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Werner Syndrome Protein, WRN, Protects Cells from DNA Damage Induced by the Benzene Metabolite Hydroquinone

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Werner syndrome (WS) is a rare autosomal progeroid disorder caused by a mutation in the gene encoding the WRN (Werner syndrome protein), a member of the RecQ family of helicases with a role in maintaining genomic stability. Genetic association studies have previously suggested a link between WRN and susceptibility to benzene-induced hematotoxicity. To further explore the role of WRN in benzene-induced hematotoxicity, we used short hairpin RNA to silence endogenous levels of WRN in the human HL60 acute promyelocytic cell line and subsequently exposed the cells to hydroquinone (HQ). Suppression of WRN led to an accelerated cell growth rate, increased susceptibility to hydroquinone-induced cytotoxicity and genotoxicity as measured by the single-cell gel electrophoresis assay, and an enhanced DNA damage response. More specifically, loss of WRN resulted in higher levels of early apoptosis, marked by increases in relative levels of cleaved caspase-7 and cleaved poly (ADP-ribose) polymerase 1, in cells treated with HQ compared with control cells. Our data suggests that WRN plays an important role in the surveillance of and protection against DNA damage induced by HQ. This provides mechanistic support for the link between WRN and benzene-induced hematotoxicity.

Key Words: **Werner syndrome protein (WRN); benzene-induced hematotoxicity; DNA damage; apoptosis and poly (ADP-ribose) polymerase 1 (PARP-1).**

Werner syndrome (WS) is a rare autosomal premature aging syndrome associated with a predisposition to cancer and genomic instability (Oshima, 2000). Fibroblasts from WS individuals have a decreased life span, an extended S phase, and display an elevated rate of chromosomal translocations and genomic deletions (Fukuchi *et al.*, 1989; Gebhart *et al.*, 1988; Salk *et al.*, 1985; Takeuchi *et al.*, 1982). WRN, the protein defective in WS, belongs to the RecQ family of helicases, which are conserved from *Escherichia coli* to humans (Karow *et al.*, 2000). Although the RecQ helicases display 3′–5′ unwinding directionality on a variety of DNA structures, WRN is unique in that it also possesses 3′–5′ exonuclease activity (Bohr *et al.*, 2000; Huang *et al.*, 1998). Furthermore, WRN has been shown to interact physically and functionally with a number of cellular proteins, and has been subsequently implicated in several DNA metabolic processes (Opresko *et al.*, 2003). More specifically, hypersensitivity of cultured WS-derived cells to DNA damaging agents that produce reactive oxygen species (ROS), such as camptothecin (Poot *et al.*, 1999) and 4-nitroquinoline-1-oxide (Kodama *et al.*, 1998; Poot *et al.*, 2002), suggests that WRN may play a role in sensing oxidative DNA damage and in the repair of DNA lesions caused by these agents.

DNA double-stand breaks (DSBs) are one of the most severe lesions caused by genotoxic agents because both DNA stands are affected and erroneous end joining can occur. The genotoxic and cytotoxic implications of DSBs thus potentially compromise the genomic integrity of a cell. DSBs are repaired *in vivo* by either non-homologous end-joining or homologous recombination (Christmann *et al.*, 2003). Recently, WRN has been implicated in DSB repair. As such, WRN has been shown to interact with Ku70/80 heterodimer and PRKDC (protein kinase, DNA-activated, catalytic polypeptide) (Karmakar *et al.*, 2002a, b; Li and Comai, 2000; Orren *et al.*, 2001) and may participate in non-homologous end-joining (Li and Comai, 2002). Additionally, recent studies have demonstrated that WRN physically interacts with the Mre11-Rad50-NBS1 complex, which functions in homologous recombination for DSB processing (Cheng *et al.*, 2004). One source of DSBs is the direct attack of DNA by ROS produced from benzene metabolites such as hydroquinone (HQ) (Winn, 2003). HQ can directly or indirectly induce DNA DSBs and chromosomal aberrations, and may contribute to the development of acute myeloid leukemia by increasing the number of genetic lesions in hematopoietic cells (Gowans *et al.*, 2005). Thus the efficient repair of DSBs by proteins like WRN would be expected to be fundamental for the maintenance of genomic stability and cancer prevention in the presence of benzene (Whysner *et al.*, 2004).

In collaboration with the National Cancer Institute and China CDC, we reported that four SNPs located in the functional domain of *WRN* were associated with benzene hematotoxicity

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in benzene-exposed workers (Shen *et al*., 2006a). To further explore WRN's role in benzene toxicity susceptibility *in vitro*, we recently used short interfering RNA in HeLa cells and determined that when exposed to the benzene metabolite HQ, depletion of WRN resulted in an increased DNA damage response in HeLa cells (Galvan *et al*., 2008). In the present study, we investigated WRN's role in benzene hematotoxicity by using short hairpin RNA (shRNA) to target and stably silence endogenous WRN levels in the acute promyelocytic cell line, HL60, treated with HQ. Using this cell model, we subsequently examined HQ's effect on cell proliferation, apoptosis, and DNA strand break production, as well as the mechanism for the DNA damage response. Our results showed that loss of WRN heightens susceptibility to HQ treatment and suggests a possible role for WRN in DNA damage repair in the presence of benzene.

MATERIALS AND METHODS

Cell culture and chemical treatments. The human HL60 acute promyelocytic cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown in Iscove's Modified Dulbecco's Medium (Gibco, San Diego, CA) with L-glutamine, 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 20% fetal bovine serum (Omega Scientific, San Diego, CA), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Omega Scientific) in standard conditions. HQ (Sigma-Aldrich, St Louis, MO) was dissolved in 1× PBS for all experiments. Cells were dosed with 1× PBS and 10, 20, or 50μM HQ at a cell density of 4–5 × 10^5 cells/ml.

Generation of shRNA and retroviral transduction. Stable WRN shRNA clones were generated using Invitrogen's BLOCK-iT RNAi Designer (Carlsbad, CA), which employs a rigorous algorithm to control for and prevent non-specific knockdown. Several 50-bp shRNA sequences targeting human WRN mRNA (NM_000553) were cloned into Invitrogen's pLenti6/BLOCK-iT-DEST lentiviral vector. The cDNA sequence of the WRN shRNA construct (sh-WRN) used in all experiments was 5′-CACCGCACCTTCTTACTGAGA-TACGCGAACGTATCTCAGTAAGAAGGTGC-3′. The nonsilencing shRNA control was modified from the nontarget shRNA control (Sigma-Aldrich), and the control sequence used for the shRNA (sh-NSC) was 5′-CACCGCAACAA-GATGAAGAGCACCAACGAATTGGTGCTCTTCATCTTGTTGC-3′. Using human embryonic kidney 293FT cells stably expressing the SV40 large T antigen, lentiviral stocks were produced, concentrated, and tittered according to the manufacturer's protocol (Invitrogen). HL60 cells were then transduced by centrifugation in 24-well plates at 970 g for 90 min at 32°C. Forty-eight hours after infection, stable cell lines were generated by selection with 6 μg/ml of the antibiotic blasticidin (selectable marker in the viral vector). Single clones resistant to blasticidin were isolated and assayed for mRNA and protein expression levels using PCR and Western blot analysis respectively. Results were compared with those for cells infected with the vector expressing nonsilencing control shRNA.

