Nitric Oxide–Dependent Downregulation of BRCA1 Expression Promotes Genetic Instability

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Abstract

Elevated levels of nitric oxide (NO) and reactive nitrogen species (RNS) may link inflammation to the initiation, promotion, and progression of cancer. Traditionally, this link has been thought to be mediated by the effects of NO/RNS in generating DNA damage. However, this damage also stimulates DNA repair responses with subsequent blocks to cell proliferation and apoptosis, thereby preventing accumulation of NO/RNS-generated mutations. In addressing this conundrum, I describe here an alternative mechanism for understanding mutagenesis by NO/RNS. Moderate NO/RNS concentrations stimulated mutagenesis not directly by generating DNA damage but indirectly by modifying the activities of DNA repair and genome stability factors without affecting cell proliferation. NO/RNS at concentrations physiologically relevant to inflammation stimulated PP2A activity, leading to dephosphorylation of RBL2, its accumulation in the nucleus, and formation of BBL2/E2F4 complexes. RBL2/E2F4 formation in turn led to a shift in BRCA1 promoter occupancy from complexes containing activator E2F1 to complexes containing repressor E2F4, downregulating BRCA1 expression. By inhibiting BRCA1 expression, NO/RNS thereby reduces the ability of cells to repair DNA double-strand breaks through homologous recombination repair, increasing the involvement of error-prone nonhomologous end joining (NHEJ). In summary, NO/RNS stimulates genetic instability by inhibiting BRCA1 expression and shifting DNA repair from high fidelity to error-prone mechanisms. Cancer Res; 73(2): 706–15. ©2012 AACR.

Introduction

Epidemiologic evidence accumulating over the years has provided the positive correlation between cancer incidence and chronic inflammation (1), and it is now a well-recognized hallmark of cancer development (2–4).

Generation of nitric oxide/reactive nitrogen species (NO/RNS) by inducible nitric oxide synthase (iNOS) is a critical feature of the inflammatory environment (5). Several studies have established that NO/RNS production leads to inflammation-stimulated carcinogenesis by generating various types of direct DNA damage (6–9). However, the DNA damage induced at high concentrations of NO/RNS also stimulates DNA repair responses with a subsequent block of cell proliferation and activation of apoptosis (10, 11). This response prevents accumulation of NO/RNS-generated mutations in subsequent cell generations and is inherently anticarcinogenic.

NO/RNS mediates cellular regulation through the posttranslational modifications of a number of regulatory proteins. The best studied of these modifications are S-nitrosylation (reversible oxidation of cysteine; refs. 12–14) and tyrosine nitration (15–17). Although tyrosine nitration is usually associated with ischemia reperfusion conditions, other physiologic conditions including chronic inflammation able to stimulate NO/RNS generation and also modulate the activity of many signal transduction proteins. Jaiswal and colleagues (8) reported NO/RNS-dependent inhibition of DNA repair during inflammatory conditions without, however, describing the exact mechanism. These results suggest that under chronic inflammatory conditions, an alternative mechanism of NO/RNS-dependent mutagenesis occurs.

The loss of BRCA1 protein function predisposes to the development of breast and ovarian cancers (18). BRCA1 contributes to cell viability in multiple ways, including homologous recombination repair (HRR) of DNA double-strand break (DSB), cell-cycle checkpoint control, mitotic spindle assembly, and regulation of chromosome segregation (19–21). The present work shows that inflammatory-relevant concentrations of NO/RNS can inhibit BRCA1 expression without affecting the cell proliferation. This inhibition significantly reduced the ability of cells to repair DNA DSBs through HRR and resulted in a moderate increase of error-prone nonhomologous end joining (NHEJ). Hence, moderate concentrations of NO/RNS, concentrations that result in low amounts of cell toxicity, stimulate genetic instability by inhibiting BRCA1 expression and shifting DNA DSB repair from high-fidelity HRR to error-prone NHEJ.

