with lentiviral expression constructs for a GFP shRNA (control) or for one of three different shRNAs targeting LSD1, and stable shRNA-expressing cell lines were established: MCF-7 shGFP and MCF-7 shLSD1-1, MCF-7 shLSD1-2, and MCF-7 shLSD1-3. (A) Western blot analyses to determine LSD1 expression levels in MCF-7 shGFP, MCF-7 shLSD1-1, shLSD1-2, and shLSD1-3 cells. Substantially reduced expression of LSD1 was detected in all three stable LSD1 shRNA-expressing MCF-7 cell lines. (B, C, and D) ChIP analyses of H3K4 methylation and H3K9 acetylation levels at the *BRCA1* (B), *RAD51* (C), and *VEGF* (D) promoters following 48-h exposure to normoxia or hypoxia in MCF-7 shGFP cells compared to those for MCF-7 shLSD1-1 cells. Representative agarose gels showing PCR amplification products corresponding to the *BRCA1* (B), *RAD51* (C), or *VEGF* (D) promoter regions are shown. (E) Quantification of H3K4 methylation levels by real-time PCR at the *BRCA1* (left), *RAD51* (middle), and *VEGF* (right) promoters following 48-h exposure to normoxia or hypoxia in MCF-7 shGFP and MCF-7 shLSD1-1 cells. Promoter occupancy levels are expressed as the fold change relative to the promoter occupancy levels of the normoxic MCF-7 shGFP cells, based on three independent ChIP assays, with error bars based on SEs. (F) Quantification of H3K9 acetylation levels by real-time PCR at the *BRCA1* (left), *RAD51* (middle), and *VEGF* (right) promoters in MCF-7 shGFP and MCF-7 shLSD1-1 cells under the same normoxic or hypoxic treatment. Promoter occupancy levels are expressed as the fold change relative to the acetylation levels of the normoxic MCF-7 shGFP cells, based on three independent ChIP assays, with error bars based on SEs. (G) Quantitative real-time PCR analysis of endogenous *BRCA1* (top) and *RAD51* (bottom) mRNA expression levels in normoxic and hypoxic MCF-7 shGFP cells and in LSD1 knockdown MCF-7 shLSD1-1 cells, normalized to 18S rRNA expression. mRNA levels are expressed as the fold change relative to those of the corresponding normoxic control cells.