Cell proliferation and apoptosis analysis. Control (HL60 sh-NSC) and WRN knockdown (HL60 sh-WRN) cell lines were seeded at 2 × 10^5 and allowed to grow under normal conditions for 7 days. Each day, cells were enumerated using a hemocytometer and the trypan blue exclusion assay to determine cell proliferation and viability. Normal cell proliferation was measured in triplicate in three independent experiments.

For analysis of apoptosis, HL60 sh-NSC and HL60 sh-WRN cells were exposed to 0, 10, 20, or 50μM HQ for 24 h. Cells were collected and stained with propidium iodide and Annexin V-fluorescein isothiocyanate according to the manufacturer's protocol (BD Pharmingen, San Diego, CA). In two independent experiments done in triplicate, at least 1 × 10^4 cells were analyzed on a Beckman Coulter EPICS XL-MCL flow cytometer using the manufacture's System II software (Beckman Coulter, Fullerton, CA).

Single-cell gel electrophoresis. The alkaline single-cell gel electrophoresis assay (Comet assay) was performed as previously described (Singh *et al*., 1988) with some modifications. Cells were exposed to HQ for 6 and 24 h prior to preparation for analysis of DNA damage in the comet assay. Five hundred randomly chosen cells per slide were scanned and analyzed automatically using CometScan imaging software (Metasystems, Germany). Cells were subsequently screened manually to exclude cells that did not meet stringent requirements (i.e., poor staining, loss of focus, or oddly shaped). The average of the mean for tail intensity (Collins, 2002), a measure of total DNA damage, was calculated from about 400 cells at the 6- and 24-h time points. All slides were coded to prevent observer bias.

Immunoblot analysis. Total cell lysates were collected from 5 × 10^6 cells using 300 μl of radioimmunoprecipitation assay lysis buffer. Protein concentrations were determined by the DC assay (Bio-Rad, Hercules, CA). Equal protein amounts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and immunoblotted for WRN (Santa Cruz Biotechnology, Santa Cruz, CA), γH2AX, cleaved poly(ADP-ribosyl)ation (PARP-1), cleaved caspase-7, cytochrome *c* (Cell Signaling Technology, Danvers, MA), and actin (Sigma-Aldrich). Proteins were visualized using the enhanced chemiluminescence method per manufacturer's protocol (Amersham Biosciences, UK). Film was exposed and developed using the Konica SRX-101 developer (Konica Minolta Medical Imaging USA, Wayne, NJ).

Data analysis. Statistical analyses of data were performed using one-way analysis of variance. Each measured protein was normalized to β-actin, the loading control, and quantified using ImageJ software (NIH, Bethesda, MD). Data was the representative or the averages of at least three independent experiments. Error bars represent SEM. $*p < 0.05$; $**p < 0.01$.

RESULTS

Stable WRN Depletion by RNA Interference Leads to an Accelerated Proliferation Rate in HL60 Cells

To investigate the effects of acute depletion of WRN in human hematopoietic cells, we used lentivirus-based RNA interference (RNAi) to knockdown WRN in HL60 cells, as lentivirus-based vectors have been shown to transduce a variety of nondividing and hard to transfect cell types (Kafri *et al*., 1997; Naldini *et al*., 1996; Uchida *et al*., 1998). After isolating single clones, transduced with either a control (HL60 sh-NSC) or WRN (HL60 sh-WRN) shRNA construct, through blasticidin selection and limiting dilution, mRNA and protein levels remained almost completely suppressed for at least a month in two clones (Clone 1 was selected for further analysis) (Fig. 1A). In contrast, HL60 sh-NSC cells constitutively expressed WRN at a level comparable to normal control cells. These results demonstrate that specific, stable suppression of WRN was achieved in the hematopoietic cell line HL-60 using a lentiviral vector.

As cells derived from WS patients have a reduced life span in vitro before entering replicative senescence (Salk, 1982), we sought to determine if the depletion of WRN by RNAi affected the normal growth characteristics of the HL60 cells, by culturing and counting both HL60 sh-WRN and HL60 sh-NSC

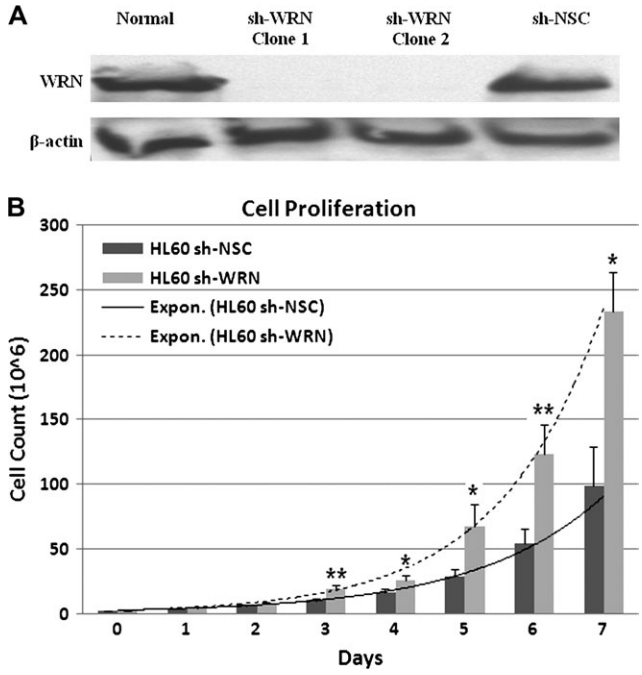

FIG. 1. Acute knockdown of WRN expression by RNAi in human hematopoietic HL60 cells leads to accelerated proliferation. (A) Immunoblot analysis showing the specific decrease of WRN protein in HL60 cells. Whole cell lysates were collected from the cells transduced with nonsilencing shRNA (HL60 sh-NSC) and shRNA constructs targeting WRN (HL60 sh-WRN). (B) Accelerated proliferation of HL60 cells following WRN depletion. HL60 sh-NSC and HL60 sh-WRN cells were cultured over a 7-day period and cell proliferation was evaluated using a hemocytometer and the trypan blue exclusion assay. Total cell numbers were plotted against day of initiation (*$p < 0.05$, **$p < 0.01$), and exponential trend lines were used to display the trends for the different proliferation patterns between the two cell lines. The data represents the average of three independent experiments.

cell lines, over a 7-day period. We found that HL60 sh-WRN cells displayed a significantly accelerated proliferation rate over the 7 days of culture compared with control HL60 sh-NSC cells (Fig. 1B), resulting in a doubling of total cell numbers by the end of the culture period. The growth characteristics of the control cells remained unaltered by the nonsilencing shRNA (data not shown).

