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14. ABSTRACT: In mice, selenium in the form of seleno-L-methionine was reported to have two effects 1) to enhance efficacy of cancer therapeutics against cancer cells; and 2) to protect bone marrow and gut epithelium from dose-limiting toxicity. We are exploring the mechanism whereby selenium can have differential effects on cancer cells versus normal cells. A key genetic alteration in cancer is p53 mutation. About 70% of all human cancers are mutant i.e. defective in p53, while bone marrow and other normal tissues are functional for p53. P53 is known to protect normal cells by a DNA repair mechanism. Cancer cells lack the DNA repair mechanism and are predicted to be sensitive to chemotherapy. A DNA repair protein that is controlled by p53 is Xpc. Thus, wildtype, p53-/-, or xpc-/- mice were used to test the hypothesis that 1) selenium evoked a DNA repair and protective response in wildtype cells including bone marrow; and 2) DNA repair was defective in cells deleted for p53 or xpc genes and these cells including cancer cells would not be protected.

15. SUBJECT TERMS
xpc, DNA repair, p53, bone marrow

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INTRODUCTION.

The data demonstrate that p53 protects normal, healthy cells from DNA damage. More importantly, the results show that p53-mediated protection from DNA damaging agents can be enhanced. We treated normal, healthy cells with selenomethionine prior to challenge with cisplatin and found that the selenium supplementation enhanced DNA repair and improved cell survival following chemotherapy. However, cells that lacked a functional copy of p53 remained sensitive to the cisplatin treatment and had no improvement in DNA repair. Furthermore, the results identify a downstream target of p53, namely Xpc, that contributes significantly to the protective response in mouse bone marrow. Xpc is a protein responsible for recognizing DNA damage, and the data reveal that mice lacking the xpc gene had substantially greater bone marrow toxicity than normal mice.

BODY.

As reported previously, preliminary data from the proposal and early data following notification of this award was published (Fischer et. al.). The work completed since the 2007 annual report is being assembled into a manuscript that will be submitted later this summer.

To evaluate any difference in sensitivity between *xpc -/-* mice and wildtype mice colorimetric MTS cell survival assays were performed (Fig. 1). The chemical reduction of the MTS compound into formazan measures dehydrogenase activity in the cells; the production of formazan is directly proportional to the number of metabolically active cells. Bone marrow from wildtype and *xpc -/-* mice was harvested and cultured *ex vivo* in complete IMDM with IL-6 and SCF then treated with carboplatin for 2 hours. The MTS colorimetric reagent was added 72 hours following the carboplatin treatment. Cell survival following carboplatin treatment was calculated relative to the untreated cells for each genotype. Bone marrow from *xpc -/-* mice was significantly more sensitive to carboplatin treatment than bone marrow from wildtype mice.

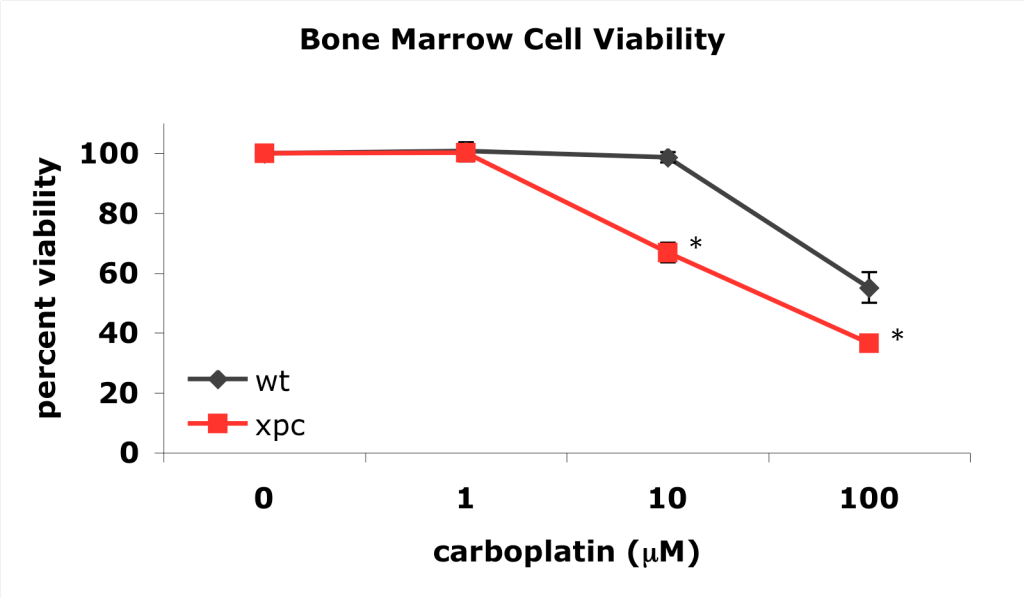


Figure 1. Bone marrow viability. Bone marrow from wildtype and *xpc* -/- mice was cultured *ex vivo* in complete IMDM with IL-6 and SCF for 16 hours. The cells were then treated with varying concentrations of carboplatin for 2 hours and plated in 96-well plates. After 72 hours the MTS substrate was added and after 24 hours the fraction of viable cells was calculated relative to the untreated cells. Shown is representative of three independent experiments. * $p < 0.006$.

Similar to the results from *ex vivo* cultures, *xpc* -/- mice were significantly more sensitive to the carboplatin regimen than wildtype mice. *Xpc* -/- and isogenic wildtype control mice were monitored for hematopoeitic toxicity during the carboplatin chemotherapy regimen described in Table 1. It is important to reiterate that the *xpc* -/- mice were given one less dose of carboplatin than the wildtype mice. A Kaplan-Meier survival plot shows animal survival during the course of carboplatin treatement (Fig. 2). While the wildtype mice began to recover, seven out of ten of the *xpc* -/- mice did not survive the treatment.

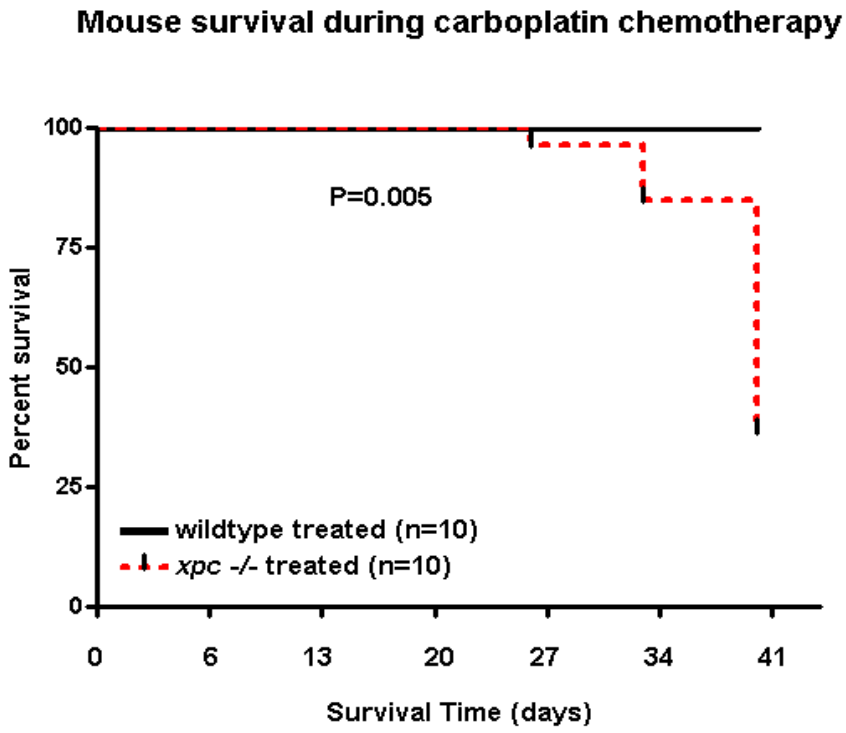


Figure 2. Kaplan-Meier survival. X*pc* -/- mice were substantially more sensitive to the carboplatin regimen than wildtype mice. Carboplatin doses were as follows: wildtype mice received 3 x 60 mg/kg and 2 x 100 mg/kg; *xpc* -/- mice received 3 x 60 mg/kg and 1 x 100 mg/kg.

Complete blood counts were taken once per week during the course of the treatment regimen, five days after administering each dose of carboplatin. At the latter time points the total white blood cells, platelets, hematocrit, and red blood cells are lower in the treated *xpc* -/- mice than the wildtype mice (Figs. 3-4). The CBC data shows that with the loss of xpc the entire hematopoietic system is more sensitive to DNA damage.

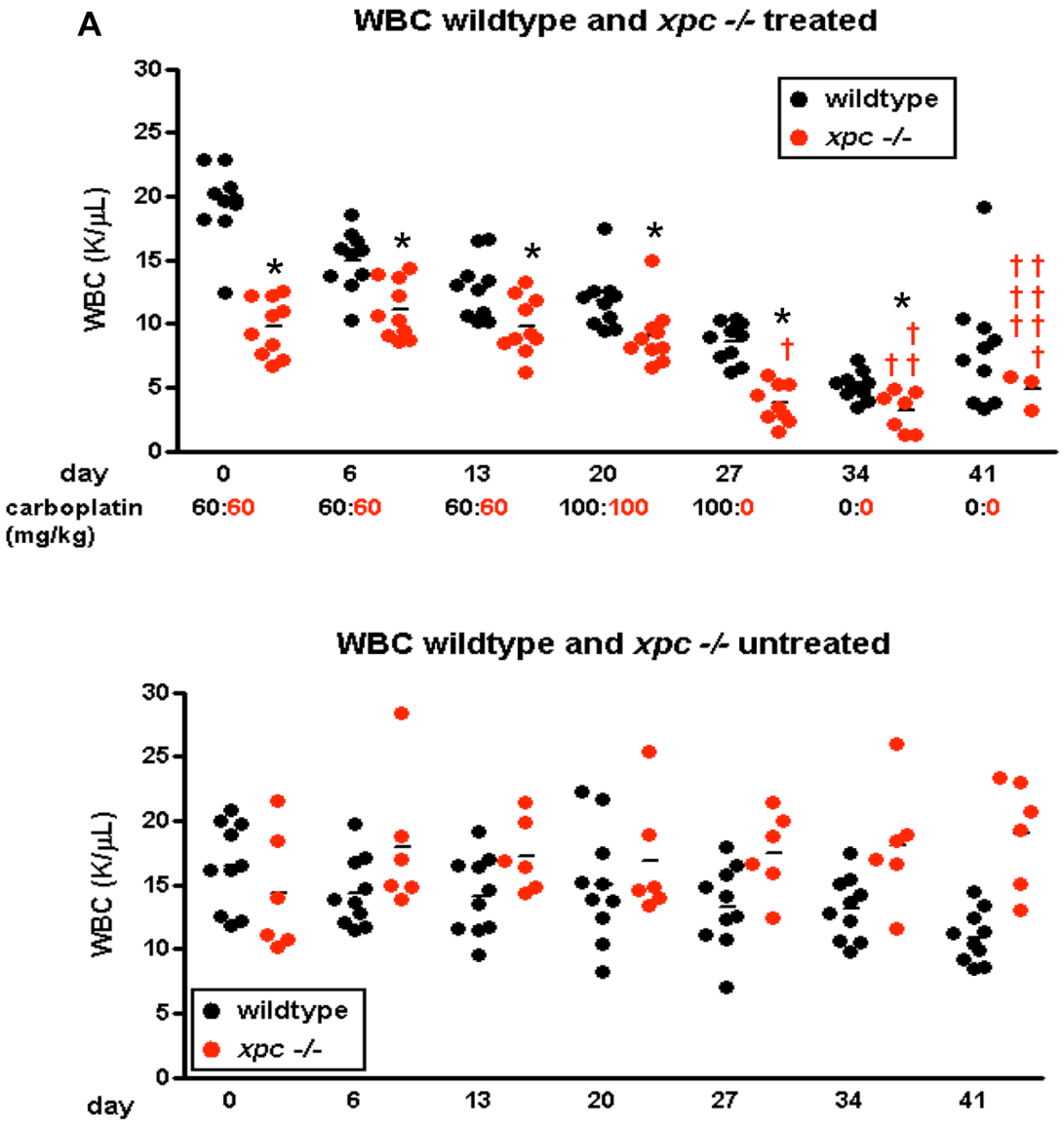


Figure 3. Total white blood cells during the carboplatin chemotherapy regimen. Doses were as indicated on the x-axis. Complete blood counts were taken once per week during the treatment cycle, five days after each dose of carboplatin was administered. **A.** Mice treated with carboplatin (wildtype n=10, *xpc* -/- n=10). **B.** Untreated control mice (wildtype n=10, *xpc* -/- n=6). * $p < 0.02$, *t* test, xpc -/- treated relative to wildtype treated. Crosses denote mice that did not survive the carboplatin regimen. Hash marks represent the mean.