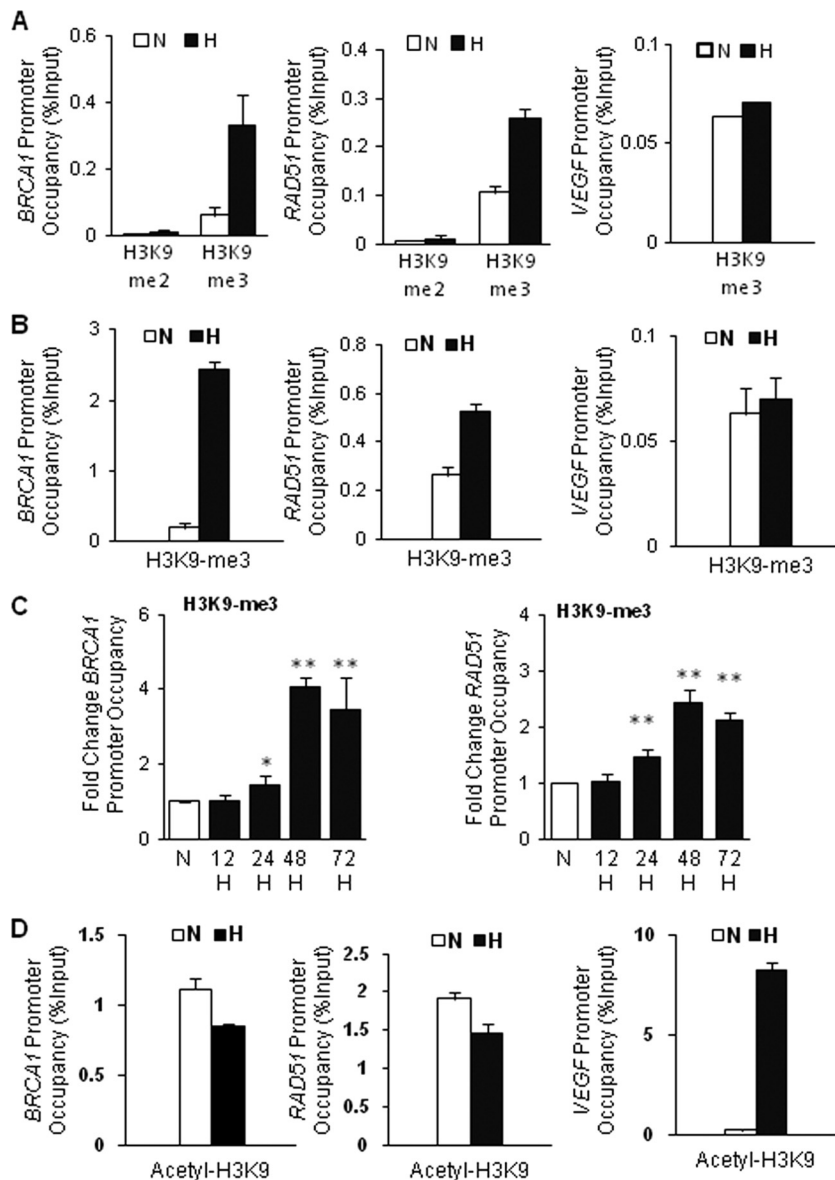


FIG. 3. Hypoxia increases H3K9 methylation and decreases H3K9 acetylation at the *BRCA1* and *RAD51* promoters. ChIP assays were performed on MCF-7 or RKO cells following exposure to normoxia (N) or hypoxia (H; 0.01% O₂) for 48 h using antibodies to the indicated H3K9 modifications at the *BRCA1*, *RAD51*, and *VEGF* promoters. (A) ChIP analysis of H3K9 methylation levels at the *BRCA1* (left), *RAD51* (middle), and *VEGF* (right) promoters as quantified by real-time PCR in MCF-7 cells. Specific antibodies that recognize either the dimethylated (H3K9me2) or trimethylated (H3K9me3) form of H3K9 were used. Relative promoter occupancies (% input) are shown with error bars based on standard errors calculated from at least three replicates. (B) ChIP analysis of H3K9 methylation levels at the *BRCA1* (left), *RAD51* (middle), and *VEGF* (right) promoters as quantified by real-time PCR in RKO cells. Specific antibodies that recognize H3K9me3 were used. Relative promoter occupancies (% input) by trimethylated H3K9 are shown with error bars based on standard errors calculated from at least three replicates. (C) Time course of hypoxia-induced H3K9 methylation at the *BRCA1* (left) or *RAD51* (right) promoter regions by ChIP analysis. MCF-7 cells were exposed to normoxia (N) or hypoxia (H) for the indicated times, and H3K9 methylation levels at the promoters were analyzed by quantification of real-time PCR of ChIP samples corresponding to the *BRCA1* or *RAD51* promoters. Promoter occupancy levels were expressed as the fold change relative to promoter occupancy levels for normoxia, based on three independent ChIP assays with error bars based on SEs. Significant differences were identified as $P < 0.05$ (indicated by *) or $P < 0.01$ (indicated by **) compared to normoxia control levels. (D) ChIP analysis of H3K9 acetylation levels at the *BRCA1* (left), *RAD51* (middle), and *VEGF* (right) promoters as quantified by real-time PCR in MCF-7 cells. Relative promoter occupancies (% input) by acetylated H3K9 are shown with error bars based on SEs calculated from at least three replicates.

ers in MCF-7 cells for this modification under normoxic and hypoxic conditions. However, there were no changes in either H3K27me1, H3K27me2, or H3K27me3 levels at the *BRCA1* or *RAD51* promoters under hypoxic conditions compared to those under normoxic conditions (data not shown). The Poly-

comb protein and histone methylase EZH2 is associated with H3K27 methylation. Consistent with the above results, we could not detect by ChIP any evidence for the presence of EZH2 at either the *BRCA1* or *RAD51* promoter in either normoxia or hypoxia (data available on request).

HIF-1 is not required for the H3K4 demethylation at the *BRCA1* and *RAD51* promoters. In prior work, we have shown that the downregulation of *RAD51* and *BRCA1* at the transcriptional level in response to hypoxia is not dependent on the hypoxia-inducible factor HIF-1 (3, 6). Nonetheless, we still asked whether HIF-1 might be required for the histone modifications that we observed at the promoters in response to hypoxia. Using an shRNA expression vector to knock down expression of HIF-1 α (data available on request), we found that H3K4 demethylation occurs at the *BRCA1* and *RAD51* promoters in response to hypoxia regardless of HIF-1 knockdown (data available on request). This result is consistent with the lack of a role for HIF-1 in regulating these factors at the transcriptional level. In contrast and in keeping with the known role of HIF-1 in stimulating VEGF expression, we observed that the hypoxia-induced increase in H3K4 methylation at the *VEGF* promoter was attenuated by HIF-1 knockdown (data available on request).