Loss of WRN Leads to Higher Levels of DNA Damage following HQ Treatment in HL60 Cells

Benzene metabolites, such as HQ, can induce DNA DSBs and other forms of DNA damage which may be causal for benzene-induced acute myeloid leukemia (Gowans *et al.*, 2005). To assess the effects of WRN depletion on DNA damage induced by HQ exposure, we used the COMET assay. Representative images of damaged and undamaged HL60 sh-NSC and HL60 sh-WRN cells are presented in Figure 2A. Analysis of both HL60 sh-NSC and HL60 sh-WRN cells at 6 and 24 h demonstrated that HQ induced a dose-dependent increase in DNA damage in both cell lines, as measured by tail intensity (Figs. 2B and 2C). However, DNA damage resulting from HQ treatment was significantly greater in the HL60 sh-WRN cells as compared with HL60 sh-NSC cells after 6 h of exposure, especially at 20μM HQ where the maximal difference in DNA damage between the two lines was detected (Fig. 2B). Although resultant tail intensities at the 24-h time point were decreased slightly for all doses of HQ compared with the 6-h time point, representative DNA damage remained significantly higher in HL60 sh-WRN cells at the 50μM dose (10-fold increase over no treatment controls) compared with HL60 sh-NSC (sevenfold increase over no treatment controls) (Fig. 2C), suggesting an inability to efficiently repair DNA damage at higher doses of HQ, in the presence of suppressed WRN.

To further substantiate our finding of increased DNA damage associated with the suppression of WRN, we measured the protein levels of phosphorylated H2AX (γH2AX), a surrogate marker for DNA damage. It is well established that in response to DNA DSBs, H2AX is rapidly phosphorylated by upstream kinases at the site of DNA damage, thus allowing for the assembly of checkpoint and DNA repair factors in many cells (Celeste *et al.*, 2002; Rogakou *et al.*, 1999; Rothkamm and Lobrich, 2003). In asynchronous HL60 cells, immunoblot analysis revealed a dose-dependent increase of γH2AX levels at 24 h (Fig. 2D) and higher levels of γH2AX protein levels in WRN-depleted cells compared with HL60 sh-NSC cells. This effect was most pronounced at the 20μM dose in response to which HL60 sh-WRN cells displayed a significant sixfold increase and HL60 sh-NSC a 1.7-fold increase of γH2AX protein levels compared with untreated control cells (data not shown). In addition, we measured WRN protein levels in both cell lines after HQ treatment. WRN remained silenced in HL60 sh-WRN cells, and whereas WRN protein levels in HL60 sh-NSC cells varied slightly by HQ dose, in general its expression was also reduced by HQ treatment (Fig. 2D), but by a much lesser degree than that of suppression by RNAi. These results further demonstrate the increased gentoxicity arising from the suppression of WRN in the presence of HQ.

Loss of WRN Enhances Levels of Early Apoptosis Mediated by Caspase-7 in HL60 Cells following HQ Treatment

Flow cytometric analysis was used to determine the percentage of apoptotic and necrotic cells in cell cultures treated with HQ for 24 h. In order to better examine early apoptosis, we limited our analysis to intact cells. As can be seen in Figures 3A and 3B, suppression of WRN expression had little effect on the percentage of early apoptotic cells in nontreated controls. However, treatment with increasing concentrations of HQ resulted in significant dose-dependent increases in the levels of early apoptosis in both cell lines and a significantly higher percentage of early apoptosis in all treated HL60 sh-WRN cell cultures compared with control cells (Figs. 3A and 3B). More specifically, treatment of control