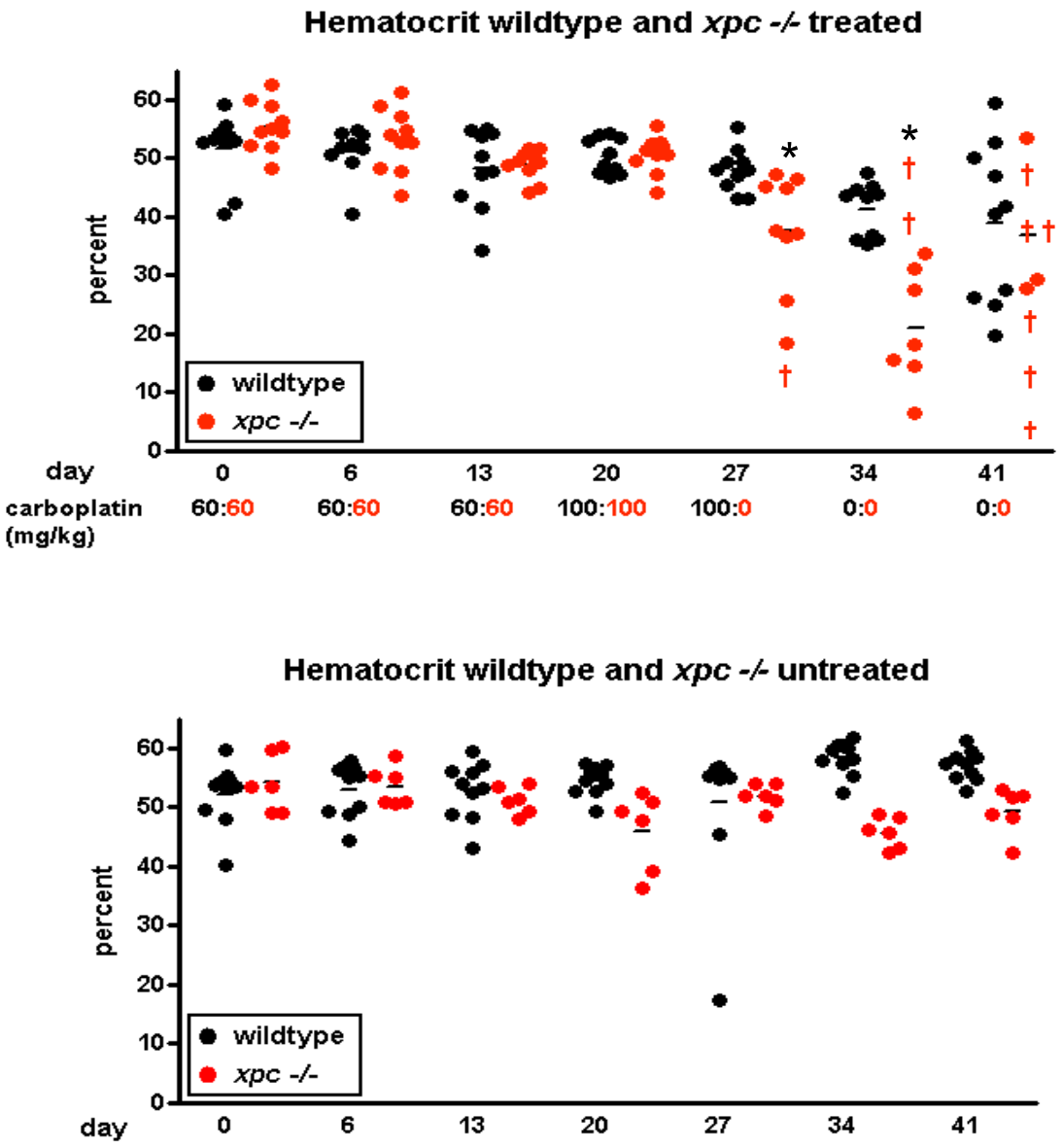


Figure 4. Hematocrit during the carboplatin chemotherapy regimen. Doses were as indicated on the x-axis. Complete blood counts were taken once per week during the treatment cycle, five days after each dose of carboplatin was administered. **A.** Mice treated with carboplatin (wildtype n=10, *xpc* -/- n=10). **B.** Untreated control mice (wildtype n=10, *xpc* -/- n=6). * $p < 0.008$, *t* test, xpc -/- treated relative to wildtype treated. Crosses denote mice that did not survive the carboplatin regimen. Hash marks represent the mean.

Necropsies were performed on the animals from this cohort in an effort to identify the proximate cause of death; a summary of the results are shown in Table 1. While the exact cause of death could not be determined, it is reasonable to conclude, based on the results of necropsy (Table 1) that myelosuppression associated with hematopoietic toxicity was a significant factor. However, other contributing factors cannot be ruled, such as toxicity to the gastrointestinal tract that did not produce gross histological changes could have contributed to the reduced survival of the *xpc* -/- mice. While many tissues are adversely affected by carboplatin chemotherapy, several studies have shown that the primary dose-limiting tissue is bone marrow [1]. It is also plausible that because the mice were immuno-compromised they may have suffered bacterial infections causing sepsis.

Wildtype	**bone marrow**	**brain**	**GI tract**	**kidney**	**heart**
untreated #4	no lesions	no lesions	no lesions	no lesions	no lesions
carboplatin #10	no lesions	mild	no lesions	no lesions	no lesions
carboplatin #12	no lesions	mild	no lesions	no lesions	no lesions
XPC -/-					
untreated #2	mild suppression	none	no lesions	no lesions	no lesions
carboplatin #7	marked suppression	mild	no lesions	no lesions	no lesions
carboplatin #8	marked suppression	moderate	no lesions	no lesions	no lesions
carboplatin #11	marked suppression	moderate	moderate inflammation and hemorrhage	no lesions	multifocal myocardial hemorrhage

Table 1. Necropsy report. *Xpc* -/- mice that received carboplatin chemotherapy had marked bone marrow suppression, whereas wildtype mice that received the treatment had no apparent suppression. The untreated *xpc* -/- animal also had mild bone marrow suppression, likely due to endogenous DNA damage. The carboplatin treated wildtype mice had mild cerebral hemorrhage, and the *xpc* -/- mice had mild to moderate cerebral hemorrhage.

The results of the necropsy study showed marked bone marrow suppression in treated *xpc* -/- mice but not in the wildtype mice. Femurs from wildtype and *xpc* -/- untreated and treated mice were sectioned and stained with DAPI or H&E to validate the effect of carboplatin on the bone marrow (Fig. 5). The femurs from *xpc* -/- mice treated with carboplatin were markedly hypocellular. Furthermore, the femur from the *xpc* -/- control animal also had hypocellular morphology; this finding correlates with the necropsy data (Table 1) and bone marrow cellularity. It is apparent that the bone marrow was substantially affected by the carboplatin treatment.

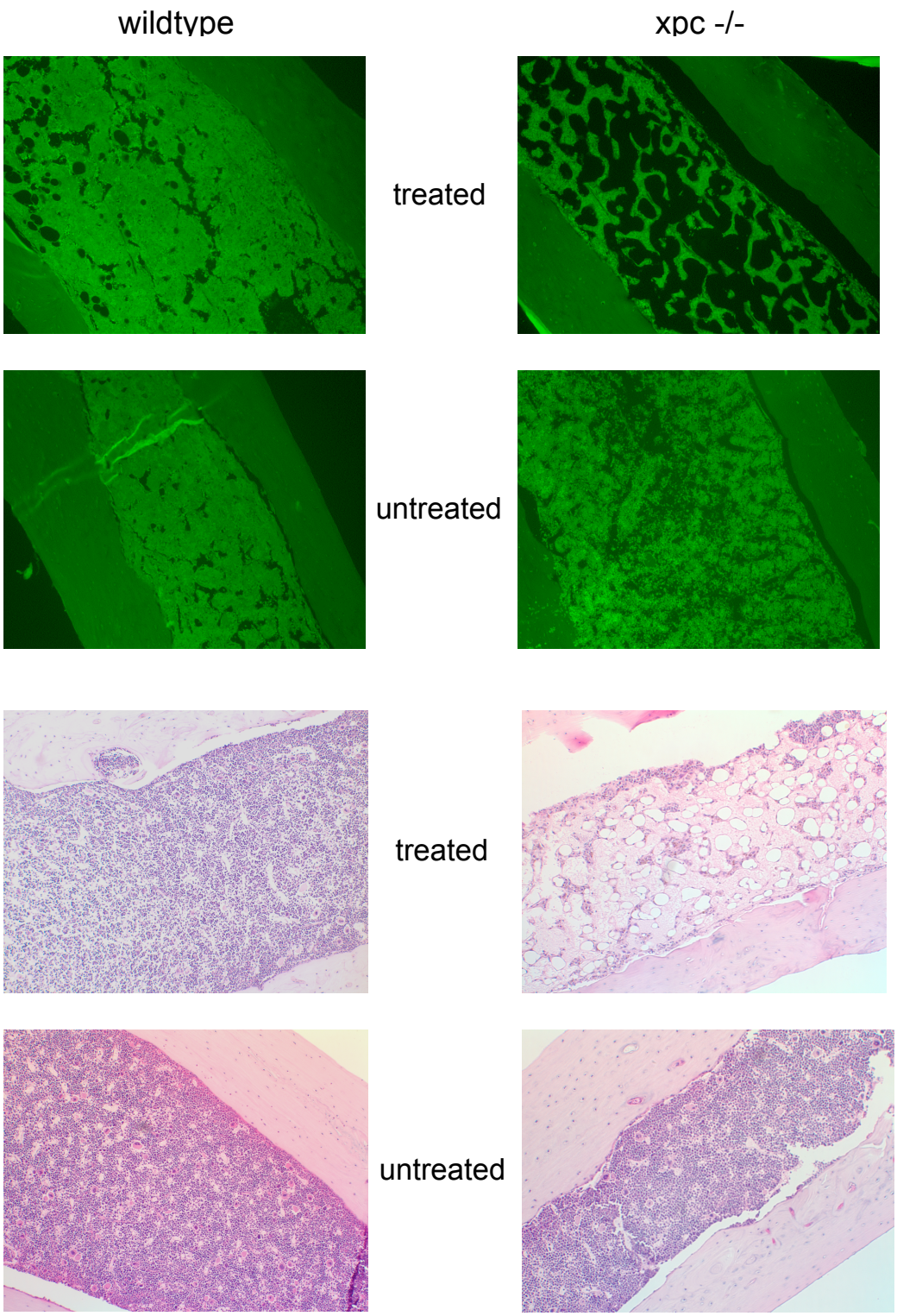


Figure 5. Bone marrow suppression. Femurs from untreated and treated wildtype and *xpc* -/- mice were sectioned and stained with A. DAPI or B. H&E. The femurs from *xpc* -/- mice treated with carboplatin were markedly hypocellular and substantially more sensitive to the treatment than the wildtype mice.

Xpc -/- mice, like the human XP patients, are known to have increased sensitivity to UV radiation and increased incidence of various tumor types. It is well documented that the Xpc protein functions in the damage recognition step of GGR. However, there is a dogma that says XPC contributes relatively little to cell survival Early experiments compared human cells to mouse cells; the human cells had normal GGR and the mouse cells lacked GGR [2]. The colony-forming ability after UV-radiation was similar, which led to the conclusion that GGR contributed little if at all to cell survival. Most early studies did not use isogenic, strain matched systems which to address the role of XPC in cell survival. Later studies employed isogenic fibroblasts of human or mouse origin lacking *XPC* or other effectors of GGR and showed that GGR contributed modestly to cell survival (2-3 fold) [2-4]. The present study is the first to examine in an isogenic system, the cell survival response of non-fibroblast primary cells in relation to GGR. The UV-mimetic agent carboplatin was used to induce bone marrow suppression. *Xpc -/-* mice were markedly sensitive to carboplatin *in vivo*.

Mice lacking normal, functional Xpc were substantially more sensitive to carboplatin chemotherapy than isogenic wildtype mice. The data from the complete blood counts reveal that the *xpc -/-* mice were consistently more affected by the carboplatin treatment than the wildtype mice. Furthermore, the CBC data reveal that all of the different lineages within the hematopoietic system were affected. Lymphocytes, neutrophils, monocytes, platelets, and red blood cells were consistently lower in the *xpc -/-* mice than the wildtype mice receiving the carboplatin.

The data also demonstrate that the untreated *xpc -/-* mice had significantly lower cellularity in several different cell populations (Gr-1, B220, CD4, CD8, Lin-) within the bone marrow relative to untreated wildtype mice. It is plausible that the basis for the reduced cellularity in the untreated mice is the contribution of endogenous DNA damage.

Perhaps the best indicator of the functional significance of Xpc loss in bone marrow is shown by the colony forming assays. The bone marrow from *xpc -/-* mice was substantially more impaired in colony forming capacity than bone marrow from wildtype mice following the carboplatin treatment. Again it is important to note that the *xpc -/-* mice had substantially reduced colony forming ability even after receiving one less dose of carboplatin than the wildtype mice. It is also worthwhile to note that while the difference in total bone marrow cellularity was not significantly different between the *xpc -/-* and wildtype groups following treatment the difference in colony forming capacity was more than 13-fold. The data from the necropsy study also shows that the *xpc -/-* mice were more affected by the carboplatin treatment than the wildtype mice.

Many studies have emphasized the role of p53 in DNA damage induced apoptosis. While it is true that normal p53 can induce apoptosis, this work highlights the importance of another, perhaps overlooked, role for p53, which is p53-mediated protection from DNA damage. Because XPC only has a role in GGR, it is typically overlooked as it is thought to contribute little to cell survival. This study demonstrates that XPC plays a substantial role in cell survival. At doses not lethal to wildtype mice, seventy percent of *xpc -/-* mice did not survive the carboplatin regimen.

KEY RESEARCH ACCOMPLISHMENTS

- Hematological data demonstrating marked sensitivity of xpc -/- mice to carboplatin chemotherapy.
- Pathological and histological data demonstrating marked sensitivity of xpc -/- mice to carboplatin chemotherapy.
- Demonstrate that contrary to previous reports XPC protein is important for cell survival *in vitro* and more importantly that it contributes substantially to mouse survival *in vivo*.

REPORTABLE OUTCOMES

- Ph.D., Microbiology and Immunology, at Indiana University-Purdue University Indianapolis, Indianapolis, IN, Cancer major, biochemistry/molecular biology minor. 2008.
- P53 And Xpc Contribute Significantly to Cellular Protection From DNA Damage. Abstract for the Department of Defense Breast Cancer Research Program 2008 Era of Hope Meeting.
- Data within the report has been submitted for publication and is currently under review.

CONCLUSION

The implication of this work for cancer therapeutics is the discovery that XPC plays a substantial role in protecting the hematopoietic system from the overwhelming DNA damage induced by carboplatin chemotherapy. The results show that myelosuppression in the *xpc -/-* mice was substantially greater than in the wildtype mice following carboplatin treatment, as indicated by the reduced bone marrow cellularity and reduced colony forming ability. There is an abundance of literature showing that p53 regulates the transcription of XPC and it is plausible that XPC plays a key role in the selenium-mediated protection of normal cells from DNA damaging chemotherapeutics. Experiments that aim to further evaluate this possibility are described in the next section.

Furthermore, patients with certain *XPC* polymorphisms may have poor performance when treated with platinum agents, which induce DNA damage primarily repaired by NER. Some *XPC* single nucleotide polymorphisms have been characterized as having reduced repair capacity compared to the common allele. It may be the reduced repair capacity that is responsible for tumorigenesis in those patients. Furthermore, several studies have demonstrated polymorphisms that are associated with elevated risk for bladder, lung, and colon cancer [5-8]. While a cancer could be more sensitive to the chemotherapy, the treatment toxicity could be equally elevated due to a polymorphism.

It is important to consider that while the majority of polymorphisms are present at low frequency in the population in general, they may be present at relatively high frequency in groups with certain types of cancer. Single nucleotide polymorphisms in *XPC* are probably not useful as a cancer prevention screen, but they may be useful as a therapeutic prognosticator.