Hypoxia induces *BRCA1* promoter silencing. *BRCA1* is frequently silenced in sporadic tumors of multiple sites (8, 14, 51, 52). To test whether hypoxia might play a role in the silencing of the *BRCA1* promoter, we established a system to select for cells in which the *BRCA1* promoter had been silenced. We engineered a construct with the 218-bp *BRCA1* proximal promoter (essentially the entire *NBR2-*BRCA1** intergenic region) driving expression of the human *HPRT* cDNA. This construct (Fig. 4A) was transfected into the mouse Dif-6 cell line, chosen because it lacks expression of endogenous *hpri*, thereby allowing both positive and negative selection for HPRT function. One stable transfectant cell line, designated BSH8, was found to express high baseline levels of HPRT. To test whether hypoxia could silence the *BRCA1* promoter in this construct, the BSH8 cells were exposed to hypoxia for 2 weeks and then subjected to selection for *HPRT*-deficient cells in medium supplemented with the purine analog 6-TG. 6-TG is toxic to cells with functional *HPRT*. The size of the fraction of surviving 6-TG-resistant clones reflects the frequency at which the *BRCA1* promoter-*HPRT* construct is inactivated. We found that hypoxia exposure led to the emergence of HPRT-negative clones at a frequency of more than 2%, 500-fold more than the background frequency arising in cells grown under standard normoxic conditions (Fig. 4B) and more than 1,000-fold greater than the typical spontaneous mutation frequencies in the *HPRT* coding region (49).

Several independent 6-TG-resistant clones were isolated for further analysis. These showed greatly reduced levels of *HPRT* mRNA expression (Fig. 4C; compare TGH22 and TGH31 to BSH8), consistent with silencing of the *BRCA1* promoter in the construct. The silencing was demonstrated to be durable, since silenced clones maintained in normoxic conditions for up to 8 weeks after hypoxic exposure retained the silenced state (data not shown). However, we found that the silenced promoters could be reactivated by treatment with the HDAC inhibitor TSA (Fig. 4C), pointing to epigenetic silencing via histone modifications.

Hypoxia-induced *BRCA1* promoter silencing is correlated with specific histone modifications. Since we showed above that short-term hypoxia causes decreased H3K4 methylation and H3K9 acetylation as well as increased H3K9 methylation at the *BRCA1* and *RAD51* promoters in MCF-7 and RKO cells,

we hypothesized that the *BRCA1* promoter silencing in the *BRCA1-HPRT* system was likely the result of similar or related histone modifications. To test this, we compared histone marks at the *BRCA1* promoter within the *BRCA1-HPRT* construct in the parental BSH8 cells with those of two 6-TG-resistant subclones, BSH8 TGH22 and BSH8 2XTGH22. qChIP analysis revealed markedly reduced H3K4 methylation at the *BRCA1-HPRT* promoter in both the BSH8 TGH22 and BSH8 2XTGH22 cell lines compared to that of the parental BSH8 cell line that highly expresses HPRT (Fig. 4D). The level of H3K9 acetylation in the promoter was also decreased in both of the silenced subclones (Fig. 4D). Similar results were found in two other silenced subclones (BSH8 TGH31 and BSH8 2XTGH31; data not shown). The H3K4 demethylation and the H3K9 deacetylation seen in the silenced clones are in keeping with the effects of short-term hypoxia on the endogenous *BRCA1* promoter in the MCF-7 cells, suggesting that these modifications play key roles in both *BRCA1* suppression and *BRCA1* silencing.

Consistent with histone modifications at the *BRCA1* promoter in silenced clones, we found that treatment of the silenced clones with the HDAC inhibitor TSA could produce reactivation of functional *HPRT* expression following treatment, manifested both by elevated levels of *HPRT* mRNA (Fig. 4C) and by the generation of azaserine- and hypoxanthine-resistant subclones, indicative of reactivated *BRCA1* promoter-*HPRT* expression constructs (Fig. 4E). These results further indicated that inactivation and silencing of the *BRCA1* promoter-*HPRT* cDNA constructs in response to hypoxia was due to durable, but still pharmacologically reversible, silencing of the promoter. We also found that treatment of the BSH8 cells with TSA immediately after the 2-week hypoxia exposure but before selection with 6-TG prevented the emergence of silenced clones (Fig. 4F). In addition, when the BSH8 TGH22 cells were treated with TSA, thereby reactivating the *BRCA1* promoter (as in Fig. 4E), the repressive histone changes were partially reversed (Fig. 4G), including not only the deacetylation of H3K9 but also the demethylation of H3K4.