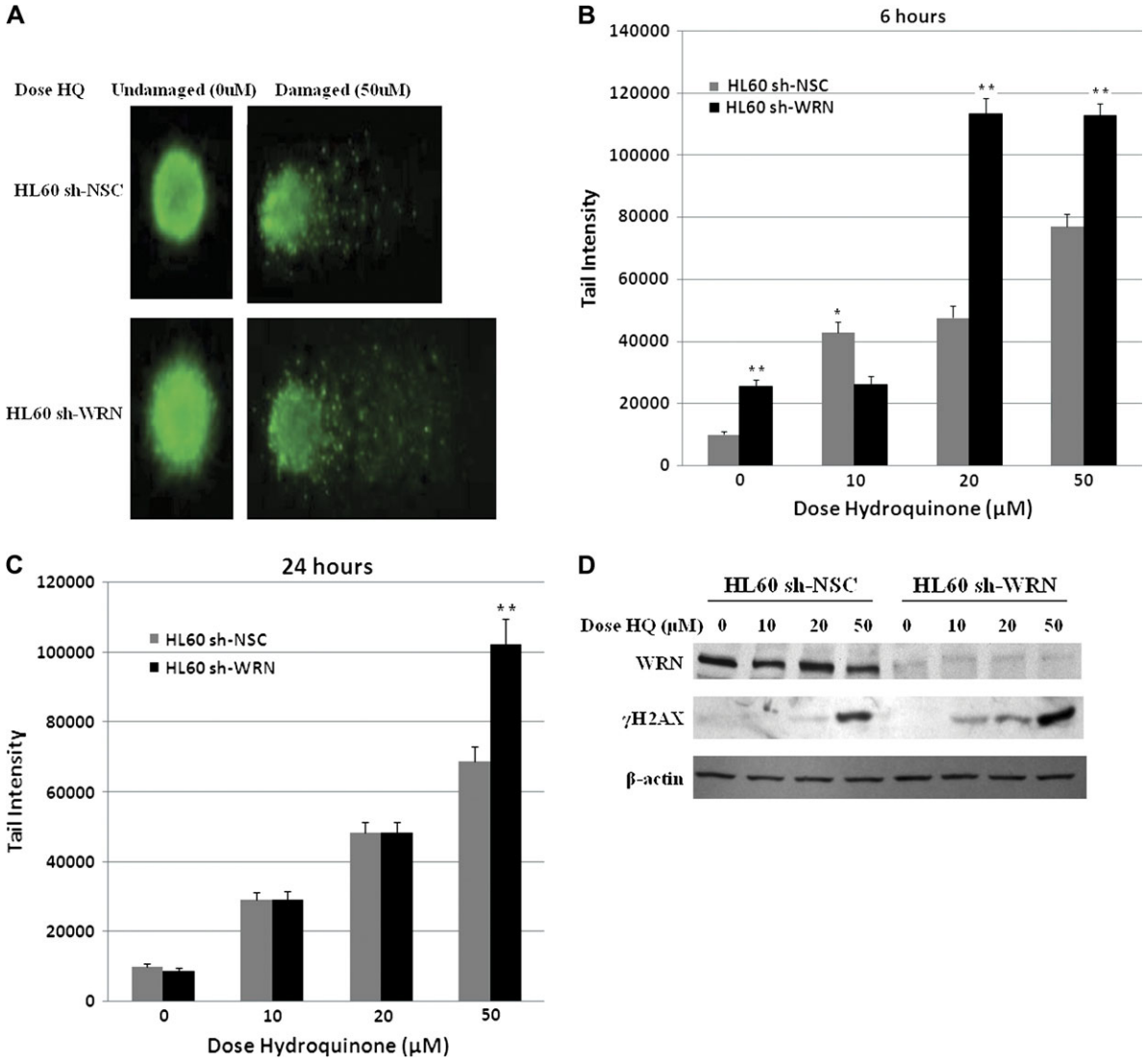

FIG. 2. WRN depletion in HL60 cells increases susceptibility to HQ-induced DNA damage. (A) Representative images of undamaged and damaged HL60 sh-NSC and HL60 sh-WRN cells with either vehicle or 50μM HQ treatment are shown for the 6-h time point. (B and C) Analysis by single-cell gel electrophoresis demonstrates that loss of WRN results in increased levels of DNA damage following 6- (B) and 24-h (C) HQ treatments. The average and standard error of the mean at the 6- and 24-h time point are shown. (D) Accumulation of phosphorylated histone γH2AX protein levels of cells exposed to HQ for 24 h correlate in a dose-dependent manner with tail intensity measurements obtained from the comet assay analysis of cells exposed to HQ for 24 h. WRN protein level in HL60 sh-NSC cells is slightly reduced by HQ treatment, and WRN level remains silenced in HL60 sh-WRN cells. Western blots are representative of three independent experiments.

cells with 50μM HQ resulted in a fivefold increase in the percentage of early apoptotic cells, whereas WRN-depleted cells resulted in a sevenfold increase over untreated HL60 sh-NSC cells. These results demonstrate that HQ produces higher levels of apoptosis in WRN-depleted cells.

Given the p53 null background of the HL60 cell line, apoptosis is most likely mediated by another pathway such as through the release of cytochrome *c*. During stress-induced apoptosis (*in vitro* and *in vivo*) cytochrome *c* is released from the mitochondria and cytosolic cytochrome *c* forms an essential part of the vertebrate "apoptosome," which is composed of cytochrome *c*, Apaf-1, and procaspase-9 (Li *et al*., 1997). The result is the activation of initiator caspase-9, which then processes and activates other effector caspases, such as caspase-3 or -7, to orchestrate the biochemical execution of cells, cleaving PARP-1, the highly utilized marker of apoptosis in many cell types (Kaufmann *et al*., 1993). In order to determine whether this apoptotic pathway was activated by HQ, cell lysates were prepared from HL60 sh-NSC and HL60 sh-WRN cells that had been untreated or treated for 24 h with HQ, and immunoblotting was performed with antibodies to cleaved caspases-3, -7, and PARP-1, and cytochrome *c*.