Materials and Methods

Antibodies, reagents, siRNAs

Primary antibodies used included anti-β-tubulin and anti-PP2Aa (Cell Signaling); anti-E2F1, anti-PP2Ac, and anti-nitro-
tyrosine (Millipore); anti-BRCA1 (Calbiochem), anti-E2F4 and anti-I-SceI (Santa Cruz Biotechnology); anti-RBL2 (BD Transduction Laboratories); anti-TBP (Abcam); and anti-VCP/p97 (Thermo Scientific). NO donors SNAP and DETA NONOate (DETA) were purchased from Cayman Chemical. SNAP and DETA were decomposed by allowing oxidation to occur at 37°C for 7 days, and both decomposed NO donors were used as additional negative controls. Okadaic acid was purchased from Fisher Scientific. siRNA FlexiTube mixtures (Qiagen) for siRNA transfection included Hs_BRCA1_13 (SI02654575), Hs_RBL2_6 (SI02664473), Hs_E2F4_5 (SI02654694), and AllStars negative control (SI03650318). Transfections with siRNAs (25 nmol/L unless a specific concentration was stated) were conducted according to the manufacturer’s recommendations.

Cell culture, transfection, subcellular fractionation, immunoprecipitation, and adenovirus treatment

Nontumorigenic human breast epithelial cells (MCF-10A), human lung adenocarcinoma epithelial cells (A549), and mouse leukemic monocyte macrophage cells (RAW264.7) were obtained from American Type Culture Collection and grown as recommended. All cell lines were used within 6 months after resuscitation. MCF-10A and RAW264.7 cells were co-cultured at a 1:1 ratio in MCF-10A-specific medium. Stable and transient transfections were conducted with Lipofectamine Plus (Invitrogen). Subcellular fractionation and immunoprecipitation were conducted as previously described (22, 23). The Ad-SceI-NG adenovirus was a generous gift of Dr. Kristoffer Valerie (Massey Cancer Center, Virginia Commonwealth University, Richmond, VA) and has been described previously (24). Adenovirus was added to culture medium at 30 virus particles (VP)/cell and incubated while rocking for 4 hours at 37°C. Virus was then removed and fresh medium was applied.

Western blotting

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were exposed to antibodies at specific dilutions. Specific protein bands were detected using infrared-emitting conjugated secondary antibodies: anti-mouse 680 Alexa Fluor (Molecular Probes) or anti-rabbit IRDye 800 (Rockland Immunochemicals), using the Odyssey Infrared Imaging System and the Application software version 2.0 from Li-Cor Biosciences.

Proliferation and phosphatase assays

Cells were plated in 96-well microplates at a density of 2 × 10^3 cells per well. After culture for 24 hours, different amounts of NO donors were added. WST-1 reagent was added at 24, 48, and 72 hours after the start of NO donor treatment, and absorbance at 450 nm was measured following the manufacturer’s instructions (Roche Molecular Systems) after 3 hours of incubation.

The PP2A Immunoprecipitation Phosphatase Activity kit (Millipore) was used according to the manufacturer’s instructions to estimate of PP2A activity.

Detection of tyrosine nitration

After sorting by flow cytometry, MCF-10A cells were centrifuged at 2,500 rpm for 5 minutes, washed 3 times with ice-cold 1× PBS, counted, and 5 × 10^6 cells resuspended in 1 mL of the ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton X-100, 10% glycerol, 1 mmol/L PMSF, 1 mmol/L Na3VO4, 100 μmol/L NaF, 0.25 mmol/L pepstatin, 0.25 mmol/L leupeptin, 0.25 mmol/L aprotinin, 1 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride, and 2 μmol/L pepstatin A). Cells were lysed in 1 mL of lysis buffer and incubated on ice for 1 hour. The cell lysates were centrifuged at 15,000 rpm for 15 minutes at 4°C, and supernatants were collected and stored at −80°C.

Figure 1. MCF-10A and A549 cell proliferation as a function of NO donor concentration. Cells were incubated with different doses of SNAP or DETA and were assayed at 24, 48, and 72 hour time points using the WST-1 cell proliferation reagent. Experimental data are presented as the mean ± SD for quadruplicate samples.
mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 2.5 mmol/L EDTA, Halt cocktail of protease inhibitors; Thermo Scientific). Cell lysates were collected after centrifugation at 12,000 rpm for 20 minutes. Ten microliters of rabbit anti-nitro-tyrosine Ab (Millipore) and 15 µL of protein A agarose (Sigma) were added to each sample for immunoprecipitation. Samples were incubated overnight at 4°C with continuous rotation. The protein A agarose beads were then washed, resuspended in 1 x Laemmli buffer, and boiled for 5 minutes. Protein bands were then resolved by SDS-PAGE, after which Western blotting was conducted with mouse anti-nitro-tyrosine Ab (Millipore).