DNA promoter methylation is not involved in hypoxic *BRCA1* promoter silencing. Because DNA hypermethylation at CpG sites in promoter regions has been associated with silenced tumor suppressor genes, including *BRCA1*, in many human cancers (14, 35, 50), we entertained the possibility that methylation at CpG sites might also be playing a role in the hypoxia-induced silencing. We tested this by methyl-specific PCR analysis in hypoxia-treated MCF-7 cells. Cells were exposed to hypoxia (0.01% O₂) for 48 h or 1% oxygen for 2 weeks or 5 cycles of 48 h with alternating normoxia and hypoxia (0.01% O₂) and then analyzed for *BRCA1* promoter methylation. As a positive control for *BRCA1* promoter methylation, the HCC38 breast cancer cell line was included (52). We did not detect any evidence of cytosine methylation in any of the hypoxia-treated cell populations (data available on request), even though we used published primers and methodologies that have previously detected methylation in human tumor samples (51). Interestingly, prior work probing for hypoxia-induced DNA methylation at the downregulated *MLH1* promoter was also negative (34).

It has been reported that the methyl DNA binding proteins, MDB2 and MeCP2, bind not just to densely methylated re-

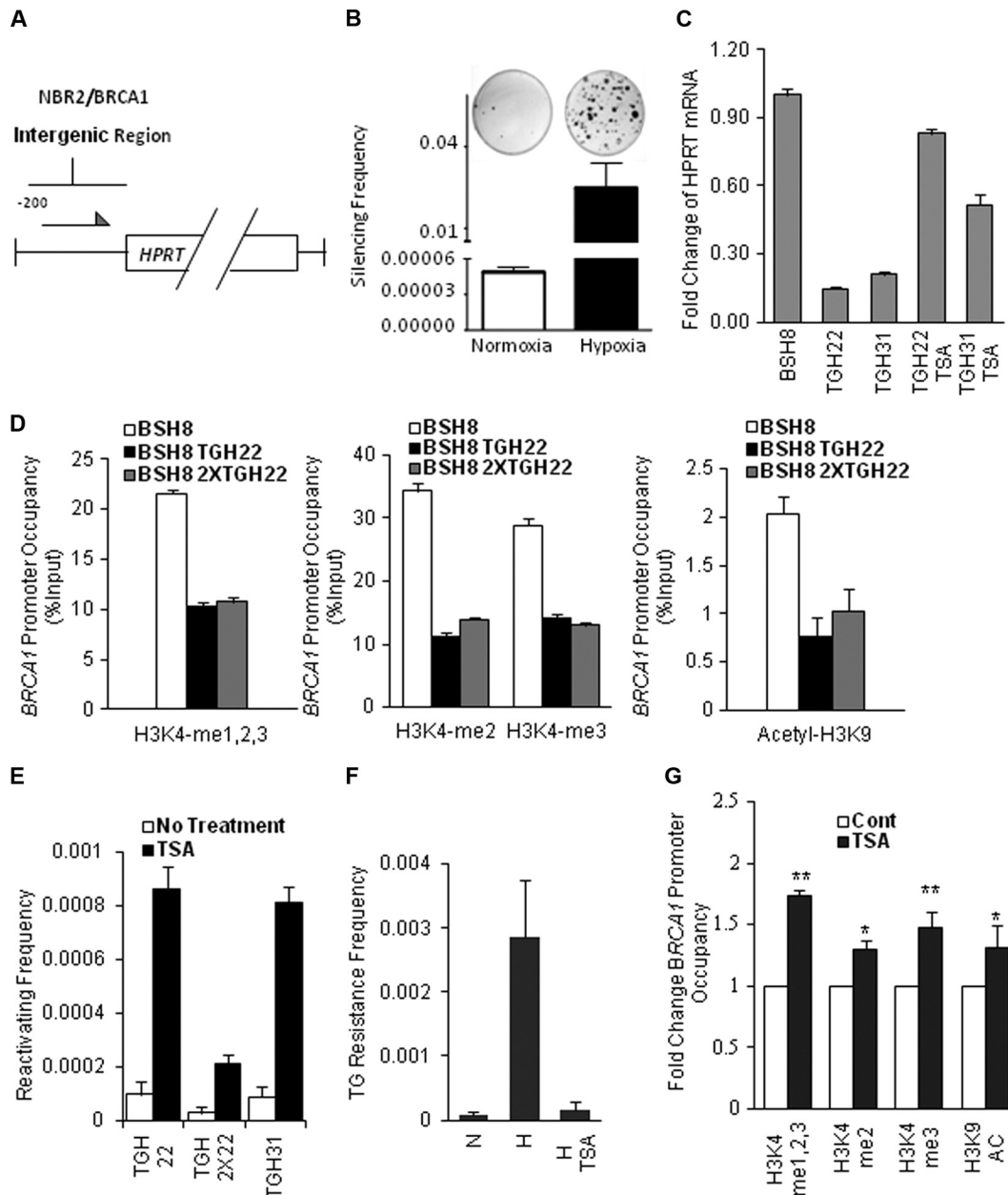


FIG. 4. Hypoxia induces silencing of the *BRCA1* promoter that is correlated with specific histone modifications. (A) Schematic of the *BRCA1-HPRT* construct that was used to express *HPRT* in Dif-6 cells, which lack endogenous *HPRT* expression. A subclone containing a stable, single-copy integrant of the *BRCA1-HPRT* expression construct was isolated and designated BSH8. (B) Frequency of 6-TG-resistant clones (indicative of silenced *BRCA1-HPRT* expression) following exposure of BSH8 cells to normoxia or hypoxia for 2 weeks and then selection in 6-TG for two additional weeks. (Top) Visualization of differential 6-TG-resistant colony formation following growth in normoxia or hypoxia and subsequent 6-TG selection. (Bottom) Quantification of the frequency of 6-TG-resistant clones arising after growth in normoxia or hypoxia. Error bars represent standard errors from three replicates. (C) Quantitative real-time PCR analysis of *HPRT* mRNA expression in two randomly selected 6-TG-resistant clones, BSH8 TGH22 and BSH8 TGH31, compared with *HPRT* mRNA expression in the parental cell line BSH8. The TGH22 and TGH31 cells were also treated with the HDAC inhibitor TSA and then analyzed for *HPRT* expression. *HPRT* mRNA levels were normalized to those for *GAPDH* and presented as the fold change relative to *HPRT* mRNA levels of the parental BSH8 cells. Error bars represent standard errors from three replicates. (D) ChIP analysis of histone modifications at the *BRCA1-HPRT* promoters in the 6-TG-resistant clones, BSH8 TGH22 and BSH8 2XTGH22. (Left) Relative promoter occupancies (% input) by all three forms of H3K4 methylation (H3K4me1,2,3) are shown with error bars based on standard errors calculated from three replicates. (Middle) Relative promoter occupancies (% input) by dimethylated H3K4 and trimethylated H3K4 are shown with error bars based on standard errors calculated from three replicates. (Right) Relative promoter occupancies (% input) by acetylated H3K9 are shown with error bars based on standard