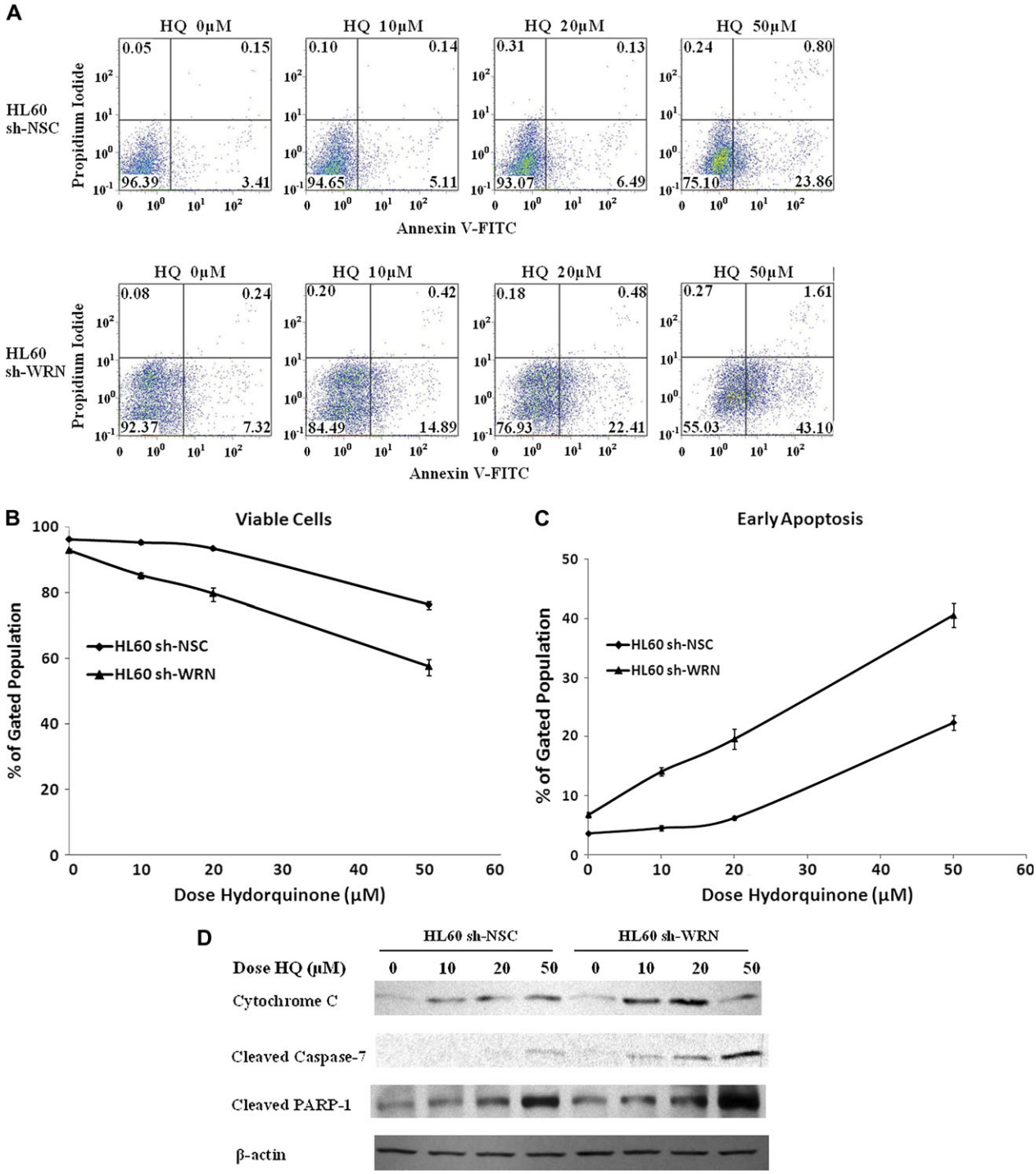

FIG. 3. HQ induces enhanced apoptotic response in WRN-depleted HL60 cells. (A) HL60 cells were exposed to HQ for 24 h, and subjected to flow cytometric analysis. A gate parameter to exclude cell debris was set to analyze intact cells for apoptosis after HQ treatment. Staining was used to discriminate among early apoptotic (lower right quadrants) and late apoptotic (upper right quadrants) cells for both cell lines. (B) Quantification of results in (A). HQ treatment led to increases in early apoptosis levels and decrease in viable cells in a dose-dependent manner in both cell lines. However, the combinational loss of WRN and HQ treatment resulted in a significantly (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) higher level of apoptosis at all doses of HQ treatment. (C) WRN depletion and HQ exposure results in the release of cytochrome *c*, which leads to increased cleaved caspase-7 and PARP-1 protein levels as measured by immunoblot analysis. β-Actin is used as a loading control. Western blots are representative of three independent experiments.

Treatment with HQ resulted in the release of cytochrome *c* into the cytosol and the subsequent cleavage of procaspase-7 to its p19 subunit (Fig. 3C), but not of procaspase 3 (data not shown). At the lower doses of 10 and 20μM HQ, HL60 sh-NSC cells displayed a threefold increase in cytosolic cytochrome *c*, whereas HL60 sh-WRN cells displayed increases of 5- and 6.7-fold, respectively, in comparison to untreated control cells, indicating an increased sensitivity to HQ in the absence of WRN. Interestingly, we did not observe a strong cytochrome *c* release in the 50μM HQ treated HL60 sh-WRN cells at 24 h. It is probably because at this highly toxic dose, apoptosis may have been initiated much earlier than 24 h after exposure and the released cytochrome *c* may have partially degraded as the apoptotic process increased levels of cleaved PARP-1 and caspase-7. Immunoblot analysis revealed a dose-dependent increase in the expression levels of cleaved PARP-1 in both cell lines, indicating the effect of HQ on apoptosis induction (Fig. 3C). However, HL60 sh-WRN cells, depleted of WRN, displayed a higher level of PARP-1 cleavage regardless of the dose of HQ, in which cleaved PARP-1 in HL60 sh-WRN cells was 1.2-, 1.4-, and 2.1-fold higher than in the control cells. Furthermore, there was a significantly elevated amount of caspase-7 cleavage at all doses of HQ in HL60 sh-WRN cells (Fig. 3C), the time at which PARP was cleaved, suggesting that caspase-7 could effectively cleave PARP *in vivo*. Our results indicate that hematopoietic HL60 cells, depleted of WRN, display an increased susceptibility to stress-induced apoptosis mediated by caspase-7. Together, the higher levels of DNA DSBs and apoptosis observed in WRN-depleted cells, lend support to a probable role for WRN in the DNA repair process.

DISCUSSION

The protein responsible for WS, WRN, belongs to the RecQ family of helicases that are believed to be important for maintaining genomic integrity by facilitating accurate detection and repair of DNA damage (Cobb *et al*., 2002). Although genetic association studies have implicated WRN in the susceptibility to benzene toxicity and risk of non-Hodgkin lymphoma (Shen *et al*., 2006a, b), the role of WRN in benzene-induced hematotoxicity has yet to be fully addressed. Previously, we reported that suppression of WRN expression enhances DNA damage in HeLa cells exposed to the benzene metabolite, HQ (Galvan *et al*., 2008). However, as the major health concerns from benzene exposure are toxicity to the blood and bone marrow and the induction of hematological malignancies, use of a cell model that is more relevant to benzene hematotoxicity would be more informative. In the present study, therefore, we investigated the effects of the benzene metabolite, HQ, on the human acute promyelocytic cell line HL60, in which we stably suppressed WRN by infection with a retrovirus expressing shRNA specifically targeting the WRN transcript. We achieved greater than 85% knockdown of WRN and analyzed the cellular responses to this stable WRN depletion alone and in conjunction with HQ treatment.

In HeLa cells, suppression of WRN expression caused a decrease in cell proliferation (Galvan *et al*., 2008). WRN depletion in HL60 cells resulted in a significant increase in cell proliferation rate, which could increase HQ genotoxicity by reducing the amount of time for repair and increasing fixation of mutation and chromosomal aberrations. Although that finding was in concordance with previous reports showing that loss of WRN leads to decreased cell proliferation and increased senescence (Grandori *et al*., 2003; Szekely *et al*., 2005), we believe that the observed increase in proliferation rate in the HL60 cells may be related to its p53 null status. Depleted of both WRN and p53, the HL60 cells responded similarly to Wrn mutant mice with a p53-deficient background, in which the synergistic effect of the Wrn and p53 mutations led to rapid tumorigenesis, including that of leukemias and lymphomas (Lebel *et al*., 2001). It has been suggested that in an already unstable p53 null cell line, the functional loss of the Wrn protein could further increase genomic instability, give rise to additional mutations, and thus accelerate tumor progression in a Wrn mutant background. Our results lend support to such a hypothesis.

Despite an increased proliferation rate, WRN depletion enhanced the cytotoxity and genotoxicity of HQ. This was demonstrated by the reduced cell survivability and significantly elevated levels of both DNA strand breaks and early apoptosis compared with controls cells transduced with nonsilencing shRNA, following HQ exposure. There was no significant change in the expression of γH2AX in both untreated cell lines. However, after HQ treatment, a synergistic induction of γH2AX level was seen at all doses compared with control cells (Fig. 2D). The mechanism underlying these cellular responses to WRN depletion and HQ treatment is most likely related to increased oxidative damage and replicative stress.