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Appendices

Selenium Protection from DNA Damage Involves a Ref1/p53/Brca1 Protein Complex

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Abstract. *Selenium, in the form of seleno-L-methionine (SeMet), induced Redox-factor-1 (Ref1) and p53 proteins in normal human and mouse fibroblasts. Ref1 and p53 are known to be associated with each other, resulting in enhanced sequence-specific DNA binding by p53 and transactivation of p53-regulated effector genes. SeMet preferentially induced the DNA repair branch of the p53 pathway, while apoptosis and cell cycle arrest were unaffected. Accordingly, pretreatment with SeMet protected normal fibroblasts from subsequent DNA damage. In the current study, Brca1 and Ref1 were shown to interact concurrently with p53 in targeting a SeMet-induced DNA repair response. Moreover, like p53 and Ref1, Brca1 was required for SeMet-mediated DNA damage protection, as brca1-/- mouse fibroblasts were not protected from UV-radiation by SeMet treatment. These findings indicate that besides p53 and Ref1, Brca1 is required for selenium protection from DNA damage. The data are consistent with selective induction of the DNA repair branch of the p53 pathway by SeMet.*

The tumor suppressor/transcription factor p53 interacts with a number of cellular proteins to coordinate its complex biological functions of apoptosis, cell cycle arrest and DNA repair. Specifically, other authors have shown that p53 interaction with Brca1 is required for the p53-mediated nucleotide excision (NER) DNA repair pathway, involving the p53-regulated effector genes *XPC, p48XPE* and *Gadd45a* (1, 2). One unanswered question is whether known p53-interacting proteins can interact concurrently with p53, as opposed to potentially exclusionary interactions. Both redox factor-1 (Ref1) and Brca1 are well-known to interact physically and functionally with p53. This is the first report demonstrating that they may do so concurrently, *i.e.* the p53 function(s) that Ref1 and Brca1 govern are compatible and not mutually exclusive.

Selenium, in the form of seleno-L-methionine (SeMet), promotes the reduction of key p53 cysteine residues 275 and/or 277, a reduction required for sequence-specific DNA binding of p53 to its target genes (3). The redox factor Ref1 is the mediator of selenium signaling to p53, since a Ref1 mutant carrying alanine substitution for cysteine 65 (the known redox center of Ref1) blocked p53 cysteine reduction in response to SeMet (4). Recently, Ref1 was shown to bind to p53, promote p53 tetramerization and enhance p53 sequence-specific DNA binding in its reduced state (5).

SeMet protected normal human or mouse fibroblasts from UV-radiation corresponding to a SeMet-inducible DNA repair response (4, 6). This repair response was p53-dependent as p53-null mouse embryo fibroblasts did not exhibit increased DNA repair and were not protected (4). Likewise, cells carrying dominant-negative Ref1 were UV-sensitive and did not elicit a DNA repair or protective response to SeMet (4). Importantly, the induction of DNA repair and DNA damage protection required a 15-h pretreatment with SeMet (4). Neither DNA repair nor protection was observed when SeMet was given concurrently with DNA damage (4, 6). The mechanism whereby SeMet induced DNA repair involves a non-genotoxic signal transduction pathway, since SeMet alone did not cause DNA damage (3, 4, 6). Rather, DNA repair proteins were elevated by SeMet pretreatment, which protected cells from subsequent DNA damage (4, 6, 7).

In determining the mechanism for SeMet-enhanced DNA repair, p53- and Ref1-containing protein complexes in SeMet-treated cells were probed under the exact conditions previously shown to induce NER and DNA damage protection (4, 6). One important p53-interacting protein, Brca1, was found to interact with p53 concurrently with Ref1, indicating that Ref1 could potentially regulate the

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Key Words: DNA-repair, cancer chemoprevention, nucleotide excision repair, redox factor-1.

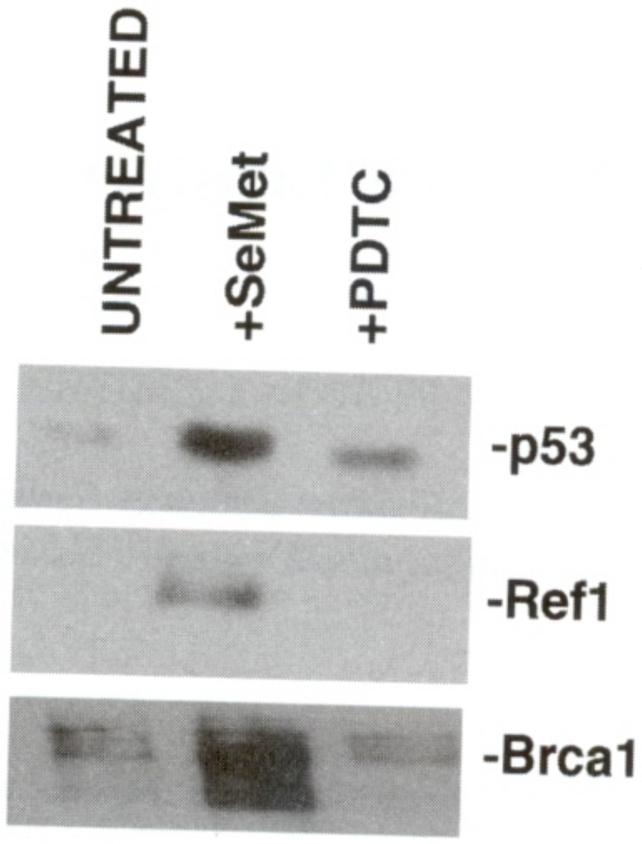


Figure 1. *Detection of Ref1 and Brca1 in p53-immune complexes of selenium-treated cells. Antibody to p53 D01/agarose conjugate was used to immunocapture p53, then p53 and p53 interacting proteins were detected by immunoblotting. Cellular Ref1 and Brca1 were pronounced in SeMet-treated cells compared to untreated cells and were decreased to absent in PDTC-treated cells. All detected proteins were endogenous to the RKO cells. The cellular treatment conditions (10 μM SeMet; 15 h) have been shown to elicit a DNA repair and protective response to subsequent DNA damage (4, 6). Pyrollidone dithiocarbamate (PDTC) blocked DNA repair and DNA damage protection in those same studies (4).*

activities of p53 and Brca1, *e.g.*, by the redox-factor-1 function. DNA repair genes regulated by transcription factors p53 and Brca1 may be sensitive to changes in cellular redox state *via* the presence of Ref1 in the complex.

Materials and Methods

Immunocapture of cellular and recombinant p53 using anti-p53 D01/agarose conjugate. Recombinant p53, Rad51 and Brca1 were purchased from Santa Cruz Biotech, Santa Cruz, CA, USA, as baculovirus-encoded proteins. Ref1 (APE/Ref1) was a bacterially-expressed histidine-tagged protein from Dr. Mark R. Kelley, Indiana University, USA. RKO cells carrying wild-type p53 were treated with SeMet (10 μM, 15 h). Untreated cells and cells treated with pyrrolidine dithiocarbamate (PDTC; Sigma, St. Louis, MO, USA) were used as controls. PDTC is known to oxidize p53 cysteine residues, an effect opposite to that of SeMet. The immune complexes were collected by overnight agitation with anti-p53 D01 agarose conjugate (Oncogene Research Products, San Diego, CA, USA). The immunoprecipitation lysis buffer consisted of 50 mM Tris/HCl pH 8, 150 mM NaCl, 1% Triton X-100 and protease inhibitors. The immune complexes were washed 4 times with lysis buffer, then boiled in SDS gel-loading buffer and subjected to electrophoresis and transfer. Proteins were detected with horseradish peroxidase conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce Inc., Rockford, IL, USA). The complexes were analyzed using mouse monoclonal antibodies for p53 (D01); APE/Ref1 (NB100-116A3, Novus Biologicals, Littleton, CO, USA), BRCA1 (Ab-1, Oncogene Research Products) and Rad51 (Ab1, NeoMarkers, Fremont, CA, USA) antibodies.

For the immunocapture of cellular p53, RKO human colon cancer cells wild-type for p53 or H1299 human lung cancer cells null for p53 were used. Endogenous wild-type p53 and bound Brca1 and Ref1 are depicted in Figure 1. A pcDNA3.1 plasmid encoding wild-type p53 was used to transfect H1299 cells by the FuGene method, together with pcDNA3.1 plasmids encoding the wild-type Ref1 or C65A Ref1 mutant. The cells were treated with 10 μM SeMet overnight and immunoprecipitation was conducted as reported in reference 4.

Chemical crosslinking of recombinant Ref1, p53 and Brca1. Recombinant proteins (1 μg) were allowed to interact at room temperature, were then treated with 5 mg/mL dimethylsuberimidate for 20 min at 37°C, in a 20 μL volume of 20 mM HEPES pH7.9, 100 mM KCl, 1 mM DTT, followed immediately by SDS/polyacrylamide gel electrophoresis and immunoblotting on nitrocellulose (8). The monoclonal antibody to APE/Ref1 (NB100) was used to detect Ref1.

Sequence-specific DNA binding of p53 (4). The prototype p53-binding site is identical to that of the p53-regulated *Gadd45a* gene (9). We showed that SeMet treatment enhanced sequence-specific p53 binding to the prototype sequence (4). In the current study, the protein complexes were large, owing to the presence of the 200 kilodalton Brca1 protein in the complex, as well as to p53 tetramerization and formation of higher-order p53-containing complexes. Therefore, an agarose conjugate in which the prototype p53-binding site was covalently coupled to agarose beads (Santa Cruz Biotech) was used. Recombinant proteins were incubated with the beads in a 25-μl volume in 20 mM HEPES pH 7.8, 100 mM KCl, 1 mM EDTA, 1 mM DTT for 20 min at 37°C, and were then washed 4 times in 1 mL of lysis buffer composed of 10 mM Tris pH 7.5, 150 mM NaCl and 1% Triton-X100. Bound proteins were dissociated from the beads by boiling in 3X SDS gel-loading buffer and were separated by electrophoresis. Bound proteins were detected by immunoblotting as above. The detection of Brca1 and Ref1 required p53, as neither was detected in controls lacking p53 (results not shown).

Cell survival assays. To evaluate the role of BRCA1 in the selenium protective response to DNA damage, brca1-/- MEFs (homozygous for the Δ exon 11 allele) were treated in the presence or absence of 10 μM SeMet for 15 h and were then exposed to UV radiation as indicated. Non-irradiated cells served as controls. The cell yield was determined after 7 days by a thiazolyl blue viability assay (Ref. 4; Figure 3).

Results

Both p53 and Ref1 proteins were elevated in cells treated overnight with 10-20 μM SeMet (4, 7), a relatively physiological and nontoxic concentration (10, 11). Both proteins are regulated by post-translational protein stabilization and p53 cysteine residues 275 and/or 277 were reduced in SeMet-treated cells (4). Although human p53 contains 12 cysteine residues, cysteines 275 and/or 277 reside in the DNA-binding domain and are known to be critical for sequence-specific binding of p53 to its downstream effector

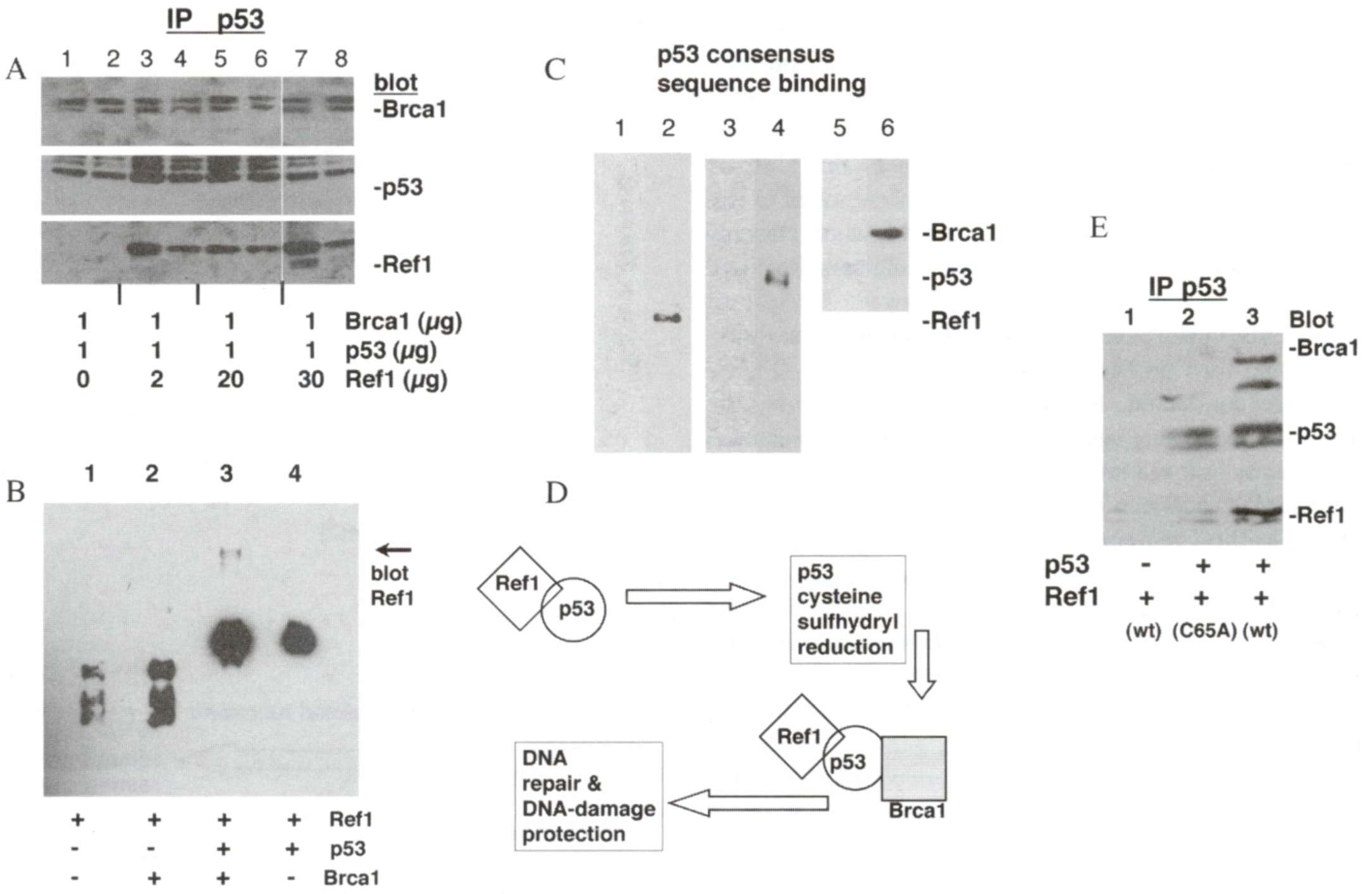


Figure 2. *Evidence of concurrent interaction of Ref1 and Brca1 with p53 using recombinant proteins. A) Excess Ref1 does not compete with Brca1 for p53 binding. Recombinant proteins were mixed as indicated and allowed to interact for 1 h at room temperature. Then, p53 was immunocaptured using antibody D01-agarose. After extensive washing in 1 mL of buffer containing 1% Triton-X100, the immunocaptured proteins were subjected to electrophoresis and immunoblotting. The amount of Ref1 added did not affect the binding of Brca1 to p53, suggesting that different p53 domains are involved in binding to Ref1 versus Brca1. Note that the amount of added Ref1 (20 μg, 30 μg) saturated the p53 binding sites. Duplicate lanes shown. B) Chemical crosslinking of putative ternary complex containing p53, Ref1 and Brca1. Recombinant proteins were allowed to interact as in A, were then crosslinked by addition of dimethylsuberimidate for 20 min at 37°C and were subjected to electrophoresis and immunoblotting. Ref1 and Brca1 alone showed no evidence of interaction (lanes 1 and 2), however, p53 addition yielded slower-migrating species where all 3 proteins were present (lane 3). Immunodetection was carried out with a Ref1 antibody, hence the higher molecular weight band was not detected in controls lacking Ref1 (not shown). C) Immunocapture of p53 by sequence-specific DNA binding results in concurrent capture of Ref1 and Brca1 in complex with p53. Recombinant proteins were used as in A and B. The consensus p53 DNA-binding sequence conjugated to agarose was used to bind p53 and p53-interacting proteins. After extensive washing in buffer containing 1% Triton X-100, electrophoretically-resolved proteins were detected by their respective antibodies. The data suggest that Brca1 and Ref1 can interact concurrently with p53 and do not compete for p53 binding, consistent with the other experiments. Lanes 1, 2, blot Ref1, p53 absent in lane 1, p53 present in lane 2; lanes 3,4, blot p53, p53 absent in lane 3, p53 present in lane 4; lanes 5,6, blot Brca1, p53 absent in lane 5, p53 present in lane 6. D) Current model of DNA damage protection by selenium involving the Ref1/p53/Brca1 complex. Key p53 cysteine residues are sulfhydryl-reduced in selenium-treated cells, a response that requires Ref1/p53 interaction (4, 9). Brca1 can interact with p53 even in the presence of excess Ref1, suggesting a ternary complex. Brca1 cooperates with p53 in driving p53-mediated DNA repair and protective response to UV-radiation (2, 18, 26, 27). E) Binding of cellular Brca1 and Ref1 to p53 are linked. Cellular p53 was immunocaptured from transfected H1299 cells null for p53, then probed for the presence of Brca1 and Ref1 in the immune complexes; lane 1, Brca1 and Ref1 not detected in p53-minus control; lane 2, little Brca1 or Ref1 detected in presence of Ref1 C65A mutant, unable to reduce p53 cysteines; lane 3, clear Brca1 and Ref1 detection in presence of wild-type Ref1. The interacting Brca1 was endogenous to the cell line. The lower band detected by the Brca1 antibody may be a truncated Brca1 protein endogenous to the cell line, or a degradation product.*