errors calculated from three replicates. (E) Reactivation of *HPRT* expression in *BRCA1* promoter-silenced clones by TSA treatment. Three 6-TG-resistant clones, BSH8 TGH22, BSH8 2XTGH22, and BSH8 TGH31, were treated with TSA or not treated and were placed under selective conditions (in the presence of 10 μ g/ml of azaserine and 10 μ g/ml of hypoxanthine) to assay for reactivation of functional *HPRT* expression. The reactivation frequency represents the frequency of colonies surviving after 2 weeks of azaserine/hypoxanthine selection. (F) The HDAC inhibitor TSA blocks the emergence of silenced clones following hypoxia treatment of BSH8 cells. The cells were grown in hypoxia or normoxia for 2 weeks. After exposure to hypoxia, BSH8 cells were treated

gions of DNA but also to isolated methyl CpG dinucleotides (45, 54). Hence, we also tested by ChIP for the presence of MDB2 and MeCP2 at the *BRCA1* promoters in MCF-7 cells exposed to hypoxia. No evidence for the recruitment of these proteins to the *BRCA1* promoter was detected (data available on request).

Finally, we tested whether inhibiting DNA methylation with 5-aza-dC treatment could reactivate the silenced *BRCA1* promoter in the BSH8 TGH22 and BSH8 2XTGH22 cells (data available on request), as we had seen with TSA treatment in Fig. 4E. No evidence of reactivation by treatment with 5-aza-dC was seen (data available on request), nor did combining 5-aza-dC treatment with TSA treatment have any additional effect beyond the substantial effect of TSA alone (data available on request). Taken together, these results indicate that the downregulation and silencing of the *BRCA1* promoter by hypoxia can be associated with specific histone modifications but are not dependent on DNA methylation.

Hypoxia-induced E2F4/p130 pathway is not required for hypoxia-induced histone modifications at the *BRCA1* promoter and is not sufficient for silencing. Histone modifications are regulated by many different enzymes, including histone methyltransferases, histone demethylases, and histone deacetylases (HDACs) (23, 41, 43, 57); however, the process by which histone-modifying enzymes are recruited to their target loci is not fully understood. Our previous work demonstrated that hypoxia-induced downregulation of both *BRCA1* and *RAD51* is mediated by binding of repressive E2F4/p130 complexes at the promoters (3, 6). We therefore hypothesized that these E2F4/p130 complexes might recruit histone-modifying enzymes to these promoters. To test this, we examined histone modifications at the *BRCA1* and *RAD51* promoters following exposure to normoxia or hypoxia in a matched pair of RKO-derived cell lines stably transfected with either a human papillomavirus (HPV) E7 cDNA construct (RKO E7) or an empty vector (RKO Neo). HPV E7 disrupts p130 function, and previous work established that E7 expression prevents downregulation of *BRCA1* and *RAD51* in hypoxia by disrupting the binding of p130/E2F4 complexes to the promoters (3, 6). However, as shown in Fig. 5A, the presence of E7 had no effect on the hypoxia-induced decreases in H3K4 methylation or increases in H3K9me3 at the *BRCA1* and *RAD51* promoters, even though E7 blocks the downregulation of these genes under the conditions tested (data not shown, but similar results were published in references 3 and 6).