Previous in vitro studies have demonstrated that peroxidatic metabolism of HQ to its semiquinone radicals can reduce dioxygen to superoxide anion radicals (Sadler *et al*., 1988) and that further subsequent redox reactions have the potential to produce large amounts of ROS (Ruiz-Ramos *et al*., 2005). ROS targets DNA, generating lesions such as oxidized bases and DNA strand breaks (rev. in Cadet *et al*., 1999). In the test tube, HQ and benzoquinone (BQ) are able to generate ROS and induce DSBs (Yu and Anderson, 1997). Several observations suggest that ROS formation may directly induce DNA double-strand breaks, for example, treatment with H_2O_2 induced DSBs (Yu and Anderson, 1997), and cellular oxygen tension caused chromosome breaks and DSBs (Karanjawala *et al*., 2002). A crucial pathway for the repair of oxidative DNA lesions is base excision repair (BER). However, these lesions can be converted to DSBs during BER and DNA replication (Haber, 1999). Thus, it is tempting to speculate that ROS can indirectly or directly generate DNA DSBs. Although DSBs are generally repaired by either homologous recombination or

non-homologous end joining, recombinational repair is not error-free and erroneous repair has been shown to lead to genomic instability and carcinogenesis (Ramel *et al*., 1996). Several benzene metabolites, including HQ, have been shown to initiate oxidative DNA damage in HL60 cells (Kolachana *et al*., 1993). As WRN has proposed roles in BER (see Lee *et al*., 2005) and recombinational repair pathways (see Bachrati and Hickson, 2003), WRN may have specific functions in relation to the processing of different DNA lesions initiated by HQ. More specifically, because WRN exonuclease arrests at certain oxidative lesions (Machwe *et al*., 2000) and has been proposed to function as a proofreader for polymerases that lack intrinsic proofreading ability (Shevelev and Hubscher, 2002), it has been suggested that WRN may act as a sensor of oxidative damage (Von Kobbe *et al*., 2004). Indeed, we detected elevated levels of DNA strand breaks after combinational loss of WRN and HQ treatment, suggesting that the HQ-induced lesions may not have been efficiently detected or repaired, thus allowing the damage to accumulate. Considering the limited redundancy between the RecQ helicases, loss of this proposed sensor function could be detrimental to genomic integrity.

Given that WS cells are defective in the homologous recombination pathway (Hickson, 2003), both cellular and biochemical evidence implicates a role for WRN in the recombinational repair of DSBs (Prince *et al*., 2001; Saintigny *et al*., 2002). A recent report showed that HQ and BQ exposure generated γH2AX (Ishihama *et al*., 2008), a well-established marker of DNA DSBs (Celeste *et al*. 2002; Rogakou *et al*. 1999; Rothkamm and Lobrich, 2003). It has been proposed that soon after DNA damage, γH2AX targets the site of DSBs, followed by the association of the Mre11/Rad50/Nbs1 complex and Nbs1 recruitment of WRN for the optimization of DNA DSB repair during HR (Cheng *et al*., 2005). Considering the role of Nbs1 in recruiting other repair proteins or signal factors during DNA DSB repair, the elevated γH2AX levels seen in the WRN-depleted cells treated with HQ may reflect the accumulation of DSBs resulting from the deficiency of WRN and the limited redundancy of repair proteins. Furthermore, WRN's involvement in non-homologous end joining (NHEJ) through the binding and recruitment to DNA by Ku70/80 (Cooper *et al*., 2000; Karmakar *et al*., 2002b; Li and Comai, 2000) again implicates its probable role in DSB repair. As WRN has reported roles in several repair mechanisms important for the recovery from lesions induced by HQ, our data lend support to WRN's role in the promotion of cellular survival and proliferation in the presence of DNA damage or oxidative stress.

The data presented here also demonstrate that the elevated apoptosis levels were mediated by cytochrome *c* release, caspase-7 activation, and PARP-1 cleavage. It was previously reported that in WS cells, p53-mediated apoptosis is attenuated (Spillare *et al*., 1999). However, given the p53 null background and the increased levels of apoptosis seen in the WRN-depleted cells in the present study, apoptosis is most likely mediated by another pathway. In the presence of single and double-strand DNA breaks, PARP-1 binds to these breaks and poly (ADP-ribosyl)ates several nuclear proteins. With a role in DNA repair, it was demonstrated that the poly(ADP-ribosyl)ation of the nuclear proteins occurred early in apoptosis, prior to the commitment to death (Simbulan-Rosenthal *et al*., 1999). In addition to DNA repair, PARP-1 can induce apoptosis or necrosis if there is sustained activation of PARP-1. However, it was recently shown that loss of WRN prevents the activation of PARP-1 in response to DNA damage caused by oxidative damage (von Kobbe *et al*., 2003). As WRN may act as a surveyor of DNA damage in BER, HR, and NHEJ, the lack of WRN may result in the loss of this monitoring system, which could lead to the activation of apoptosis in response to the oxidative damage resulting from HQ treatment. As such, we propose that the mechanism by which HQ treatment induces apoptosis is through the release of cytochrome *c*. Although it is still unclear how cytochrome *c* is released, much evidence has accumulated suggesting that its release from the mitochondria is an important step in the apoptotic pathway (see Reed, 1997, for review). Although we believe that cytochrome *c* release led to Apaf-1 binding, cleavage of procaspase-9, followed by activation of procaspase-7, and PARP-1 cleavage and inactivation, further work is necessary to determine the sequence of events.

In conclusion, our findings suggest that WRN plays an important role in the surveillance and protection of genomic integrity. Depending on the type of genotoxic stress, WRN may help to activate the appropriate pathways and as such may act at the junction of several DNA metabolic processes, including DNA damage response. As HQ has been shown to induce DNA breaks in HL60 cells, the inability to correct these breaks could lead to chromosomal rearrangements and genetic instability as typical for cells derived from WS patients. Hence, benzene exposure and loss of WRN may contribute to the development of acute myeloid leukemia by allowing for the accumulation of such genetic lesions in hematopoietic cells or through the suppression of hematopoietic progenitor cells. Although our studies suggest that WRN may be an important player in the HQ-induced damage response, further functional studies are required to fully elucidate WRN's role in benzene hematotoxicity.

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REFERENCES

Bachrati, C. Z., and Hickson, I. D. (2003). RecQ helicases: Suppressors of tumorigenesis and premature aging. *Biochem. J.* **374,** 577–606.

Bohr, V. A., Cooper, M., Orren, D., Machwe, A., Piotrowski, J., Sommers, J., Karmakar, P., and Brosh, R. (2000). Werner syndrome protein: Biochemical properties and functional interactions. *Exp. Gerontol.* **35,** 695–702.

Cadet, J., Delatour, T., Douki, T., Gasparutto, D., Pouget, J. P., Ravanat, J. L., and Sauvaigo, S. (1999). Hydroxyl radicals and DNA base damage. *Mutat. Res.* **424,** 9–21.

Celeste, A., Petersen, S., Romanienko, P. J., Fernandez-Capetillo, O., Chen, H. T., Sedelnikova, O. A., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, M. J., *et al.* (2002). Genomic instability in mice lacking histone H2AX. *Science (New York, N.Y.)* **296,** 922–927.

Cheng, W. H., Sakamoto, S., Fox, J. T., Komatsu, K., Carney, J., and Bohr, V. A. (2005). Werner syndrome protein associates with gamma H2AX in a manner that depends upon Nbs1. *FEBS Lett.* **579,** 1350–1356.