Fluorescence-based DSB repair assay and flow cytometry

The role of NO/RNS-dependent BRCA1 downregulation in DSB repair was studied using a GFP-based DSB repair system in MCF-10A and A549 cell lines as described previously (24, 25, 26) For more details of DSB assay and flow cytometry methods, see Supplementary Data and Supplementary Figs. S1 and S2.

NHEJ DRGFP-based assay

NHEJ events were detected by a PCR-based assay using GFP primers directed to sites flanking the I-SceI restriction site. At different time points after Ad-SceI-NG infection of MCF-10A/DRGFP cells (incubated with different concentrations of DETA), genomic DNA was isolated and used as a template for PCR with primers in a reaction volume of 50 µL. The sequences of primers were as follows: DRGFP-R: AAGTCGGCTGCTCTTATGG; DRGFP-F: TTGGGAAAAGATTCCAGATCC. PCRs were carried out by using the PCR 2xMaster Mix (Promega) with PCR Sprint Thermal Cycler (Thermo Scientific). After PCR amplification, a half volume of PCR products was digested for 6 hours at +37°C with 10 units of each of I-SceI and BglI (NEB). The nondigested controls and digested samples were separated on a 1.5% agarose gel. Cleaved products constitute HRR and SSA and DNA remaining and digested samples were separated on a 1.5% agarose gel. after which Western blotting was conducted with mouse anti-nitro-tyrosine Ab (Millipore).

DNA and RNA extraction, real-time quantitative PCR

Total DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen). Total RNA was isolated from the cultured cells following the manufacturer’s instructions with the RNaseasy kit (Qiagen). cDNA samples from breast cancer cells were amplified in triplicate from the same starting material of total RNA following the manufacturer’s instructions (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems). Samples were amplified using TaqMan MGB FAM dye-labeled probes from Applied Biosystems in an ABI7900HT model real-time PCR machine. The following probes were used: Hs99999901_s1 (18S rRNA) and Hs00173233_m1 (BRCA1).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay kit (Millipore) was used following the manufacturer’s instructions. ChIP assay primers for the proximal region of BRCA1 promoter: BRCA1-F: GATTGGGACCTCTTCTTACG; BRCA1-R: TACCAGAGCAGGGTTGAA (27, 28). ChIP assay antibodies: E2F1 (clone KH20&KH95, Upstate Biotech), E2F4 (clone C-20, Santa Cruz), and RBL2/p130 (clone C-20, Santa Cruz).

Results

Cell proliferation and NO/RNS concentrations

Previous reports show that NO/RNS production correlates with NO donor concentrations and time of incubation (29). Measurements, using an NO-specific electrode, of actual NO concentrations during cell exposure to 125 to 500 µmol/L of DETA indicated relatively constant NO concentrations in the 150 to 400 nmol/L range (29–31). The NO concentrations produced in vivo at sites of colonic crypt chronic inflammation and airway inflammation were below 300 nmol/L and below 400 nmol/L, respectively (30, 32). In comparison, normal in vivo NO concentrations in the absent of inflammation are unlikely to exceed 50 nmol/L (33). Hence, production of NO from 50 to 350 µmol/L DETA is comparable with those produced in vivo at sites of chronic inflammation.

MCF-10A and A549 were incubated with a range of concentrations of 2 different NO donors, DETA (half-life, 20 hours) and SNAP (half-life ~ 6 hours), to study the effects of physiologically relevant NO/RNS concentrations. At 24, 48, and 72 hours of incubation, the level of cell proliferation was estimated...
by a WST-1 assay (Fig. 1). Both NO donors, in a dose range 20 to 100 μmol/L, moderately stimulated proliferation. At concentrations of 200 μmol/L or higher, the NO donors were progressively more toxic and show dose-dependent inhibition of proliferation, which was consistent with a previous report (29). Hence, inflammatory-relevant concentrations of NO donors can be divided into a low toxicity subgroup (50–200 μmol/L) and a high toxicity subgroup (200–350 μmol/L). On the basis of these results, subsequent experiments used 100 to 200 μmol/L SNAP or DETA.