The above results suggest that active E2F4/p130 complexes are not required to produce the repressive histone modifications at the *BRCA1* promoter that are associated with silenc-

ing. We next sought to test the converse: whether such complexes are sufficient to promote these histone changes. We knew from previous work that inhibitors of the enzyme PARP could cause *BRCA1* and *RAD51* suppression and also do so via an E2F4/p130-dependent pathway (15), in partial similarity to the effect of hypoxia. Interestingly, we found that treatment of cells with the PARP inhibitor PHEN did not induce any changes in H3K4 methylation, H3K9 methylation, or H3K9 acetylation at the *BRCA1* promoter (Fig. 5B; also data available on request), even though PHEN treatment suppresses expression of *BRCA1* and induces E2F4/p130 binding to the promoter (data not shown here but published in reference 15). Taken together, the above results suggest that E2F4/p130 complexes are neither necessary nor sufficient for the hypoxia-induced histone modifications at the *BRCA1* promoter.

Because inhibition of PARP causes downregulation of *BRCA1* in a manner somewhat similar to the effect of hypoxia, we also tested whether prolonged treatment of the BSH8 cells (containing the *BRCA1* promoter-*HPRT* construct) with PHEN could promote *BRCA1* promoter silencing. The BSH8 cells were exposed to PHEN for 2 weeks and then selected for possible *BRCA1*-*HPRT* silencing by the addition of 6-TG to the medium, as above. Unlike the prolonged hypoxia exposure, the PHEN treatment did not produce any 6-TG-resistant clones above the background (Fig. 5C). Hence, our data suggest that while hypoxia-induced E2F4/p130 complexes can suppress *BRCA1* expression, they do not, by themselves, mediate *BRCA1* silencing.

DISCUSSION

The work presented here shows that hypoxia-induced downregulation of *BRCA1* and *RAD51* is associated with specific histone modifications, including H3K4 demethylation, H3K9 methylation, and H3K9 deacetylation, with the opposite changes seen in the promoter of the hypoxia-inducible gene *VEGF*. In testing a number of histone-modifying enzymes, we identified LSD1 as the histone demethylase responsible for H3K4 demethylation at the *BRCA1* and *RAD51* promoters in response to hypoxic stress. By using a cell line with the *BRCA1* promoter driving a selectable marker, we found that hypoxia can cause epigenetic silencing of the promoter at frequencies of up to 2% in a cell population. Importantly, the hypoxia-induced silencing was associated with the same histone modifications at the *BRCA1* promoter as seen after acute hypoxic exposure (H3K4 demethylation and H3K9 deacetylation), providing a mechanistic link between downregulation of the promoter and its silencing in response to hypoxia.

with 100 nM TSA or not treated for 24 h when first removed from hypoxia. The cell populations (normoxia for 2 weeks, hypoxia for 2 weeks, or hypoxia for 2 weeks plus TSA for 24 h) were then placed in normoxia under selective conditions in the presence of 2.5 $\mu\text{g/ml}$ of thioguanine for an additional 2 weeks. The frequency of 6-TG-resistant clones was determined as for panel B. (G) TSA partially reverses certain histone modifications that were produced by hypoxia in the *BRCA1* promoter-silenced clones. The 6-TG-resistant clone TGH22 was treated with TSA and placed under selection for functional *HPRT* expression and therefore for *BRCA1* promoter reactivation in the presence of 10 $\mu\text{g/ml}$ of azaserine and 10 $\mu\text{g/ml}$ of hypoxanthine. ChIP analyses were performed to examine histone modifications at the *BRCA1*-*HPRT* promoters in the silenced TGH22 cells compared to those in the TGH22-derived cells with reactivation of the *BRCA1* promoter following TSA treatment. Promoter occupancy is expressed as the fold change relative to histone modifications in the TGH22 cells, based on three independent ChIP experiments, with error bars based on standard errors. Significant differences compared to control levels were identified as $P < 0.05$ (indicated by *) or $P < 0.01$ (indicated by **).