Cheng, W. H., von Kobbe, C., Opresko, P. L., Arthur, L. M., Komatsu, K., Seidman, M. M., Carney, J. P., and Bohr, V. A. (2004). Linkage between Werner syndrome protein and the Mre11 complex via Nbs1. *J. Biol. Chem.* **279,** 21169–21176.

Christmann, M., Tomicic, M. T., Roos, W. P., and Kaina, B. (2003). Mechanisms of human DNA repair: An update. *Toxicology* **193,** 3–34.

Cobb, J. A., Bjergbaek, L., and Gasser, S. M. (2002). RecQ helicases: At the heart of genetic stability. *FEBS Lett.* **529,** 43–48.

Collins, A. R. (2002). The comet assay. Principles, applications, and limitations. *Methods Mol. Biol.* **203,** 163–177.

Cooper, M. P., Machwe, A., Orren, D. K., Brosh, R. M., Ramsden, D., and Bohr, V. A. (2000). Ku complex interacts with and stimulates the Werner protein. *Genes Dev.* **14,** 907–912.

Fukuchi, K., Martin, G. M., and Monnat, R. J., Jr. (1989). Mutator phenotype of Werner syndrome is characterized by extensive deletions. *Proc. Natl. Acad. Sci. U. S. A.* **86,** 5893–5897.

Galvan, N., Lim, S., Zmugg, S., Smith, M. T., and Zhang, L. (2008). Depletion of WRN enhances DNA damage in HeLa cells exposed to the benzene metabolite, hydroquinone. *Mutat Res.* **649,** 54–61.

Gebhart, E., Bauer, R., Raub, U., Schinzel, M., Ruprecht, K. W., and Jonas, J. B. (1988). Spontaneous and induced chromosomal instability in Werner syndrome. *Hum. Genet.* **80,** 135–139.

Gowans, I. D., Lorimore, S. A., McIlrath, J. M., and Wright, E. G. (2005). Genotype-dependent induction of transmissible chromosomal instability by gamma-radiation and the benzene metabolite hydroquinone. *Cancer Res.* **65,** 3527–3530.

Grandori, C., Wu, K. J., Fernandez, P., Ngouenet, C., Grim, J., Clurman, B. E., Moser, M. J., Oshima, J., Russell, D. W., Swisshelm, K., *et al.* (2003). Werner syndrome protein limits MYC-induced cellular senescence. *Genes Dev.* **17,** 1569–1574.

Haber, J. E. (1999). DNA recombination: The replication connection. *Trends Biochem. Sci.* **24,** 271–275.

Hickson, I. D. (2003). RecQ helicases: Caretakers of the genome. *Nat. Rev. Cancer* **3,** 169–178.

Huang, S., Li, B., Gray, M. D., Oshima, J., Mian, I. S., and Campisi, J. (1998). The premature ageing syndrome protein, WRN, is a 3′–>5’ exonuclease. *Nat. Genet.* **20,** 114–116.

Ishihama, M., Toyooka, T., and Ibuki, Y. (2008). Generation of phosphorylated histone H2AX by benzene metabolites. *Toxicol. In Vitro* **22,** 1861–1868.

Kafri, T., Blomer, U., Peterson, D. A., Gage, F. H., and Verma, I. M. (1997). Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* **17,** 314–317.

Karanjawala, Z. E., Murphy, N., Hinton, D. R., Hsieh, C. L., and Lieber, M. R. (2002). Oxygen metabolism causes chromosome breaks and is associated with the neuronal apoptosis observed in DNA double-strand break repair mutants. *Curr. Biol.* **12,** 397–402.

Karmakar, P., Piotrowski, J., Brosh, R. M., Jr.., Sommers, J. A., Miller, S. P., Cheng, W. H., Snowden, C. M., Ramsden, D. A., and Bohr, V. A. (2002a). Werner protein is a target of DNA-dependent protein kinase in vivo and in vitro, and its catalytic activities are regulated by phosphorylation. *J. Biol. Chem.* **277,** 18291–18302.

Karmakar, P., Snowden, C. M., Ramsden, D. A., and Bohr, V. A. (2002b). Ku heterodimer binds to both ends of the Werner protein and functional interaction occurs at the Werner N-terminus. *Nucleic Acids Res.* **30,** 3583–3591.

Karow, J. K., Wu, L., and Hickson, I. D. (2000). RecQ family helicases: roles in cancer and aging. *Curr. Opin. Genet. Dev.* **10,** 32–38.

Kaufmann, S. H., Desnoyers, S., Ottaviano, Y., Davidson, N. E., and Poirier, G. G. (1993). Specific proteolytic cleavage of poly(ADP-ribose) polymerase: An early marker of chemotherapy-induced apoptosis. *Cancer Res.* **53,** 3976–3985.

Kodama, S., Kashino, G., Suzuki, K., Takatsuji, T., Okumura, Y., Oshimura, M., Watanabe, M., and Barrett, J. C. (1998). Failure to complement abnormal phenotypes of simian virus 40-transformed Werner syndrome cells by introduction of a normal human chromosome 8. *Cancer Res.* **58,** 5188–5195.

Kolachana, P., Subrahmanyam, V. V., Meyer, K. B., Zhang, L., and Smith, M. T. (1993). Benzene and its phenolic metabolites produce oxidative DNA damage in HL60 cells in vitro and in the bone marrow in vivo. *Cancer Res.* **53,** 1023–1026.

Lebel, M., Cardiff, R. D., and Leder, P. (2001). Tumorigenic effect of nonfunctional p53 or p21 in mice mutant in the Werner syndrome helicase. *Cancer Res.* **61,** 1816–1819.

Lee, J. W., Harrigan, J., Opresko, P. L., and Bohr, V. A. (2005). Pathways and functions of the Werner syndrome protein. *Mech. Ageing Dev.* **126,** 79–86.

Li, B., and Comai, L. (2000). Functional interaction between Ku and the Werner syndrome protein in DNA end processing. *J. Biol. Chem.* **275,** 28349–28352.

Li, B., and Comai, L. (2002). Displacement of DNA-PKcs from DNA ends by the Werner syndrome protein. *Nucleic Acids Res.* **30,** 3653–3661.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* **91,** 479–489.

Machwe, A., Ganunis, R., Bohr, V. A., and Orren, D. K. (2000). Selective blockage of the 3′–>5’ exonuclease activity of WRN protein by certain oxidative modifications and bulky lesions in DNA. *Nucleic Acids Res.* **28,** 2762–2770.

Naldini, L., Blomer, U., Gage, F. H., Trono, D., and Verma, I. M. (1996). Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11382–11388.