RNS-dependent activation of PP2A, RBL2 dephosphorylation, and BRCA1 downregulation

Studies by Ohama and colleagues recently showed one mechanism for NO/RNS-dependent stimulation of PP2A activity (34). Tyrosine nitration of PP2Ac released the PP2Aβ scaffolding subunit from the VCP/p97-PP2Ac complex thereby increasing PP2A enzymatic activity. In the present study, treatment of MCF-10A cells with exogenous NO/RNS donor SNAP also stimulated tyrosine nitration of the PP2Ac subunit (Fig. 2A) and a dose-dependent dissociation of PP2Aβ subunit from the VCP/p97 complex.
from the PP2Ac-VCP/p97 complex (Fig. 2B). The SNAP-induced PP2Ac nitration and PP2Aa dissociation from the PP2Ac-VCP/p97 complex stimulated PP2A activity as shown for both MCF-10A and A549 cells (Fig. 2C). About 100 to 200 µmol/L of the decomposed SNAP did not affect PP2A activity (data not shown).

It was previously reported that 2 members of E2F group, activating E2F1 and repressive E2F4, bind the BRCA1 proximal promoter (27, 28). Transcriptional regulation by these factors is dependent on their interactions with members of the Dp family of nuclear factors and with the hypophosphorylated forms of the repressive pocket proteins Rb, RBL1/p107, and RBL2/p130 (35, 36). Repression of BRCA1 is regulated via dephosphorylation of RBL2 with subsequent stimulation of RBL2/E2F4-repressive complex formation and increase of RBL2/E2F4 occupancy at the BRCA1 proximal promoter (27, 28, 37). The phosphorylation status of the RBL2 protein is regulated by PP2A (38). In the present work, I tested whether...
NO-Dependent Downregulation of BRCA1 Expression

NO/RNS-dependent stimulation of PP2A activity affects RBL2 phosphorylation and BRCA1 protein levels. After 6 hours of incubation, SNAP (100 μmol/L) stimulated significant RBL2 dephosphorylation and decreased BRCA1 protein expression in the MCF-10A and A549 cell lines (Fig. 3A). The same regimen of the treatment with decomposed SNAP did not affect RBL2 and BRCA1 proteins (data not shown). Incubation with the different doses of SNAP showed that RBL2 dephosphorylation and BRCA1 downregulation are dose-dependent (Supplementary Fig. S3). No significant changes were observed in E2F1, E2F4, and PP2Ac protein expression after treatment with SNAP (Fig. 3A).

The same result was achieved by stimulation of the endogenous iNOS. MCF-10A and RAW264.7 cells were co-cultured and iNOS in the RAW264.7 cells stimulated by incubation with 0.5 μg/mL of lipopolysaccharide (LPS) for 18 hours. After iNOS stimulation, RAW264.7 cells were labeled with anti-mouse F4/80 antibody, and nonlabeled MCF-10A cells were isolated by flow cytometry (Supplementary Fig. S2). A sufficient amount of NO/RNS, produced by activated iNOS in RAW264.7 cells, stimulated total tyrosine nitration, RBL2 dephosphorylation, and BRCA1 protein downregulation in co-cultured MCF-10A cells (Fig. 3B).

Stimulatory E2F1 and repressive E2F4 proteins simultaneously bind to the proximal *BRCA1* promoter and regulate *BRCA1* expression (28). E2F4 is a weak repressor in the absence of the pocket protein binding. RBL2 (35). RBL2 dephosphorylation and its nuclear accumulation leads to the formation of RBL2/E2F4 inhibitory complex, increasing E2F4 occupancy at the *BRCA1* proximal promoter, resulting in a dynamic shift of promoter occupancy from stimulatory E2F1 to repressive E2F4 (27, 28). To test whether NO/RNS-dependent RBL2 dephosphorylation stimulates the same effect, MCF-10A and A549 cells were incubated with 100 μmol/L SNAP for 6 hours and nuclear extracts analyzed for RBL2/E2F4 complex formation, E2F1/E2F4 *BRCA1* promoter occupancy, and *BRCA1* mRNA expression. SNAP treatment stimulates significant RBL2 nuclear accumulation and RBL2/E2F4 inhibitory complex formation in both cell lines (Fig. 3C). SNAP treatment also stimulated a moderate nuclear accumulation of E2F4 but had no effect on E2F1 nuclear accumulation.