gene sequences (3). Immunoprecipitation and immunoblot experiments of extracts of SeMet-treated cells were conducted. A p53-antibody/agarose conjugate was used to capture p53 and then the membranes were probed for the interacting proteins Ref1 and Brca1. Both Ref1 and Brca1 were detected in complex with p53 in SeMet-treated cells (Figure 1). As a control, pyrrolidine dithiocarbamate (PDTC), known to oxidize p53 cysteine residues (12), was used. PDTC completely blocked the interaction of p53 with Ref1 and Brca1 (Figure 1).

To ascertain that the protein interactions we observed were direct interactions, recombinant proteins of bacterial or baculovirus origin were used. The recombinant proteins were incubated together and the immune complexes were then captured by p53-antibody/agarose beads. Blots were probed for Brca1 and Ref1 proteins as in Figure 1. Brca1 was not displaced from p53, even in the presence of excess Ref1 (Figure 2A). As a control, Rad51, which strongly competed for interaction with p53, displacing both Ref1 and Brca1 (results not shown), was employed. Reciprocal experiments, in which recombinant protein complexes were formed and then probed for the presence of Ref1 (Figure 2B), were conducted. Rather than immunocapture, the chemical crosslinking agent dimethylsuccinimide was used to covalently link the interacting proteins (8). No evidence of crosslinking between Ref1 and Brca1 alone was found, in as much as the 2 proteins are not known to interact (Figure 2B). However, the presence of p53 caused a larger protein complex to be detected. The putative ternary complex containing p53, Brca1 and Ref1 was detected only in lanes where all 3 proteins were present (Figure 2B).

Most p53-regulated cellular functions depend on p53 interaction with downstream effector gene sequences. A consensus p53-binding site has been derived from the study of a number of p53-dependent gene elements (13). We used a consensus p53-binding double-strand oligonucleotide coupled to agarose to capture p53 and any p53-bound proteins (Figure 2C). Recombinant proteins were mixed as above, then immunocapture was conducted in an excess volume of a buffer containing 1% Triton X-100. The beads were washed extensively in the same buffer and the proteins were detected by immunoblotting. Both Brca1 and Ref1 were detected in the p53 complexes (Figure 2C). EMSA assays were conducted using the radiolabelled free double-strand p53-binding sequence as in (4). However, the high molecular weight of the putative ternary complex caused most of the protein to remain in the wells (results not shown). We chose the less complex assay shown in Figure 2C to demonstrate that Ref1 and Brca1 can concurrently interact with p53 and do not compete for p53 binding in the context of p53 sequence-specific DNA binding.

The data point to a model (Figure 2D) in which Ref1 and Brca1 bind cooperatively to p53. Although recombinant Brca1 binds p53, irrespective of the presence or absence of Ref1 (Figure 2A), cellular Brca1 bound to p53 in the presence of wild-type but not mutant Ref1 (Figure 2E). The C65A Ref1 mutant affects the redox center of Ref1 and cannot promote p53 cysteine 275/277 sulfhydryl reduction. Likewise, PDTC, a known oxidizer of p53 cysteines 275/277, blocked or decreased Brca1 and Ref1 interaction with p53 (Figure 1). Recombinant proteins were already in the cysteine-sulfhydryl reduced form, owing to the presence of DTT (Figure 2A).

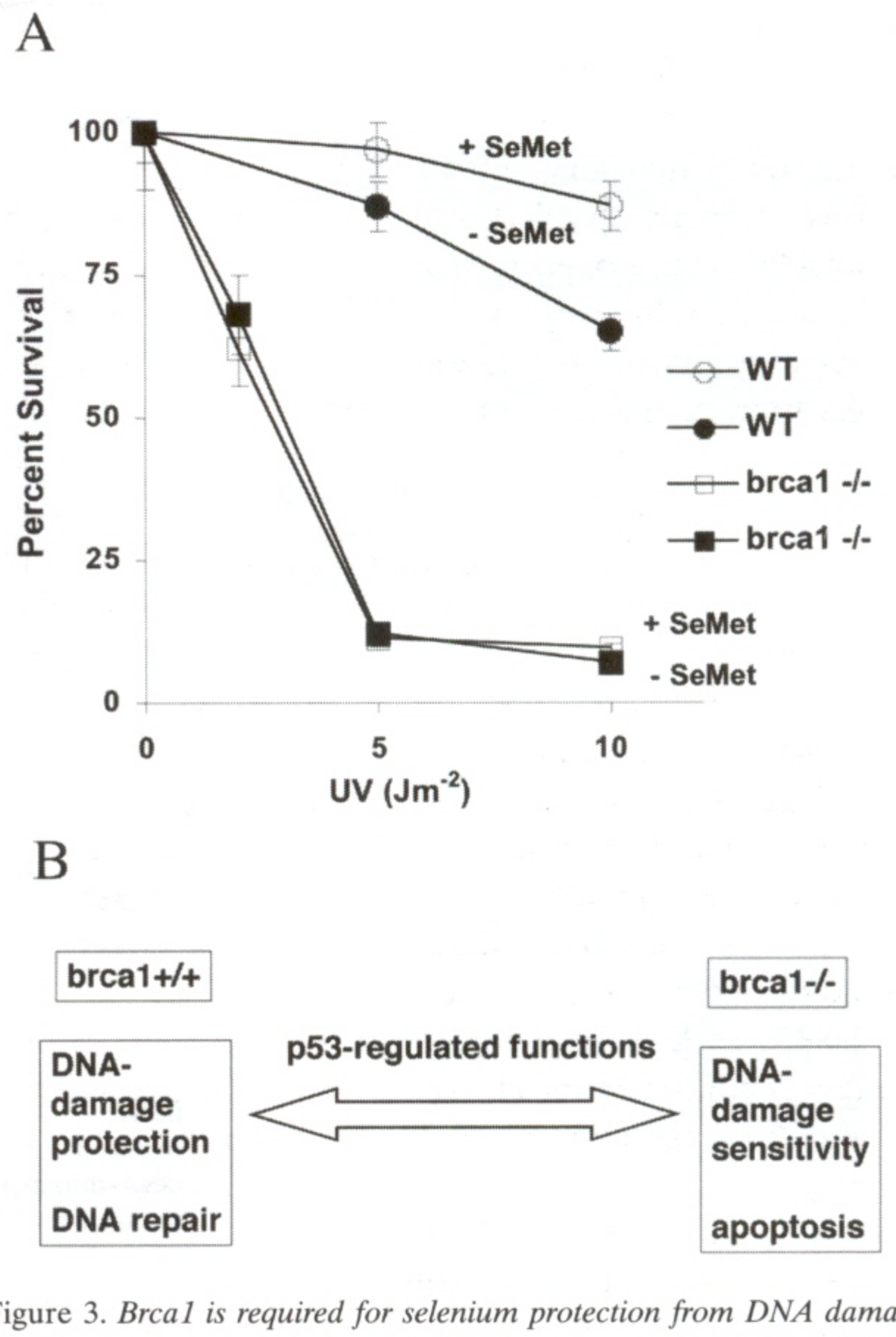


Figure 3. *Brca1 is required for selenium protection from DNA damage. A) Primary, low-passage, brca1-/- MEFs were treated with 10 μM SeMet for 15 h and were then exposed to UV radiation as indicated. The cell yield was determined after 7 days. SeMet did not protect brca1 -/- MEFs from UV-radiation. The mean±SD of 3 independent determinations is shown. B) Model depicting a role for Brca1 in directing p53 in DNA repair and DNA damage protection. In the absence of Brca1, p53-mediated apoptosis is favored over DNA damage protection, i.e. Brca1 is a key component of the p53-mediated protective response to UV-radiation (15, 18, 26, 27).*

The above findings suggest that, in addition to p53 and Ref1 (4), Brca1 might also be involved in DNA damage protection by SeMet. We used brca1-/- mouse embryo fibroblasts to test the hypothesis. Similar to our earlier findings using p53-/- mouse fibroblasts (4), SeMet did not protect brca1-/- fibroblasts from UV-radiation (Figure 3). Thus, the SeMet-enhanced DNA repair and protective responses observed in normal fibroblasts require not only p53 and Ref1, but also Brca1.

Discussion

The tumor suppressor p53 has multiple cellular functions that are regulated by protein-protein interactions. Quite a number of proteins are known to interact with p53 and to regulate p53 functions, leading to distinct cellular end-points

pertaining to cell survival, including apoptosis, DNA repair and cell cycle effects (14). It is extremely unlikely that the many p53-interacting proteins are able to bind p53 simultaneously, thus some interactions are probably mutually exclusive. Interactions that are mutually exclusive would presumably mediate opposing (*i.e.* mutually exclusive) effects on p53 function. Interactions that occur concurrently would be compatible in the regulation of p53 function toward a singular functional end-point, *i.e.* DNA repair (15).

We showed that p53-dependent DNA repair was the predominant pathway whereby selenium protected normal fibroblasts from DNA damage, and that the redox factor Ref1 was required to convey selenium signal transduction to p53 (4, 6). Specifically, Ref1 binds to p53 causing the reduction of sulfhydryl groups on key p53 cysteines 275 and/or 277 (4). Mutation of Ref1 cysteine 65 to alanine blocked p53 cysteine reduction in response to selenium, possibly involving the selenoenzyme thioredoxin reductase (3, 4, 16). Selenium enhanced the sequence-specific DNA binding and trans-activation of DNA repair genes by p53, with no evidence of cell cycle arrest or apoptosis in normal fibroblasts, at least in response to seleno-L-methionine (SeMet) at concentrations below 45 μM (4, 6, 17). The use of higher (non-physiological) SeMet concentrations or other chemical forms of selenium may lead to apoptosis or other functional consequences (7). Thus, SeMet, at relatively physiological nontoxic concentrations, selectively enhanced the DNA repair branch of the p53 pathway and protected mouse or human fibroblasts from DNA damage in the form of UV-radiation (4, 6). Both DNA repair and DNA damage protection were p53-dependent and both required a 15-h SeMet pretreatment prior to DNA damage (4).

We focused on the DNA repair branch of the p53 pathway. One p53-interacting protein known to regulate the DNA repair branch of the p53 pathway is Brca1 (2, 18). Therefore, the finding that Ref1 and Brca1 can interact concurrently with p53 supports the hypothesis, among the many alternative p53 functions, that the DNA repair branch of the p53 pathway was preferentially activated in selenium-treated cells (4, 6).

The data also suggested that cells lacking either p53, Ref1, or Brca1 would be defective in mounting a protective response to selenium treatment. We showed previously that selenium protection from DNA damage required p53 and Ref1 (4). Here, we showed that Brca1 is additionally required (Figure 3). Besides NER, Brca1 participates in other DNA repair pathways, about which less is known mechanistically (19). For example, Brca1 is required for Rad51 protein assembly at sites of double-strand break DNA damage (20, 21). Curiously, p53 also binds to Rad51 and p53-/- MEFs exhibited a phenotytpe of rad51 "stalling" at double-strand break sites (22, 23). Brca1 functions are complex and may involve DNA damage signaling *via* the poly-ADP-ribose polymerase (PARP) signaling system (24). It is likely that different domains of Brca1 are involved in DSB repair, as compared to its role in transcription of DNA repair genes (2), given that Rad51 and Brca1 interactions with p53 were mutually exclusive in the current study. Of course, the role of Brca1 in DSB repair may be a direct one, while its role in NER requires transcription of the NER genes *XPC, p48XPE* and *Gadd45a* (15, 17, 25). Moreover, NER occurs in the G1- and G2-phases of the cell cycle, while Rad51 functions mainly in the S-phase. More studies are needed for mechanism-driven approaches to cancer prevention and therapy, such as employing selenium in conjunction with DNA repair targets.