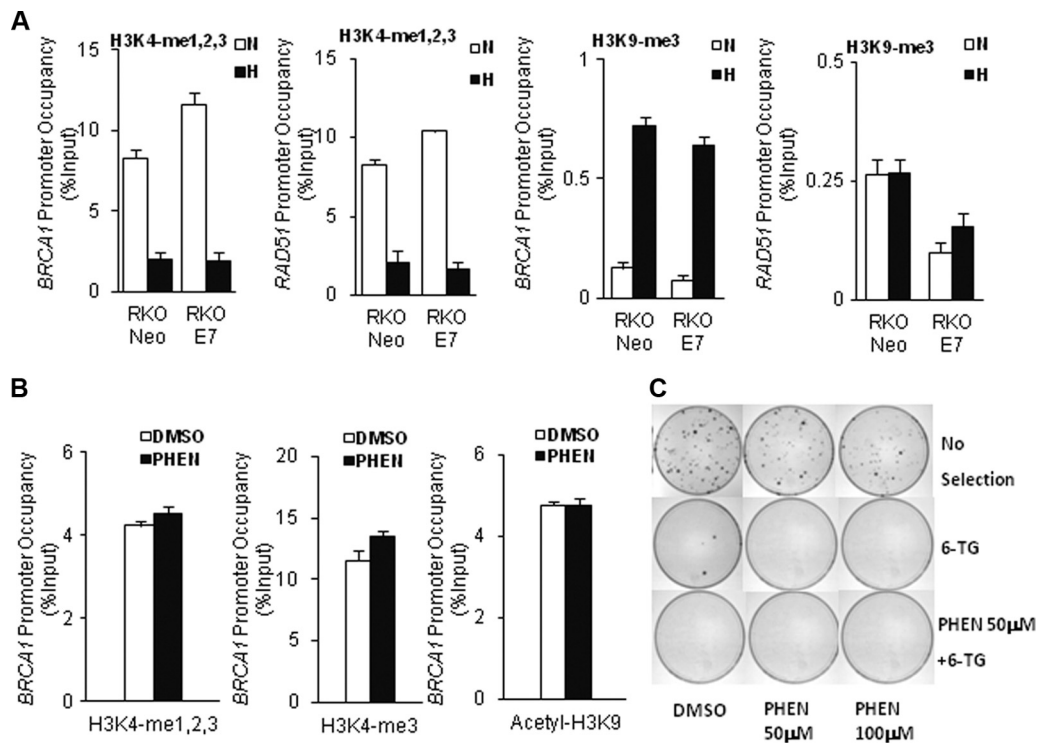


FIG. 5. The hypoxia-induced E2F4/p130 pathway is not required for hypoxia-induced histone modifications at the *BRCA1* promoter and is not sufficient for silencing. (A) ChIP analyses for the presence of the indicated histone modifications at the *BRCA1* or *RAD51* promoters were performed in RKO cells expressing either E7 or a vector control (Neo) following a 48-h exposure to normoxia (N) or hypoxia (H). ChIP analyses of levels of H3K4 methylation (left two graphs) and levels of H3K9 methylation (right two graphs) are shown for the *BRCA1* and *RAD51* promoters, as quantified by real-time PCR. Relative promoter occupancies (% input) are shown, with error bars based on standard errors calculated from three replicates. (B) ChIP analyses of histone modifications at the *BRCA1* promoter. Relative levels of *BRCA1* promoter occupancy (% input) by the indicated factors is shown with error bars based on standard errors calculated from three replicates. (C) Visualization of 6-TG-resistant clones arising from BSH8 cells treated with PHEN or dimethyl sulfoxide (DMSO) (as a vehicle control). BSH8 cells were treated with PHEN at 50 μ M or 100 μ M or with DMSO (0.001%) for 2 weeks. Cells were then selected in 2.5 μ g/m of 6-TG or 6-TG plus 50 μ M PHEN for two additional weeks. The cells without selection were used to determine cloning efficiencies.

The hypoxia-induced silencing was found to be reversible by treatment with an HDAC inhibitor (TSA), consistent with the role of histone modifications in the silencing. In contrast, hypoxia-induced silencing of the *BRCA1* promoter was not associated with promoter DNA methylation, as silenced clones showed neither methylation at CpG sites nor binding of methyl DNA binding proteins to the promoter, and 5-aza-dC treatment of the silenced clones was unable to reverse the silencing. Manipulation of the E2F4/p130 pathway via ectopic HPV E7 expression revealed that this complex is not necessary for the hypoxia-mediated histone modifications at H3K4 and H3K9. In addition, although inhibition of PARP downregulates *BRCA1* and does so through E2F4/p130 (15), it does not induce the same histone modifications as hypoxia and does not cause promoter silencing. Taken together, the above results suggest that short-term downregulation of *BRCA1*, in the absence of coordinated histone modifications, is not sufficient, by itself, to produce long-term epigenetic silencing of the promoter. The difference between hypoxic stress and PARP inhibition points to a key role for specific hypoxia-induced histone modifications in the epigenetic repression and silencing of the *BRCA1* promoter.