Opresko, P. L., Cheng, W. H., von Kobbe, C., Harrigan, J. A., and Bohr, V. A. (2003). Werner syndrome and the function of the Werner protein; what they can teach us about the molecular aging process. *Carcinogenesis* **24,** 791–802.

Orren, D. K., Machwe, A., Karmakar, P., Piotrowski, J., Cooper, M. P., and Bohr, V. A. (2001). A functional interaction of Ku with Werner exonuclease facilitates digestion of damaged DNA. *Nucleic Acids Res.* **29,** 1926–1934.

Oshima, J. (2000). The Werner syndrome protein: An update. *Bioessays* **22,** 894–901.

Poot, M., Gollahon, K. A., Emond, M. J., Silber, J. R., and Rabinovitch, P. S. (2002). Werner syndrome diploid fibroblasts are sensitive to 4-nitroquinoline-

N-oxide and 8-methoxypsoralen: Implications for the disease phenotype. *FASEB J*. **16,** 757–758.

Poot, M., Gollahon, K. A., and Rabinovitch, P. S. (1999). Werner syndrome lymphoblastoid cells are sensitive to camptothecin-induced apoptosis in S-phase. *Hum. Genet*. **104,** 10–14.

Prince, P. R., Emond, M. J., and Monnat, R. J., Jr. (2001). Loss of Werner syndrome protein function promotes aberrant mitotic recombination. *Genes Dev*. **15,** 933–938.

Ramel, C., Cederberg, H., Magnusson, J., Vogel, E., Natarajan, A. T., Mullender, L. H., Nivard, J. M., Parry, J. M., Leyson, A., Comendador, M. A., *et al*. (1996). Somatic recombination, gene amplification and cancer. *Mutat. Res*. **353,** 85–107.

Reed, J. C. (1997). Cytochrome c: Can't live with it–can't live without it. *Cell* **91,** 559–562.

Rogakou, E. P., Boon, C., Redon, C., and Bonner, W. M. (1999). Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J. Cell. Biol*. **146,** 905–916.

Rothkamm, K., and Lobrich, M. (2003). Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. *Proc. Natl. Acad. Sci. U. S. A*. **100,** 5057–5062.

Ruiz-Ramos, R., Cebrian, M. E., and Garrido, E. (2005). Benzoquinone activates the ERK/MAPK signaling pathway via ROS production in HL-60 cells. *Toxicology* **209,** 279–287.

Sadler, A., Subrahmanyam, V. V., and Ross, D. (1988). Oxidation of catechol by horseradish peroxidase and human leukocyte peroxidase: Reactions of o-benzoquinone and o-benzosemiquinone. *Toxicol. Appl. Pharmacol*. **93,** 62–71.

Saintigny, Y., Makienko, K., Swanson, C., Emond, M. J., and Monnat, R. J., Jr. (2002). Homologous recombination resolution defect in Werner syndrome. *Mol. Cell. Biol*. **22,** 6971–6978.

Salk, D. (1982). Werner's syndrome: A review of recent research with an analysis of connective tissue metabolism, growth control of cultured cells, and chromosomal aberrations. *Hum. Genet*. **62,** 1–5.

Salk, D., Au, K., Hoehn, H., and Martin, G. M. (1985). Cytogenetic aspects of Werner syndrome. *Adv. Exp. Med. Biol*. **190,** 541–546.

Shen, M., Lan, Q., Zhang, L., Chanock, S., Li, G., Vermeulen, R., Rappaport, S. M., Guo, W., Hayes, R. B., Linet, M., *et al*. (2006a). Polymorphisms in genes involved in DNA double-strand break repair pathway and susceptibility to benzene-induced hematotoxicity. *Carcinogenesis* **27,** 2083–2089.

Shen, M., Zheng, T., Lan, Q., Zhang, Y., Zahm, S. H., Wang, S. S., Holford, T. R., Leaderer, B., Yeager, M., Welch, R., *et al*. (2006b). Polymorphisms in DNA repair genes and risk of non-Hodgkin lymphoma among women in Connecticut. *Hum. Genet*. **119,** 659–668.

Shevelev, I. V., and Hubscher, U. (2002). The 3' 5' exonucleases. *Nat. Rev. Mol. Cell. Biol*. **3,** 364–376.

Simbulan-Rosenthal, C. M., Rosenthal, D. S., Iyer, S., Boulares, H., and Smulson, M. E. (1999). Involvement of PARP and poly(ADP-ribosyl)ation in the early stages of apoptosis and DNA replication. *Mol. Cell. Biochem.* **193,** 137–148.

Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell. Res*. **175,** 184–191.

Spillare, E. A., Robles, A. I., Wang, X. W., Shen, J. C., Yu, C. E., Schellenberg, G. D., and Harris, C. C. (1999). p53-mediated apoptosis is attenuated in Werner syndrome cells. *Genes Dev*. **13,** 1355–1360.

Szekely, A. M., Bleichert, F., Numann, A., Van Komen, S., Manasanch, E., Ben Nasr, A., Canaan, A., and Weissman, S. M. (2005). Werner protein protects nonproliferating cells from oxidative DNA damage. *Mol. Cell. Biol*. **25,** 10492–10506.

Takeuchi, F., Hanaoka, F., Goto, M., Yamada, M., and Miyamoto, T. (1982). Prolongation of S phase and whole cell cycle in Werner's syndrome fibroblasts. *Exp. Gerontol*. **17,** 473–480.

Uchida, N., Sutton, R. E., Friera, A. M., He, D., Reitsma, M. J., Chang, W. C., Veres, G., Scollay, R., and Weissman, I. L. (1998). HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc. Natl. Acad. Sci. U. S. A*. **95,** 11939–11944.

von Kobbe, C., Harrigan, J. A., May, A., Opresko, P. L., Dawut, L., Cheng, W. H., and Bohr, V. A. (2003). Central role for the Werner syndrome protein/poly(ADP-ribose) polymerase 1 complex in the poly(ADP-ribosyl) ation pathway after DNA damage. *Mol. Cell. Biol*. **23,** 8601–8613.

Von Kobbe, C., May, A., Grandori, C., and Bohr, V. A. (2004). Werner syndrome cells escape hydrogen peroxide-induced cell proliferation arrest. *FASEB J*. **18,** 1970–1972.

Whysner, J., Reddy, M. V., Ross, P. M., Mohan, M., and Lax, E. A. (2004). Genotoxicity of benzene and its metabolites. *Mutat. Res*. **566,** 99–130.

Winn, L. M. (2003). Homologous recombination initiated by benzene metabolites: A potential role of oxidative stress. *Toxicol. Sci*. **72,** 143–149.

Yu, T. W., and Anderson, D. (1997). Reactive oxygen species-induced DNA damage and its modification: A chemical investigation. *Mutat. Res*. **379,** 201–210.