A ChIP assay was used to determine whether the treatment with NO donor induces changes in the binding activity of the E2F1, E2F4, and RBL2 proteins to the *BRCA1* promoter. Following incubation with SNAP, ChIP assay results revealed a significant increase in promoter occupancy by RBL2 and E2F4 and a significant decrease in promoter occupancy by E2F1 (Fig. 3D). These effects were observed in both MCF-10A and A549 cell lines.

To further elucidate the mechanism by which NO/RNS repress *BRCA1* gene expression, mRNA levels of *BRCA1* were measured after incubation with SNAP. Results of quantitative real-time PCR (qRT-PCR) showed decreases in *BRCA1* mRNA expression of 2.5-fold (*P = 0.002*) and 2.2-fold (*P < 0.001*), respectively, for the MCF-10A and A549 cell lines.

In summary, these data provide evidence for a mechanism of NO/RNS-dependent inhibition of *BRCA1* gene expression. Figure 3F schematically illustrates this mechanism.

**NO/RNS reduces HRR, but not NHEJ**

To estimate the impact of RNS-dependent *BRCA1* downregulation on the level of HRR and NHEJ, MCF-10A and A549 clones stably transfected with a DR-GFP reporter construct (MCF-10A/DRGFP and A549/DRGFP) were generated. The reporter construct and the fluorescence-based assay for measuring the frequency of HRR at a single chromosomal DSB have been described (refs. 25, 39; Supplementary Fig. S1A). In cells incubated with different doses of an NO donor, as well as in control cells, infection with an F-SceI expression adenovirus (Ad-SceI-NG) generates equal amount of F-SceI and *BcgI* from uncleaved original DR-GFP construct. The 590-bp band in the I-SceI digest represents the product amplified from cells that have undergone HRR and have lost both restriction sites. B, NHEJ level (590-bp band) at different times following Ad-SceI-NG infection of MCF-10A/DRGFP cells incubated with different concentrations of DETA. Figure 5.

![Diagram](image_url)

**Figure 5.** NHEJ level in MCF-10A/DRGFP cells after treatment with different doses of DETA. A, after expression of I-SceI in MCF-10A/DRGFP cells, repair of the DSB can proceed through HRR or NHEJ. The level of NHEJ is measured by PCR amplification and digestion with I-SceI and BcgI. The 590-bp DNA fragment in the I-SceI digest represents the original PCR product amplified from cells that have undergone HRR or imprecise NHEJ. The 440-bp DNA fragment in the I-SceI + BcgI digest represents the product amplified from cells that have undergone HRR or from uncleaved original DR-GFP construct. The 590-bp band in the I-SceI + BcgI digest represents the product amplified from cells that have undergone imprecise NHEJ and have lost both restriction sites. B, NHEJ level (590-bp band) at different times following Ad-SceI-NG infection of MCF-10A/DRGFP cells incubated with different concentrations of DETA. Nontreated/noninfected cells were used as a negative control (top).
downstream GFP fragment (iGFP) as a template for HRR (ref. 40; Supplementary Fig. S1A and S1B). The percentage of GFP-positive cells after infection with Ad-SceI-NG represents the level of HRR in the test.

Initially, I tested whether downregulating BRCA1 expression by targeted siRNA inhibition also attenuated the level of HHR. MCF-10A/DRGFP cells were transfected with 12.5, 25, or 50 nmol/L of BRCA1 siRNA. Transfection with 50 nmol/L of nonspecific siRNA was used as a positive control. BRCA1 protein levels were measured by Western blotting 48 hours after siRNA transfection (Supplementary Fig. S1C). Cells were infected with Ad-SceI-NG 48 hours and after transfection, and after additional 48 hours, GFP expression was analyzed by flow cytometry. After transfection with BRCA1 siRNA, a dose-dependent decrease of HRR was observed, with a 9.4-fold decrease at 50 nmol/L of siRNA (Supplementary Fig. S1D).