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Chemotherapeutic selectivity conferred by selenium: a role for p53-dependent DNA repair

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Abstract

Selenium in various chemical forms has been the subject of cancer chemoprevention trials, but, more recently, selenium has been used in combination with DNA-damaging chemotherapeutics. Specifically, selenium protected tissues from dose-limiting toxicity and, in fact, allowed delivery of higher chemotherapeutic doses. At the same time, selenium did not protect cancer cells. Therefore, we seek to define the genetic basis for the observed selectivity of selenium in combination chemotherapeutics. The tumor suppressor p53 is mutated in the vast majority of cancers, but is by definition wild-type in nontarget tissues such as bone marrow and gut epithelium, tissues that are often dose-limiting due to DNA damage. We used primary, low-passage mouse embryonic fibroblasts that are wild-type or null for *p53* genes to test differential effects of selenium. Seleno-L-methionine, nontoxic by itself, was used to pretreat cell cultures before exposure to UV radiation or UV-mimetic cancer chemotherapy drugs. Seleno-L-methionine pretreatment caused a DNA repair response, which protected from subsequent challenge with DNA-damaging agents. The observed DNA repair response and subsequent DNA damage protection were p53 dependent as neither was observed in p53-null cells. The data suggest that (*a*) p53 may be an important genetic determinant that distinguishes normal cells from cancer cells, and (*b*) combinatorial chemotherapeutics that act by p53-dependent mechanisms may enhance chemotherapeutic efficacy by increasing the chemotherapeutic window distinguishing cancer cells from normal cells. [Mol Cancer Ther 2007;6(1):355–61]

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Introduction

The majority of investigation with selenium has emphasized cancer chemoprevention, and there are a number of large prevention clinical trials, many focusing on prostate cancer (1). The potential role for selenium in cancer chemotherapeutics is an area that has shown significant promise in preclinical and small clinical trials, but this potential has been overshadowed by the prevention studies. Compelling preclinical work has shown that nude mice bearing human tumor xenografts that received daily seleno-L-methionine (SeMet) supplementation before and during chemotherapy better tolerated increasing doses of irinotecan. Dose escalation allowed elimination of previously chemoresistant tumors (2). A clinical trial using selenium supplementation during chemotherapy has been initiated based on these results (3). Furthermore, phase I trials have shown that SeMet can be administered in very high doses without significant toxicity (4, 5).

There have been relatively few clinical trials investigating the effect of selenium supplementation during cancer chemotherapy; nevertheless, the results have been positive. Forty-one patients undergoing cisplatin chemotherapy were randomized into two groups, and the group that received selenium showed significantly higher WBC counts on day 14 after initiation of chemotherapy (6). Furthermore, consumption of granulocyte colony-stimulating factor and volumes of blood transfusion were significantly less in the selenium-supplemented group. An ovarian cancer study was done with 62 women undergoing cisplatin and cyclophosphamide combination chemotherapy and half of the patients received selenium supplementation (7). The group that received selenium showed significantly reduced neutropenia as well as increased WBCs from the second to third chemotherapy cycle. The authors also report that with selenium supplementation, there seemed to be a significant decrease in all cited side effects: nausea, vomiting, hair loss, etc. It was also noted that serum and tissue selenium levels in the control group decreased during the chemotherapy regimen whereas levels in the study group increased. Neither of these studies observed any loss of chemotherapeutic efficacy in association with selenium supplementation.

Of the major types of DNA repair, nucleotide excision repair (NER) is the repair pathway responsible for removing bulky lesions. For example, 6-4 photoproducts and cyclobutane pyrimidine dimers caused by UV radiation are repaired by NER. Similarly, platinum-DNA adducts formed by platinum-containing cancer chemotherapeutics are repaired by NER (8, 9). NER is divided into two distinct pathways: global genomic repair and transcription-coupled repair. Both pathways have three

basic steps: recognition of the damaged lesion, excision of the lesion, and resynthesis. The pathways differ in the initial recognition step but use the same proteins for the subsequent steps.

The damage recognition step of NER is rate limiting. For global genomic repair, regulation of this step is controlled by p53. Cells that have defective p53, such as those from patients with Li-Fraumeni syndrome, have defective global genomic repair but retain proficient transcription-coupled repair (10–13). p53 regulates the rate-limiting step in global genomic repair through transcriptional control of the DNA damage recognition proteins xeroderma pigmentosum complement groups C (XPC) and E (XPE). It has been shown that p53 transcriptionally regulates p48/XPE/DDB2, and forced overexpression of p48/XPE/DDB2 enhances global genomic repair (14–17). Likewise, XPC mRNA and protein expression is increased in a p53- and DNA damage–dependent manner (18). It has also been shown that within minutes of UV irradiation, p48 and XPC proteins localize to the damaged sites and that p48 enhances XPC binding (15). Several studies highlight the analogous repair of UV-damaged DNA and damage caused by platinum chemotherapeutics. $XPC^{-/-}$ cells are defective in the repair of cisplatin damage, and it has been shown that XPC protein is required for cisplatin damage recognition (19, 20).

A role for selenium in DNA repair was first noticed when selenium treatment was shown to enhance host cell reactivation of a UV-damaged reporter plasmid template (21). It was later shown that selenium could only modulate DNA repair in cells with normal p53 (22). Selenium protection from DNA damage requires redox factor 1 (Ref1), which interacts with p53 and reduces key p53 cysteine residues (22, 23). The selenoprotein thioredoxin reductase is also required for p53 cysteine reduction (24). A dominant-negative Ref1 mutant blocked SeMet-induced transactivation by p53 (22). The reduced conformation of p53, promoted by SeMet, induces its transcription factor activity and the transcriptional activation of proteins responsible for recognition of DNA damage. Furthermore, the subsequent results show that SeMet elevates DNA repair and protects cells from DNA damage in the absence of cell cycle arrest or apoptosis. A potential rationale for this differential activity by p53 is likely due, in part, to posttranslational effects. It has been shown that different chemical forms of selenium have different effects on p53 phosphorylation, which alter the cellular response (25–27).

Selective modulation of NER has significant implication for patients being treated with DNA-damaging chemotherapeutic agents. The following results show that bone marrow and gut epithelium exhibited enhanced DNA repair following selenium treatment. The DNA repair activity of the cancer cells was unaffected. This effect may allow patients to receive more intense treatment without exacerbating unpleasant side effects. Experiments using matched isogenic cell lines, as well as tumors and genetically normal tissues, show that a selenium-inducible DNA repair response protects from DNA damage and is p53 dependent. Selenium treatment did not protect or increase DNA repair in p53-deficient cells.

Materials and Methods

Chemotherapeutic Drugs

Cisplatin (purchased from Sigma, St. Louis, MO) was dissolved in DMSO as a 10 mmol/L stock solution. Carboplatin was used in some experiments instead of cisplatin, and results were identical. Oxaliplatin (purchased from HandiTech, Houston, TX) was dissolved in sterile water as a 10 mmol/L stock solution. All chemotherapeutics were frozen in small aliquots and stored at −20°C. Final concentrations in tissue culture medium were as indicated. Interleukin-6 and stem cell factor were purchased from PeproTech (Rocky Hill, NJ).

Cell Lines and Treatments

Mouse embryonic fibroblasts (MEF) of wild-type and $p53^{-/-}$ genotypes were of low passage from our frozen stocks as previously described (28). MEF were from a C57/129 genetic background. Noncancer cells were IEC6 rat gut epithelial cells (American Type Culture Collection, Rockville, MD) and primary mouse bone marrow cells (C57/129). Bone marrow cells were stimulated with interleukin-6 (200 units/mL) and stem cell factor (100 ng/mL) for 24 h, then treated with SeMet (10 μmol/L) for 15 h, followed by DNA damage by cisplatin or oxaliplatin at the concentrations indicated. Cancer cell lines of human origin A253 and FaDu were from a previous study (2, 29). Both are squamous cell carcinoma of head and neck lines and carry mutant *p53* genes. FaDu carries a R248L mutant p53 allele (30), whereas A253 carries deletions in both p53 alleles (31). Xeroderma pigmentosum XPA cells defective in DNA repair served as a negative control for some experiments, as previously described (28). Cell lines were likewise treated with SeMet (10 μmol/L, 15 h) and then with DNA-damaging chemotherapeutic drugs at concentrations and durations indicated. MEF were grown in DMEM (4.5 g/L glucose) plus 10% fetal bovine serum. Other cell lines were maintained in RPMI 1640 plus 10% fetal bovine serum, except for bone marrow, which was maintained in Iscove's modified Dulbecco's medium plus 20% fetal bovine serum, interleukin-6 (200 units/mL), and stem cell factor (100 ng/mL).

Cell Survival

Cell yield was determined by thiazolyl blue assay 7 days after DNA-damaging treatments. This assay can be applied to all cell lines irrespective of their colony-forming ability, a consideration for the MEF and other primary cells, which do not form colonies. Cells were plated at ~1,000 per well in 96-well culture plates, allowed to attach for 24 h, and treated with SeMet (10 μmol/L, 15 h) and then with DNA-damaging drugs for 2 h. Drugs were removed by washing the wells in culture medium with aspiration, then medium was replaced for the 7-day duration. On day 7, 50 μL of 2 mg/mL thiazolyl blue reagent were added to each well

and plates returned to incubator for 4 h to allow formation of a blue precipitate. The amount of blue precipitate was proportional to the number of viable cells by visual inspection. Precipitates were dissolved in DMSO and quantified by a Tecan plate reader at a wavelength of 592 nm. Data were normalized to control cells that did not receive DNA damage and expressed as percent cell yield relative to untreated controls. Data were averaged from three or more independent determinations, with wells in multiples of six in each experiment. Additionally, clonogenic cell survival was determined in some data sets. Clonogenic cell survival was conducted as described (32).

Unscheduled DNA Synthesis

DNA repair synthesis or unscheduled DNA synthesis was determined as previously described (28). Cells were treated with SeMet (15 h, 10 μmol/L), then with DNA-damaging agents to induce unscheduled DNA synthesis. The prototype DNA-damaging agent was UV radiation (20 J m^{-2}, 254 nm), which served as a positive control to induce unscheduled DNA synthesis (28). XPA cells served as a negative control because they are severely defective in nucleotide excision DNA repair (NER, <1% of normal) yet they are healthy cells unless exposed to DNA damage. After UV radiation, cellular DNA was labeled with tritiated thymidine (10 μCi/mL) in tissue culture medium for 3 h. Cisplatin (100 μmol/L) or oxaliplatin (100 μmol/L) was delivered to cells for 5 h concurrent with tritiated thymidine uptake. Cells were fixed on glass slides in ethanol, then processed for autoradiography. S-phase nuclei were strongly labeled by the tritiated thymidine and were excluded from analysis. Non–S phase nuclei, primarily in the G_1 phase of the cell cycle, exhibited DNA repair synthesis (unscheduled DNA synthesis). The number of DNA repair sites per nucleus was determined, and ≥200 nuclei were assayed for each data set.

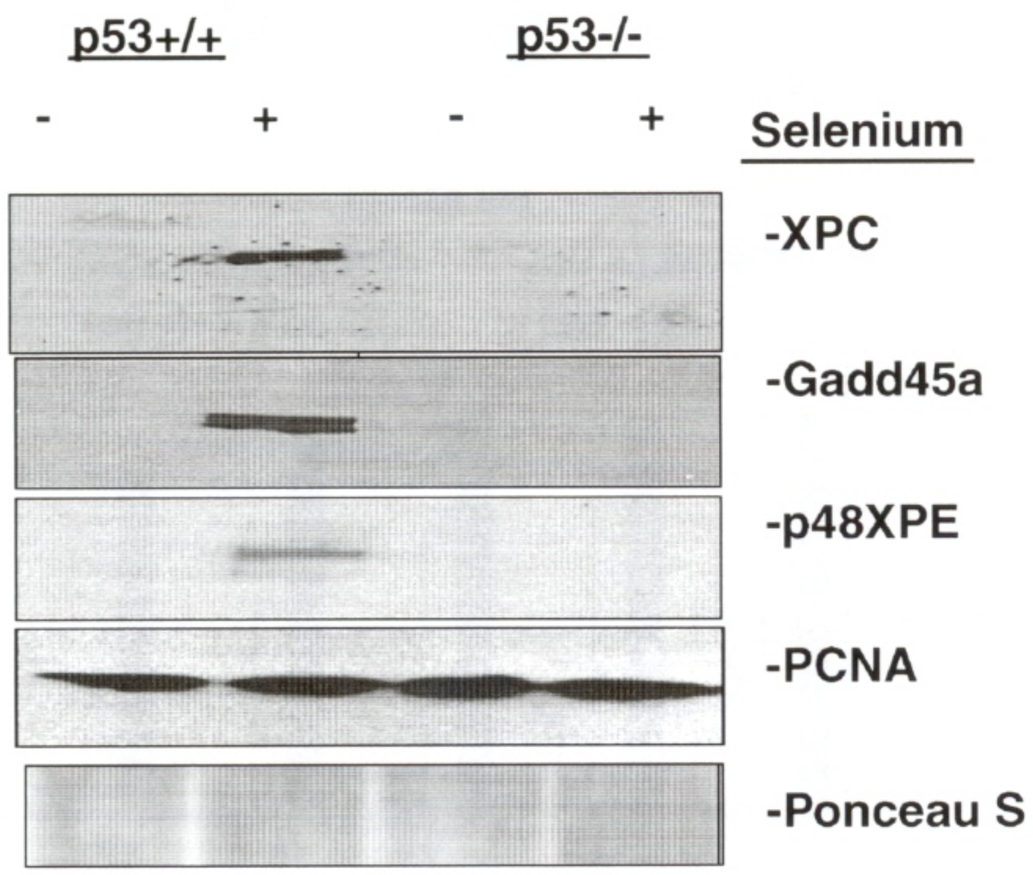


Figure 1. SeMet treatment (10 μmol/L, 15 h) caused elevated expression of p53-dependent DNA repair proteins XPC, XPE, and Gadd45a, which compose the ''DNA repair branch'' of the p53 pathway. Immunoblots were conducted with wild-type and $p53^{-/-}$ MEF. DNA repair proteins were not detected in $p53^{-/-}$ MEF. Proliferating cell nuclear antigen (*PCNA*) immunoblot and Ponceau S staining served as loading controls.