The most dramatic histone modifications induced by hypoxia

at the *BRCA1* and *RAD51* promoters are H3K4 demethylation and H3K9 methylation. H3K4 demethylation has been previously seen in hypoxic repression of the tumor suppressor *RUNX3* in gastric cancer cells (29), while H3K4 methylation has been linked to hypoxia-activated genes, such as *EGR1* (20) and *VEGF* (Fig. 1C). Prompted by these observations, we tested for the potential roles of candidate histone demethylases, *JARID1B/PLU-1*, *JARID1A/RBP2*, and *LSD1* (*KDM1*), in *BRCA1* regulation in response to hypoxia. We found that shRNA-mediated knockdown of *LSD1* but not of *PLU-1* or *RBP2* abrogated the hypoxia-induced demethylation of H3K4 at the promoters, establishing *LSD1* as the key mediator of epigenetic regulation of *BRCA1* and *RAD51* in response to hypoxic stress.

In prior work, we had shown that exposure of cells to hypoxia can stimulate E2F4/p130 binding to the *BRCA1* and *RAD51* promoters, which leads to suppression of gene expression (3, 6). However, our present results suggest that this hypoxia-induced pathway is not coordinated with hypoxia-induced histone modification at those loci. We showed that E7-mediated disruption of p130 does not prevent hypoxia-induced H3K4 demethylation or H3K9 methylation at the *BRCA1* and *RAD51* promoters. With respect to the *BRCA1* silencing pro-

duced by hypoxia in *BRCA1* promoter-*HPRT*-transfected cells, we showed that histone modifications happen at silenced *BRCA1* promoters that are similar to those at the hypoxia-downregulated endogenous *BRCA1* promoter. Together, our results suggest that regulation of *BRCA1* by hypoxia occurs through at least two mechanisms: activation of the E2F/p130 pathway to mediate acute downregulation (1) and induction of repressive histone modifications, especially H3K4 demethylation by LSD1, as a step toward epigenetic silencing (2).

Silencing of *BRCA1* has previously been associated with promoter DNA hypermethylation at CpG islands, based on observations in human tumor specimens (8, 14, 21, 35, 50). However, we could not detect any cytosine methylation either at the endogenous *BRCA1* promoter in MCF-7 cells after hypoxia exposure or at hypoxia-induced, silenced *BRCA1* promoters in BSH8-derived cells. Also, treatment of cells in which the *BRCA1* promoter was already silenced by prior hypoxic stress with the DNA methylation inhibitor 5-aza-dC had no effect on *BRCA1* promoter reactivation frequencies, in contrast to the potent effect of the HDAC inhibitor TSA. These observations point to a pathway of gene silencing initially driven by histone modifications and are in keeping with those of other recent studies (1, 26, 29, 38).

Nonetheless, while our results are consistent with a model in which progressive changes in promoter-associated histones are the key drivers of silencing at the *BRCA1* promoter, they do not rule out subsequent accumulation of DNA CpG methylation combining to mediate even more stringent silencing. Interestingly, H3K9 methylation has been recognized as a signal for DNA methylation (18, 46), and unmethylated H3K4 is believed to be required for *de novo* DNA methylation (37). As shown here, hypoxia can induce both H3K9 methylation and H3K4 demethylation at the *BRCA1* promoter, so this could provide the foundation for subsequent DNA methylation. It is possible that the hypoxic conditions used here, by themselves, are not a strong enough stressor to induce cytosine methylation. Other tumor microenvironmental factors, such as low pH, nutrient deprivation, and cytokine exposure, might be needed to combine with hypoxia to drive eventual DNA methylation, but this remains to be determined.

Overall, our work demonstrates that hypoxia induces histone modifications at the *BRCA1* and *RAD51* promoters and can drive *BRCA1* epigenetic silencing. Interestingly, *RAD51* can be downregulated by hypoxia in a manner similar to that for *BRCA1*, but there is essentially no evidence for *RAD51* silencing in human tumors. We speculate that this reflects the severe growth disadvantage that the absence of *RAD51* would place on human cells, consistent with reports that full *RAD51* knockout is lethal to cells (44). In contrast, *BRCA1* knockout is not lethal to cells; in fact, cancer cells in which the *BRCA1* promoter is silenced would lack the genome maintenance and tumor suppressor functions of the gene and so could in theory develop a growth advantage that would lead to expansion during tumor progression. This could explain the frequent observation of silenced *BRCA1* genes in human cancers.

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