Next, HRR was then measured in MCF-10A/DRGFP and A549/DRGFP cells treated with NO donors. Incubation with 100 or 200 μmol/L of SNAP inhibited HRR 1.9-fold and 7.4-fold, respectively, for MCF-10A/DRGFP cells and 2.3-fold and 4.0-fold, respectively, for A549/DRGFP cells. Incubation with 100 or 200 μmol/L of DETA was more effective at inhibiting HRR, with a 3.4-fold and 24.7-fold respective reduction in HRR for MCF-10A/DRGFP cells and a 2.7-fold and 20.9-fold respective reduction in HRR for A549/DRGFP cells (Fig. 4). Incubation with 100 to 200 μmol/L of decomposed NO donors (DETA or SNAP) did not affect HRR level in both cell lines (data not shown).

To determine whether NHEJ is also affected by RNS, the DSB repair products were analyzed by a PCR assay (Fig. 5A). MCF-10A/DRGFP cells were incubated with of DETA (100 or 200 μmol/L) and 6 hours after the start of this incubation were infected with Ad-SceI-NG. At different time points after Ad-SceI-NG infection, total DNA was extracted from the cells and the 590-bp genomic region surrounding the I-SceI site in the DR-GFP construct was amplified by PCR. The PCR amplification products are indicative of the types of repair for HRR, the I-SceI restriction site is replaced by BcgII site, whereas the imprecise repair of NHEJ is characterized by the absence of restriction sites for I-SceI and BcgII (Fig. 5A). To determine the level of NHEJ in each sample, the PCR amplification products were cleaved with I-SceI and BcgII (Fig. 5B). For each sample, uncleaved 590-bp DNA fragment represented the level of NHEJ repair, whereas 440-bp cleaved DNA fragment represented the level of HRR and original DR-GFP construct in which DSB was not generated.

Cells incubated with DETA showed a higher level of NHEJ than did the nontreated controls. Not only did incubation with DETA increase the level of NHEJ, but also the rate of repair. At
12 hours, both samples incubated with 100 to 200 μmol/L of DETA showed significant levels of NHEJ, whereas in controls, the first indication of NHEJ (uncleaved DNA fragment after restriction with I-Sce1 + BclI) appeared at 24 hours.

**NO/RNS-stimulated RBL2 dephosphorylation and BRCA1 downregulation are cell-cycle–independent**

MCF10A cells were incubated with 100 to 200 μmol/L DETA and cell-cycle distributions analyzed by flow cytometry (Fig. 6). A 6-hour incubation with 100 or 200 μmol/L DETA resulted in moderate accumulations of G0-G1 cells (65.7% and 70.3%, respectively, vs. 52.5% in control). After 12 hours with 100 μmol/L DETA or a 24-hour incubation with 200 μmol/L DETA, the cells entered into S-phase. This was followed by G2–M accumulation at 24 and 48 hours after treatment with 100 and 200 μmol/L DETA, respectively.

Both RBL2 dephosphorylation and BRCA1 expression downregulation were apparent by 6 hours of incubation with 100 or 200 μmol/L DETA, and these effects continued throughout a 24-hour incubation at either DETA concentration (Fig. 6). At the lower DETA concentration, BRCA1 expression and RBL2 phosphorylation levels returned to near normal by 48 hours. At 200 μmol/L DETA, the decreased BRCA1 expression and RBL2 phosphorylation persisted for at least 48 hours.

DETA-induced cell-cycle redistributions did not correlate with BRCA1 expression or RBL2 phosphorylation. For example, at the 12-hour time point where cells accumulated in G2, BRCA1 expression and RBL2 phosphorylation were downregulated, whereas at 24 hours, BRCA1 expression inhibition and RBL2 dephosphorylation persisted with cells entering the S- and G2–M cell-cycle phases. The absence of any correlation with cell cycle phase is also apparent at the 48-hour time point after incubation with 200 μmol/L DETA.

**Inhibition of PP2A activity or RBL2/E2F4 proteins expression attenuates RNS-dependent BRCA1 downregulation**

The above experiments indicate that RNS-dependent downregulation of BRCA1 expression pathway involves 3 proteins PP2A, RBL2, and E2F4. Hence, blocking the activity or expression of the one of these proteins should inhibit the whole pathway and prevent RNS-dependent BRCA1 downregulation.