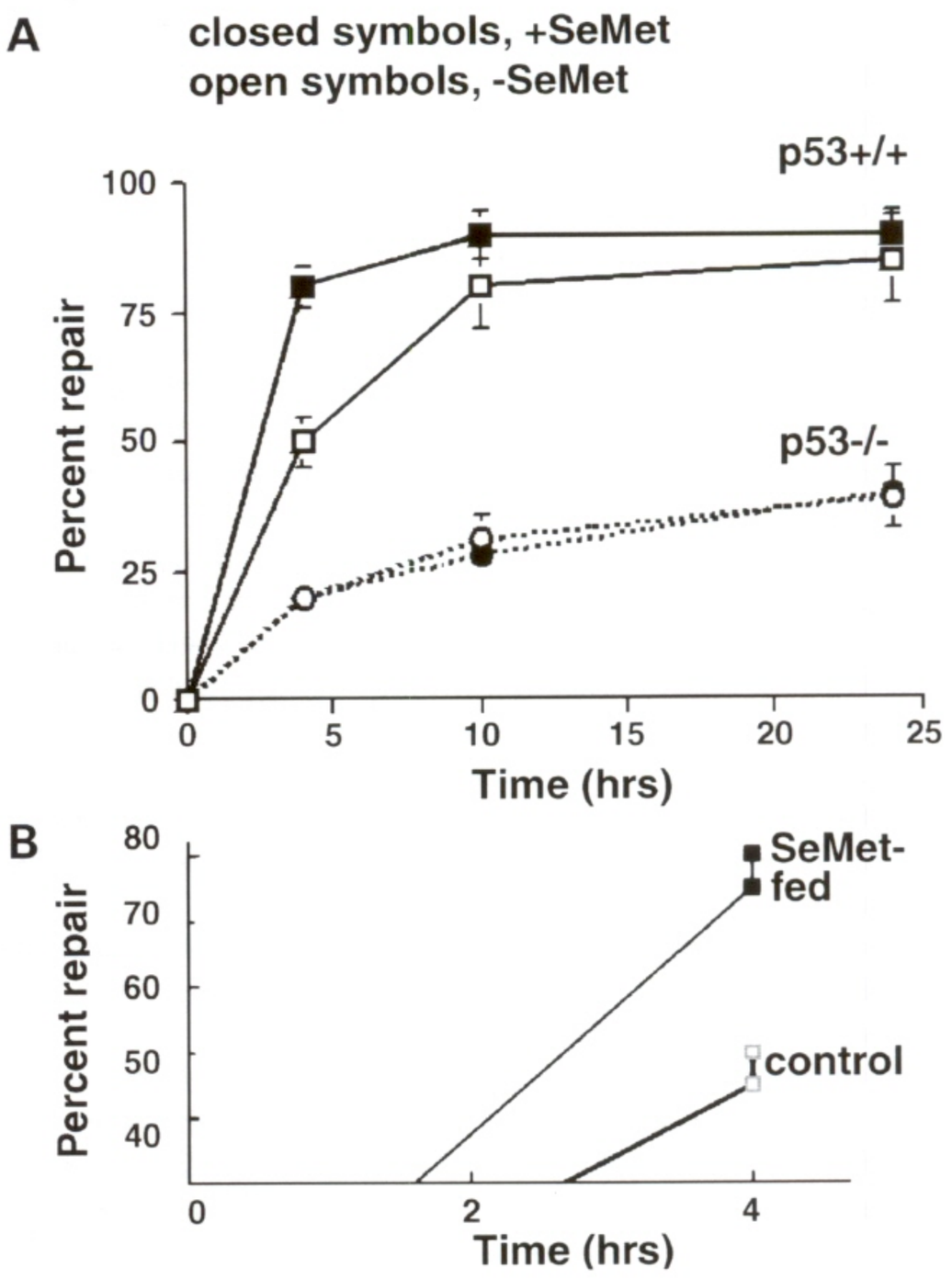


Figure 2. **A,** SeMet treatment (10 μmol/L, 15 h) increases the rate of repair of UV-induced DNA damage. MEF were treated with SeMet, then with UV radiation (20 J m^{-2}, 254 nm), and allowed indicated times for removal of UV lesions. An antibody to 6-4 photoproducts was used to assay 6-4 photoproduct removal from genomic DNA. The rate of 6-4 photoproduct removal was enhanced by selenium in wild-type MEF, but $p53^{-/-}$ MEF were unaffected. *Points,* mean of three independent determinations; *bars,* SD. $P < 0.04$, Wilcoxon rank-sum test. Note the slow rate of lesion removal in $p53^{-/-}$ MEF. **B,** *in vivo* evidence for a DNA repair response to SeMet. Feeding of mice with 200 μg/d 200 μg/d SeMet × 5 wk leads to increased DNA repair. Removal of 6-4 photoproducts was determined as in **A**.

Results

SeMet and Protein Expression

In cells treated with SeMet overnight, p53 is reduced to its transcriptionally active conformation and induces expression of NER damage recognition factors. XPC and p48XPE proteins are the main contributors to damage recognition in NER. Wild-type and $p53^{-/-}$ MEF were treated overnight with SeMet. The selenium-induced expression of damage recognition proteins is p53 dependent. Wild-type cells treated with SeMet had increased

expression of several proteins known to be involved in NER DNA damage recognition whereas $p53^{-/-}$ cells showed no change in expression of these factors (Fig. 1). Proliferating cell nuclear antigen and Ponceau S staining served as loading controls.

SeMet and Repair Rate

SeMet induces expression of damage recognition factors and has been shown to protect from DNA damage. Using an antibody to 6-4 photoproducts, a prototypical UV-inducible lesion, and cells exposed to UV radiation, the rate of repair can be assayed by monitoring the persistence of damaged lesions. Following overnight SeMet treatment, cells with wild-type p53 have fewer lesions at the indicated times (Fig. 2A). Furthermore, persistence of lesions in $p53^{-/-}$ cells is not affected. Untreated cells serve as controls. Repair rates following SeMet treatment are expressed relative to untreated controls.

To ascertain if a DNA repair response to SeMet occurs *in vivo*, mice were given 200 μg/d SeMet orally for 5 weeks. Total bone marrow cells were UV irradiated and then incubated in tissue culture for 4 h to repair. Removal of 6-4 photoproducts was determined. Repair rates by SeMet feeding are shown relative to control mice (Fig. 2B).

SeMet and Chemotherapy

Selenomethionine protects wild-type MEF from UV radiation or cisplatin (Fig. 3). $p53^{-/-}$ MEF were not protected. Cells were pretreated with 10 μmol/L selenomethionine for 15 h before DNA-damaging treatments. Cell survival was determined after 7 days by thiazolyl blue assay. Data of cell yield after 7 days are expressed relative to controls not treated with selenomethionine and controls not treated with DNA-damaging agents. The results shown for UV radiation are similar to those previously published (22). The similar results for UV and cisplatin treatment reiterate the requirement for p53-mediated NER for both types of damage. Cisplatin concentrations were as indicated. The implication is that p53 status is a molecular determinant that mediates DNA repair by selenium. The following experiments address this possibility.

SeMet and DNA Repair

The above findings show that SeMet treatment induces expression of NER damage recognition factors and elevates the rate of repair. Furthermore, SeMet protected wild-type, but not $p53^{-/-}$, cells from DNA damage. The unscheduled DNA synthesis assay was used to assay DNA repair *in vitro*. The method is illustrated in Fig. 4A. Isogenic wild-type and p53-deficient MEF (Fig. 4B) were treated with selenomethionine and various DNA-damaging agents and then unscheduled DNA synthesis was evaluated. Additionally, the effect of selenomethionine on DNA repair was evaluated in primary rat gut epithelial cells (IEC6), primary murine bone marrow, and two of the human squamous cell carcinoma of the head and neck (A253 and FaDu) cell lines used for xenografts in Cao et al.'s (2) study (Fig. 4C). Cells were treated with a variety of DNA-damaging agents: UV, cisplatin, or oxaliplatin. Wild-type MEF, rat gut epithelial cells, and murine bone marrow with genetically normal p53 show a significant increase in unscheduled DNA synthesis when treated with selenomethionine before DNA damage $P < 0.02$ (t test). Cells lacking functional p53 [A253 (p53 mut), FaDu ($p53^{-/-}$), and $p53^{-/-}$ MEF] were unresponsive to selenomethionine and showed no increase in unscheduled DNA synthesis.

SeMet Metabolites

Besides being used as seleno-amino acids for selenoprotein synthesis, low molecular weight metabolites of selenium compounds can mediate some biological responses. We used methyl selenenic acid as a representative SeMet metabolite. Although methyl selenenic acid showed some evidence for a DNA repair response at <1 μmol/L concentration (27), apoptosis predominated at methyl selenenic acid concentrations >1 μmol/L (Fig. 5). The DNA repair and protective effect of SeMet are therefore not likely due to low molecular weight metabolites.

Discussion

Clinical trials have shown that selenium supplementation during chemotherapy may partially alleviate the

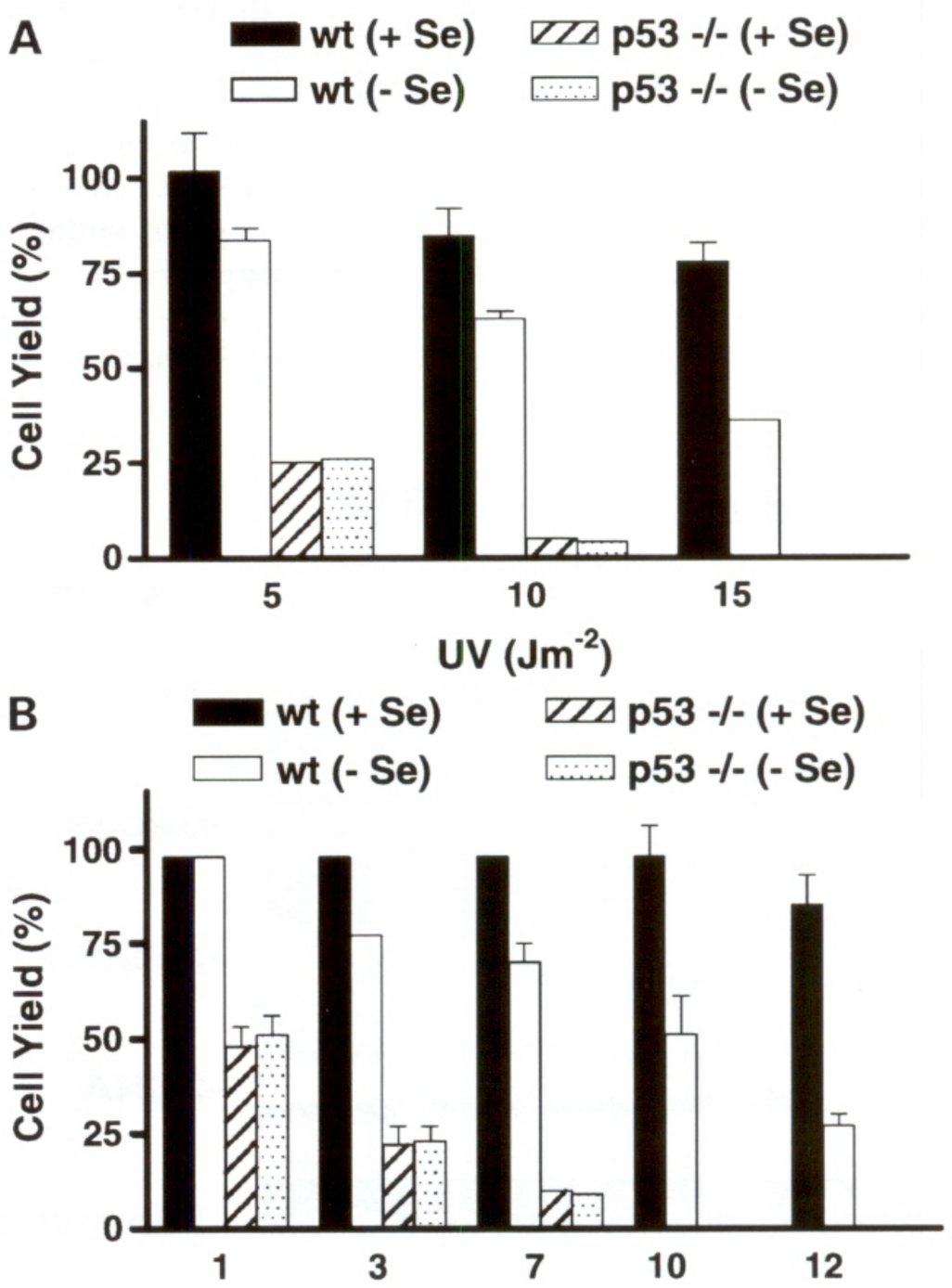


Figure 3. SeMet treatment (10 μmol/L, 15 h) promotes cell survival in wild-type, but not $p53^{-/-}$, MEF. MEF were treated with SeMet, then with 254-nm UV radiation (**A**) or cisplatin (**B**). Cell survival was determined after 7 d by thiazolyl blue assay. *Columns*, mean of three independent determinations; *bars*, SD. $P < 0.04$, t test.

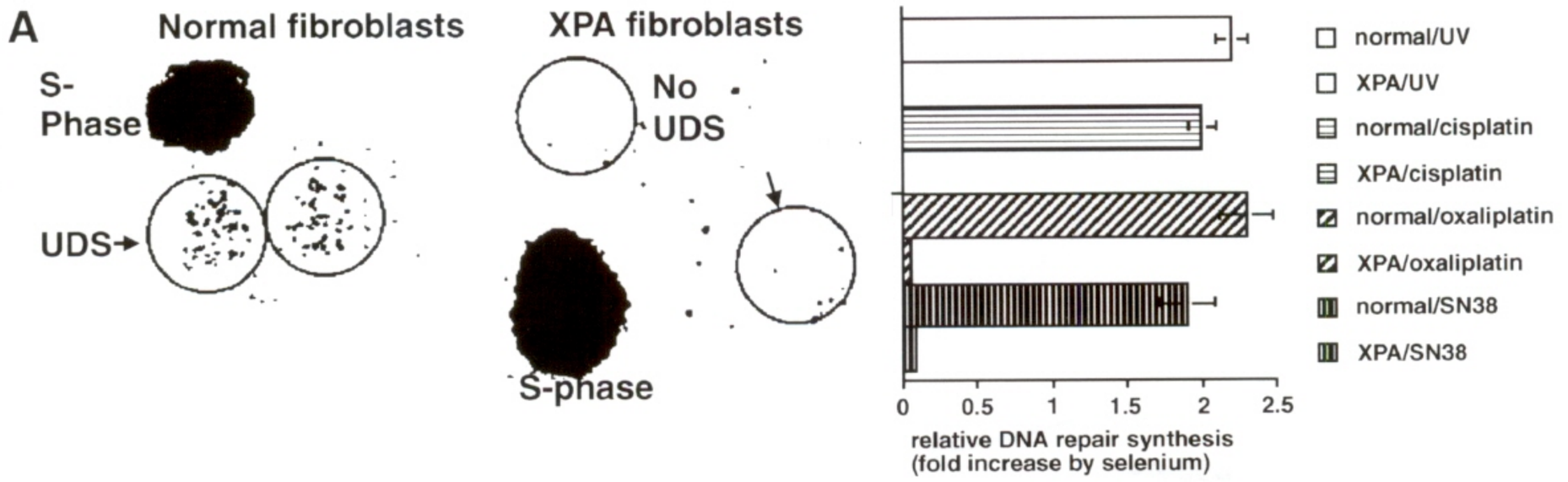


Figure 4. SeMet enhanced global genomic DNA repair as unscheduled DNA synthesis in wild-type, but not $p53^{-/-}$, MEF. **A,** illustration of methods and controls. Controls (normal human fibroblasts and DNA repair–defective XPA fibroblasts) were UV irradiated (20 J m^{-2}, 254 nm) and incubated in the presence of tritiated thymidine for 3 h, during which time the tritium label was incorporated into NER repair patches. Slides were processed for autoradiography. S-phase nuclei were excluded from analysis. By definition, unscheduled DNA synthesis (*UDS*; or repair synthesis) is confined to G_1 and G_2 nuclei. The number of tritium grains per nucleus is a direct measure of sites of repair synthesis. Cells not treated with DNA-damaging agents showed little or no unscheduled DNA synthesis (28). **B,** MEF treated with UV radiation (20 J m^{-2}, 254 nm), cisplatin (50 μmol/L), or oxaliplatin (1 μmol/L) for 4 h concurrent with tritiated thymidine labeling. SeMet was added to the medium 15 h before DNA-damaging treatments. Shown is relative repair synthesis (SeMet treated divided by SeMet untreated for each respective sample) in G_1 nuclei; *bars,* SD. At least 200 nuclei were determined per data point. SeMet induced NER in wild-type MEF ($P < 0.01$, *t* test). SeMet did not significantly induce NER in $p53^{-/-}$ MEF. **C,** SeMet induced NER in normal mouse bone marrow and in primary rat gut epithelial cells ($P < 0.01$, *t* test) but did not significantly induce NER in p53-mutant cancer cell lines A253 and FaDu.

dose-limiting and poor quality-of-life side effects. In two studies, selenium supplementation significantly reduced myelotoxicity, and in one study, selenium reduced other side effects attributed to the toxicity to rapidly proliferating nontarget tissues (6, 7, 33). The findings herein show that selenium supplementation elevates expression of proteins responsible for recognition of DNA damage. The increased expression of recognition factors is concomitant with an increase in the rate of DNA repair and overall DNA repair synthesis. However, all of these selenium-inducible observations are absent in a p53-null background. That is, selenium did not induce expression of key NER recognition factors or alter the rate or overall level of DNA repair in the p53-null cells or tumor cell lines tested. The conclusion is that selenium selectively protects genetically normal cells from DNA-damaging chemotherapeutics, while simultaneously offering no detectable protection to cells either completely lacking p53 or possessing only mutant p53. This is important considering that p53 is the most widespread genetic alteration in human cancer, with as many as 70% of tumors having a mutant p53 phenotype. One caveat is that some cancers with wild-type p53 may not be ideally suited for selenium therapies.

The results suggest a potential mechanism for selenium-inducible protection from chemotherapy in the clinical trials highlighted above and in the context of chemoprevention. In the nontarget tissues, an increase in the basal levels of NER damage recognition factors following selenium supplementation promotes an increase in the basal rate of NER, which can better tolerate the additional damage from chemotherapy. The elevated DNA repair synthesis in cells from nontarget tissues in this report, combined with the data from an earlier study showing selenium enhancing cure rates of xenograft tumors in nude mice (2), supports the proposed mechanism of selectivity.

The notion that p53 is an important marker for differentiating tumor cells from normal cells is not new. It is important to note, however, that a safe, reliable therapy that takes advantage of this widely known fact remains to be identified. The widespread p53 mutations in

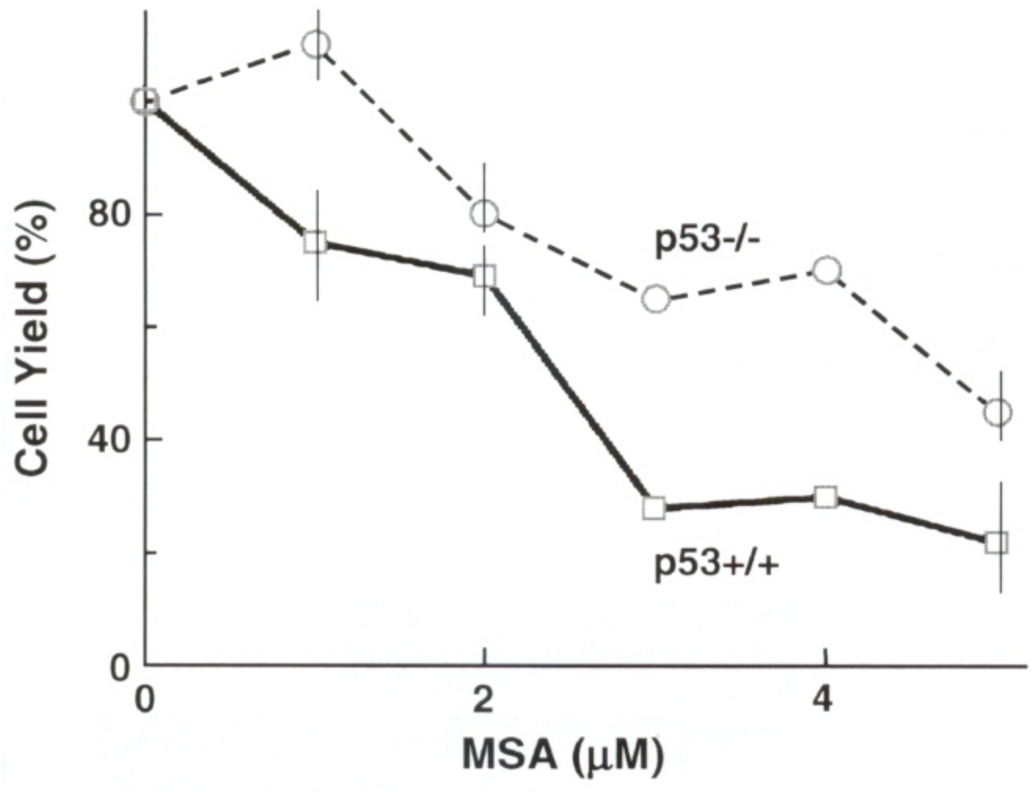


Figure 5. The selenium metabolite methyl selenenic acid did not induce a protective response in MEF. Rather, p53-mediated apoptosis predominated at methyl selenenic acid (*MSA*) concentrations >1 μmol/L. $p53^{+/+}$ and $p53^{-/-}$ MEF were treated with indicated concentrations of methyl selenenic acid for 4 h. Cell survival was determined after 5 d in culture. $p53^{+/+}$ MEF were preferentially sensitive to methyl selenenic acid ($P < 0.02$, Wilcoxon rank-sum test).

human cancer should be a benchmark for developing novel therapies. However, the inherent heterogeneity of tumors and their unpredictable responses to therapeutic strategies require extensive testing of tumor tissue. Whereas cells with altered p53 should be more sensitive to agents whose damage is repaired by the p53-regulated NER pathway, tumor cells have acquired other growth advantages, which may abrogate this potential weakness (34). A typical proposal for improving chemotherapeutic efficacy attempts to sensitize tumor cells by targeting their greatest defenses (e.g., apoptotic, cell cycle, and DNA repair targets). A strategy that protects normal cells instead is perhaps more reliable. Selenium supplementation has recently been shown in clinical trials to be nontoxic at very high doses (4). In fact, ongoing trials are attempting to reach levels of at least 15 μmol/L, which shows that the concentrations used in the present study are physiologically relevant (5). The results of this study present a safe potential method of improving chemotherapeutic selectivity that focuses on the genetically normal, nontarget tissues, which may be a more promising foundation for novel therapeutic strategies.

In the United States, serum selenium concentrations of 1 μmol/L are fairly common (1). At 1 μmol/L concentration, both seleno-amino acids exemplified by SeMet and metabolic by-products of SeMet exemplified by methyl selenenic acid may contribute to DNA repair (27). At concentrations exceeding 1 μmol/L, such as in this study, methyl selenenic acid induced apoptosis, which would mask any DNA repair response (Fig. 5). Therefore, it is likely that DNA repair and DNA damage protection observed *in vitro* (refs. 21, 22, and this study) and *in vivo* (Fig. 2B) at selenium concentrations in the 15 μmol/L range are due to selenoproteins (e.g., thioredoxin reductase). Note, however, that the apoptotic response evoked by methyl selenenic acid also involves p53, as p53-wild-type MEF were preferentially sensitive to methyl selenenic acid (Fig. 5). DNA repair or apoptotic responses would each be important in chemotherapy, albeit mediated by different selenium chemical forms.

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The Xpc DNA repair gene markedly affects cell survival in mouse bone marrow and involves the Cul4a/Cdt1 checkpoint

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Abbreviations: Xpc, protein encoded by the *xeroderma pigmentosum* XPC gene; G-NER, global genomic nucleotide excision DNA repair; TC-NER, transcription-coupled nucleotide excision DNA repair; WBC, white blood cells

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Abstract

The Xpc protein is a key DNA-damage recognition factor that is required for global genomic nucleotide excision repair (G-NER). In contrast to transcription-coupled NER (TC-NER), G-NER has been reported to contribute little if at all to cell survival after DNA damage. Previous studies were conducted using fibroblasts of human or mouse origin. Since the advent of *xpc*-/- mice, no study has focused on the bone marrow of these mice. We used carboplatin to induce DNA damage in *xpc*-/- and strain-matched wildtype mice. Using several independent methods *xpc*-/- bone marrow was about 10-fold more sensitive to carboplatin than the wildtype. Importantly, 12/20 *xpc*-/- mice died while 0/20 wildtype mice died. We conclude that G-NER, and Xpc specifically, can contribute substantially to cell survival. The data challenge the dogma that G-NER contributes little to cell survival. The data are important in the context of cancer chemotherapy, where *XPC* gene status and G-NER may be determinants of response to DNA-damaging agents including carboplatin. Additionally, the Cul4a and Cdt1 cell cycle checkpoint proteins show differences in ubiquitin modification in comparing *xpc*-/- and wild-type bone marrow cells. The data suggest a defect in cell cycle checkpoint signaling in *xpc*-/- mice involving a Cul4a/Cdt1/Xpc cell cycle checkpoint defect.

Introduction

The *xeroderma pitgmentosum XPC* gene is defective in a subset of human patients exhibiting the cancer-prone disease *xeroderma pigmentosum* which results from defective nucleotide excision DNA repair (NER). *XPC* patients are sensitive to sunlight and UV-radiation-induced DNA damage and skin cancers. They also exhibit internal cancers with advanced age. The gene products encoded by *XPC* and other *XP* genes A-F have been characterized biochemically [1]. Specifically, the Xpc protein is required and is rate-limiting for global-NER (G-NER). Xpc is not required nor apparently involved in transcription-coupled NER (TC-NER). Thus, studies of Xpc are good models for G-NER separate from TC-NER [1].

Mice lacking *xpc* genes (*xpc*-/- mice) were generated some ten years ago [2]. A number of studies of carcinogenesis and mutagenesis have been conducted, consistent with the cancer-prone human genetic disease discussed above [2-4]. No studies have examined bone marrow nor the cell survival response to DNA damage in *xpc*-/- mice.

Bone marrow is often dose-limiting in response to cancer chemotherapy drugs including carboplatin. While cell survival is a complex endpoint, the goal of chemotherapy is to sensitize cancer cells while retaining bone marrow cellularity. Agents that can protect bone marrow will be important adjuncts to chemotherapy. Presently, the cytokine GM-CSF is administered which causes proliferation of bone marrow myeloid stem/progenitor cells and thus re-populates bone marrow after chemotherapy [5]. The idea of G-NER as a protective mechanism in bone marrow or any other tissue is novel. The *xpc* gene protects bone marrow from carboplatin-induced DNA damage.

Materials and Methods

Mice. Mice originated from A.T Sands *et al*, 1995 [2], and were purchased from Taconic Farms and bred at Indiana University under license agreement as B6;129s7-XPC$^{tml/Brd}$ mice. Female mice were 10 weeks old at the time of initiating the experiments. Carboplatin (Sigma) was dissolved in sterile water and administered intraperitoneally at weekly intervals over six weeks, at 60 mg/Kg body weight. WBC counts were by weekly tail vein blood collection in EDTA-treated hematocrit tubes [6]. Counting was done using a Hemavet 950 (Drew scientific, Dallas, TX). Statistical analysis was conducted using a t-test using Microsoft Office 2003 or GraphPad Prism software. All mouse treatments were in accord with protocols approved by the Institutional Animal Care and Use Committee at Indiana University. Mice were sacrificed after 41 days using approved methods. Mice that appeared moribund before 41 days were sacrificed by veterinary staff.

DNA repair assay. To confirm the DNA repair defect in *xpc*-/- bone marrow, we used an immunoassay in which genomic DNA from UV-irradiated bone marrow cells, at 0, 4, 8, or 16 hrs after UV-irradiation, was fixed to 96-well microtiter plates at 10 ng per well. An antibody to 6-4 photoproducts (Trevigen Inc, Gaithersburg, MD, USA) was used to detect removal of lesions. Immunoassays were developed with peroxidase secondary antibody and ABTS chromogenic substrate and read at 405 nm in a Tecan Spectra plate reader. Immunoblotting of Xpc was with a rabbit polyclonal antibody (Santa Cruz Biotech, CA, USA).

Histochemical staining. Femurs were fixed in 10% buffered formalin overnight then paraffin embedded and thin sectioned for histological examination by a veterinary pathologist. Slides were stained with hematoxylin and eosin for morphological analysis or DAPI (4',6-diamidino-2-

phenylindole) to visualize intact nuclear DNA. Slides were visualized by a Nikon HB-10101AF fluorescence microscope using a 100X objective. Digital photographs were captured.

Colony-forming assays. Bone marrow was collected from femurs, fibulas, and iliac crests. Total bone marrow cells were then added to complete methylcellulose medium consisting of IMDM liquid medium (Sigma), 20% fetal bovine serum, interleukin-6 (200 units per mL), and stem cell factor 100 ng per mL (Stem Cell Technologies, Vancouver, Canada). Triplicate 35mm culture dishes were seeded with 10^5 cells and placed in a humidified incubator at 37°C for 10 days at which time colonies were counted manually as in [7].

MTS assay of cell viability. Bone marrow of wildtype and *xpc*-/- genotypes was cultured in 96-well plates in IMDM medium with cytokines as above, then treated *in vitro* with increasing concentrations of carboplatin for 2 hrs. Cell yield was determined after 72 hrs in culture by adding MTS (Promega, Madison, WI, USA) to the wells and reading the absorbance at 490 nm in a Tecan Spectra plate reader. Cells that did not receive carboplatin served as controls for each genotype representing 100% cell yield.

Cell cycle analysis. Bone marrow of wildtype and *xpc*-/- genotypes were cultured in IMDM containing cytokines as above for 48 hrs to stimulate proliferation, then treated with carboplatin for an additional 15 hrs. Untreated cultures served as controls for each genotype. Cells were fixed in 70% ethanol then analyzed on a Becton-Dickinson FacsScan for propidium iodide (PI) content. To analyze only proliferating cells, 10 μM bromodeoxyuridine (BrdU) was added to the cultures during the 48 hr growth period. Cycling cells were first gated for BrdU then with PI. The data represent 15,000 or more cells per data point.

Immunoblotting of cell cycle proteins. Initially whole cell lysates were used to probe for differences in cell cycle proteins between wildtype and *xpc*-/- bone marrow. Given apparent differences in Cdt1 and Cul4a, we purified total ubiquitinated proteins on a ubiquitin-binding resin (Pierce Chemical, Rockford, IL, USA). Cell lysates corresponding to 10^7 viable cells per sample were prepared in RIPA lysis buffer to which 50 μL of resin was added and rotated overnight at 4°C. The resin was collected by centrifugation and then boiled 15 min in SDS/gel loading buffer, and subject to electrophoresis in 4-20% polyacrylamide gels (InVitrogen, Carlsbad, CA, USA). Immunodetection was on nitrocellulose membranes using rabbit anti-Cul4a (34897; Abcam, Cambridge MA USA) or rabbit anti-Cdt1 (sc-28262; Santa Cruz Biotech, USA).