To determine whether inhibiting PP2A activity abrogates NO/RNS-dependent BRCA1 downregulation, MCF-10A cells were preincubated with 0.3 nmol/L okadaic acid (2 hours before the start of incubation with SNAP). Although OA is a specific inhibitor of both PP2A and PP1, at this dose, OA selectively inhibits the activity of PP2A. Preincubation with OA inhibited SNAP-stimulated dephosphorylation of RBL2 and downregulation of BRCA1 (Fig. 7A). Targeted siRNA inhibition of RBL2 or E2F4 expression also prevented SNAP-dependent BRCA1 downregulation (Fig. 7B and C).

Subsequent experiments tested whether attenuation of NO/RNS-dependent BRCA1 downregulation prevented reduction of HRR. MCF-10A/DRGFP cells were transfected either with control siRNA, RBL2 siRNA, or E2F4 siRNA. Incubation with 100 μmol/L SNAP was started 24 hours after transfection and the cells analyzed for HRR as described above (Fig. 7). Cells transfected with control siRNA showed a 2.6-fold decrease of HRR level after incubation with SNAP compared with cells not treated with SNAP. Cells transfected with RBL2 siRNA showed a 1.2-fold decrease (P < 0.001) of HRR level after incubation with SNAP compared with untreated controls. Cells transfected with E2F4 siRNA showed a 1.4-fold decrease (P = 0.002) of HRR level after incubation with SNAP relative to untreated controls. In summary, these data show that PP2A inhibition, or the blocking of RBL2, or E2F4 expression inhibits NO/RNS-dependent BRCA1 downregulation, thereby preventing the NO/RNS-dependent decrease in HRR.

**Discussion**

The generation of NO/RNS under inflammatory conditions provides a critical link between inflammation and cancer initiation, promotion, and progression (6, 33). NO/RNS production is often associated with contradictory effects on cell proliferation and cytotoxicity, variably promoting and inhibiting apoptosis in normal and tumor cells (10, 26, 41). Wink and
colleagues have examined these contradictory observations and have proposed a set of 5 graduated levels of NO/RNS cellular responses that range from the promotion of cell survival and proliferation at low concentrations of NO/RNS to the promotion of cell-cycle arrest and apoptosis at high concentrations of NO/RNS (10).

While high concentrations of NO/RNS can cause direct DNA damage and stimulate DNA DSBs, there is an emerging appreciation for determining the role of lower NO/RNS concentrations in signaling pathways related to apoptosis, cell-cycle, and other facets of cell functions. The present work shows a mechanism whereby inflammatory-relevant, low concentrations of NO/RNS inhibit BRCA1 expression without affecting cell proliferation. This inhibition of BRCA1 expression significantly reduces the ability of cells to fix the DNA DSB through HRR with a moderate increase of error-prone NHEJ. Hence, inflammatory relevant concentrations of NO/RNS (associated with low cell toxicity and lack of interference with cell proliferation) stimulate genetic instability by inhibiting BRCA1 expression and shifting DNA DSB repair from high-fidelity HRR to error-prone NHEJ.

The key step of the mechanism described in the present work is the dephosphorylation of the RBL2 protein and the subsequent formation of the RBL2/E2F4 inhibitory complex. The RBL2/E2F4 complex has been previously shown to bind to the promoters of cell-cycle–dependent genes and to suppress cell proliferation (42). However, to be able to suppress cell proliferation, RBL2 and E2F4 proteins have to be assembled into the DREAM complex with DP1 and 5 MuvB-like proteins (43). In contrast, the present article shows that formation of RBL2/E2F4 does not affect the cell cycle. Hence, it is possible that low toxic NO/RNS concentrations stimulate formation of RBL2/E2F4 inhibitory complex without its recruitment into the DREAM complex. Future investigations might shed light on how different concentrations of NO/RNS affect the DREAM complex formation.

The proposed model of NO/RNS-generated genetic instability is not restricted to the inflammatory environment. Different types of NOs are activated after ionizing radiation (IR) and under hypoxia (44, 45). PP2A activation and stimulation of RBL2/E2F4 inhibitory complex formation were also shown after IR and under hypoxia (27, 28, 46). Aging, another condition connected to carcinogenesis, is also characterized by increased activity of NOs and total protein nitration (47). Further investigation should determine whether all these states (IR, hypoxia, and aging), which are critical for carcinogenesis and tumor development, have decreased genetic stability modulated through the same mechanism described in the present article.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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