Additional experiments utilized a cell-free ubiquitin conjugation system (Boston Biochem, Cambridge, MA, USA). To the HeLa S-100 lysate supplied with the kit was added indicated amounts of purified Xpc protein. Ubiquitinated proteins were detected by immunoblotting as above. The PC-10 antibody was used to detect PCNA and ubiquitinated PCNA (Santa Cruz Biotech).

Results

Mice of wild-type and *xpc*-/- genotypes were first assayed for Xpc expression and global NER activity. Bone marrow was used directly for western blotting using an Xpc antibody. Bone marrow was additionally cultured 24 hrs, UV-irradiated, then subjected to an immunoassay for removal of DNA damage. Removal of DNA damage was 80% complete in wildtype mice after 16 hrs, while *xpc*-/- mice were completely defective in removal of the lesions (Fig. 1). Thus, the *xpc*-/- mice exhibit the expected defective NER phenotype.

Twenty mice of each genotype were divided into treatment groups and administered carboplatin at weekly intervals. Mice were 10 weeks old at the time of initiating the experiment.

By day 41 of carboplatin regimen, 12/20 *xpc*-/- mice had died unexpectedly. No deaths were observed in wild-type mice (results not shown). During the course of carboplatin treatments, peripheral white blood cell (WBC) counts were measured weekly [6]. Peripheral WBC counts in *xpc*-/- mice were significantly lower than in wildtype mice (results not shown). Remaining mice were sacrificed on day 41 using approved protocols. Mice were evaluated by a veterinary pathologist. The most significant pathology was marked hypocellularity in bone marrow in 10/10 mice examined, which was visibly striking in *xpc*-/- mice (Fig. 2, A & B).

To quantify and further characterize the hypocellularity in *xpc*-/- bone marrow, we conducted colony-forming assays and cell viability assays using a vital dye. A hallmark of myeloid stem/progenitor cell populations is their colony-forming ability when placed in culture and stimulated with cytokines. Colony-forming assays of bone marrow were conducted as in [ref. 7]. The data were plotted as total colonies per femur (Fig. 3A), as well as total colonies per 10^6 bone marrow cells (results not shown). Irrespective of the plotting method, *xpc*-/- mice that received carboplatin exhibited a 10-12-fold decrease in colony-forming units compared to wild-type after 10 days in culture (Fig. 3A). We also used the vital stain thiazolyl blue to measure cell viability after 4 days in culture. An advantage of the thiazolyl blue (or MTS) method is that a wide range of carboplatin concentrations can be assayed in contrast to *in vivo* carboplatin treatments. Using this method, a ten-fold difference between *xpc*-/- and wild-type was observed consistent with the other data (Fig. 3B). Interestingly, a modest (three-fold) but significant decrease in bone marrow cellularity was observed in *xpc*-/- even in mice that did not receive carboplatin treatment (Fig. 3A). The modest decrease may be due to endogenous DNA damage which is predicted to accumulate in *xpc*-/- mice [8], although the mice were only ten weeks old at the start of the experiments, and 16 weeks old at termination of the experiments.

We reasoned that the *xpc*-/- DNA repair defect might additionally affect the cell cycle. The majority (80%) of bone marrow cells when freshly harvested were in the G1 phase of the cell cycle irrespective of genotype (results not shown). By propidium iodide staining alone, no clear cell cycle defect of *xpc*-/- bone marrow or difference from wildtype was observed in any cell cycle phase. To further analyze the cell cycle, we grew the cells for 48 hrs in the presence of bromodeoxyuridine (BrdU), thereby labeling proliferating cells. Cells were then treated with 10 μM carboplatin for 15 hrs, fixed and stained for propidium iodide. Cells were gated for BrdU positivity, then for propidium iodide staining. A modest but reproducible decrease in the G1 phase was observed in *xpc*-/- compared to wildtype (Fig. 4). A strong G2 arrest by carboplatin was observed in both genotypes, which was however more pronounced in the *xpc*-/- than in wildtype (Fig. 4).

We examined several key cell cycle regulatory proteins for differences between *xpc*-/- and wildtype, most of which including G1 cyclins and Rb were unremarkable (results not shown). Interesting results were obtained though for the Cul4a and Cdt1 checkpoint proteins. Cdt1 regulates the licensing of replication origins in G1 [9-11]. Ubiquitination of Cdt1 by the ubiquitin ligase Cul4a occurs after DNA damage, is an important DNA damage signaling mechanism, and causes pre-replication complexes to dissociate from chromatin [9-11]. Cul4a is auto-ubiquitinated. We purified cellular proteins on a ubiquitin-binding resin to enrich for ubiquitinated proteins. Higher-molecular weight ubiquitinated forms of Cul4a and Cdt1 were observed in wildtype that were absent in *xpc*-/- bone marrow (Fig. 5A). Given evidence that the presence or absence of Xpc appeared to affect ubiquitination of Cul4a and Cdt1 in mouse bone marrow, we asked if the presence or absence of Xpc protein would affect ubiquitination of target substrates *in vitro*. Using a ubiquitin-conjugating HeLa S-100 extract, we titrated increasing concentrations of recombinant Xpc to the reactions. Cul4a activity was highly efficient in the S-

100 extract and was not affected by the addition of exogenous Xpc (results not shown). Xpc itself was efficiently ubiquitinated by the extract, and in fact competed with Cdt1 as a substrate. Unexpectedly, PCNA was ubiquitinated in concert with Xpc addition (Fig. 5B). Thus, one may conclude that in an S-100 extract Cdt1 appears to be a relatively weak substrate compared to other substrates Xpc and PCNA. The relative utilization of Cul4a substrates thus appears to be altered in *xpc*-/- mice (Fig. 5A) and one can alter substrate utilization by recombinant Xpc *in vitro* (Fig. 5B). We do not exclude the possible involvement of E3 ubiquitin ligases other than Cul4a.

Discussion

It has long been known that, in contrast to TC-NER, G-NER contributes little if at all to cell survival after DNA damage [12]. Early studies were conducted on fibroblasts of mouse or human origin which differed in G-NER but were non-isogenic, concluding that G-NER contributed little if at all to cell survival [12]. Later studies used isogenic fibroblasts which showed only modest (2-3 fold) differences in cell survival after DNA damage [13, 14]. The present study is the first to address G-NER and cell survival in a whole mouse and moreover is the first to examine bone marrow in *xpc*-/- mice. *Xpc*-/- mice lacking G-NER exhibited approximately tenfold greater sensitivity to carboplatin-induced DNA damage compared to strain-matched wildtype mice. The data indicate that the *xpc* gene and G-NER can contribute substantially to cell survival. The cell survival endpoint was biologically relevant as bone marrow myelosuppression was dose-limiting for carboplatin, consistent with other studies [15] and the majority of *xpc*-/- mice died during the course of the experiments.

Although it seems intuitive that mutant genotypes affecting DNA repair such as *XPC* would exhibit cell cycle alterations, surprisingly few studies have examined this issue. The E3 ubiquitin ligase Cul4a is a critical signaling mechanism for DNA damage, and targets DNA

repair and cell cycle arrest responses to DNA damage [16-19]. Both Xpc and Cdt1 are known Cul4a substrates mediating DNA repair and cell cycle arrest, respectively. In the absence of Xpc, DNA damage accumulates and cell death is one potential outcome (Figs. 1-3). However, *xpc*-/- cells that continue to proliferate may have adapted to the absence of Xpc. A mechanism suggested in this study is by attenuation of Cul4a-mediated signaling leading to decreased Cdt1 modification and therefore a bypass of G1/S cell cycle arrest (Figs. 4, 5).

This study is the first to report myelosuppression associated with the Xpc-defective genotype. Human patients with *xeroderma pigmentosum XPC* mutant alleles have not been reported to exhibit blood disorders. The patients typically develop skin cancers well prior to age 20, and develop internal cancers with aging. Similarly, *xpc*-/- mice develop spontaneous lung cancers at about one year of age [4]. We intentionally used mice as young as possible for the current study. Perhaps human *XPC* patients would exhibit myelosuppression were they to live to advanced age. On the other hand, the mice are completely null for *XPC* genes, while the human mutant *XPC* alleles probably exhibit variable penetrance.

Recent studies point to a role for Xpc in repair of endogenous DNA damage, that is, base damage owing to oxidation [8]. Consistent with the cited study, colony-forming ability of *xpc*-/- bone marrow was decreased compared to wildtype even in the absence of exogenous DNA damage (i.e. carboplatin; Fig. 3A). As base damage is removed by the separate base-excision DNA repair pathway (BER), it is conceivable that Xpc might play a role in signaling base damage. A catalytic role for Xpc in BER was suggested by the finding that Xpc enhanced the activity of the DNA glycosylase OGG1 [8]. It is likely that endogenous base damage is responsible for the late (one year) onset of lung cancers in *xpc*-/- mice, and may contribute to the accruement of mutagenic damage in human *XPC* patients as well. Here, we find a possible contribution of endogenous base damage to bone marrow cellularity.

In summary, the *xpc* gene protects mouse bone marrow from exogenous and probably also endogenous DNA damage with cell survival as an endpoint. The biological relevance is indicated by the fact that 12/20 *xpc*-/- mice receiving carboplatin died of bone marrow failure while 0/20 wildtype mice died. The Xpc protein appears to be closely linked to the Cul4a/Cdt1 cell cycle checkpoint, relevant to DNA damage signaling and probably contributes to the cell death mechanism.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Figure Legends

Fig. 1. Global-NER defect in *xpc*-/- mice. Xpc protein was undetectable in *xpc*-/- mice (*inset*). Removal of DNA damage was markedly slow in *xpc*-/- mice, consistent with the known rate-limiting role of Xpc in global-NER. Pooled bone marrow of three or more mice was used for the experiments.

Fig. 2. Histological evaluation of wild-type and *xpc*-/- mice in carboplatin-treated and saline-control groups, day 41. A) Hematoxylin-eosin staining of formalin-fixed femur sections. Marked hypocellularity was observed in *xpc*-/- mice receiving carboplatin. **B)** DAPI staining. Marked hypocellularity was observed in *xpc*-/- mice receiving carboplatin. The data are representative of at least three mice of each treatment group and genotype.

Fig. 3. Quantification of bone marrow hypocellularity in *xpc*-/- mice compared to wild-type. A) Colony-forming assays of bone marrow harvested from the respective genotypes and treatment groups. Bone marrow was harvested and grown in complete methylcellulose medium containing IL-6 and SCF for 10 days. Total colonies per femur is shown. *Xpc*-/- bone marrow in the carboplatin-treated group was decreased 10-12 fold compared to wild-type ($P < 0.008$ by t-test). Each set of experiments utilized bone marrow from three or more mice of each genotype. **B)** Assay of cell yield in bone marrow cells treated with carboplatin *in vitro*. Bone marrow of wildtype and *xpc*-/- genotypes was cultured for 24 hrs, then treated with indicated concentrations of carboplatin for 2 hrs. Cell yield after 72 hrs in culture is shown ($P < 0.006$ by t-test). The data shown were averaged from three experiments.

Fig. 4. Evidence of G1 cell cycle checkpoint defect in *xpc*-/- bone marrow; the plot shows relative cell cycle distribution after carboplatin treatment. Bone marrow was stimulated with cytokines and cultured for 48 hrs in the presence of 10 μM bromodeoxyuridine (BrdU) to label proliferating cells, then fixed and stained with propidium iodide. An antibody to BrdU was used to gate the BrdU-labeled population, which were then assayed for propidium iodide content. Values for carboplatin-treated bone marrow were divided by values for untreated bone marrow conducted side by side. The data represent three pooled mice of each genotype, assayed in two separate experiments. A modest but significant decrease in G1 population was observed in *xpc*-/- mice compared to wildtype mice ($P < 0.05$ by t-test).

Fig. 5. A) Immunodetection of Cul4a and Cdt1 cell cycle checkpoint proteins in wildtype and *xpc*-/- bone marrow. Cul4a is an E3 ubiquitin ligase that ubiquitinates Cdt1 and is also auto-ubiquitinated during DNA damage. Immunoblots were first conducted using 50 μg of total cell lysates (lanes 1 and 2). Equal amounts of cellular proteins, 5 mg, were then affinity-purified on a ubiquitin-binding resin and immunoblotting of bound proteins was conducted. The higher molecular-weight ubiquitinated forms of Cul4a and Cdt1 differ between the two genotypes (lanes 3 and 4). The data suggest a defect in Cul4a and Cdt1 ubiquitin modification in *xpc*-/- mice. To clearly identify ubiquitinated Cul4a and Cdt1, we used *in vitro* ubiquitin-conjugated proteins. Omission of ubiquitin from the reaction shows that mainly ubiquitinated Cul4a is detected by the Cul4a antibody (lanes 5 and 6, *upper panel*). Plasmid-encoded Cdt1 protein was also used as a marker (lanes 5 and 6, *lower panel*). **B) Titration of recombinant Xpc in an *in vitro* ubiquitin-conjugating system.** Purified recombinant Xpc was added in amounts indicated, in nanograms. Ubiquitin conjugation was carried out following the manufacturers protocol. Cdt1 and PCNA proteins endogenous to the S-100 extract were immunodetected by

western blotting, in addition to the input Xpc protein. Cdt1 may be a weak substrate for ubiquitination compared to PCNA or the input Xpc. If Cdt1 is a weaker substrate *in vivo*, it may indicate a mechanism whereby cells can continue to cycle despite DNA damage.

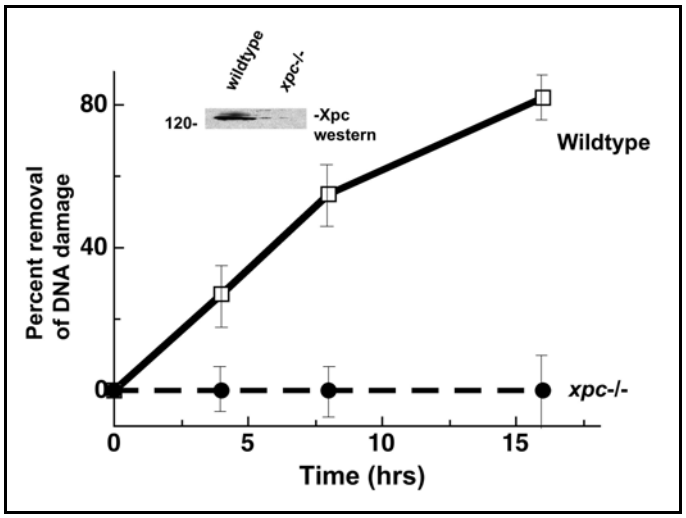

wildtype
xpc-/-
120-
-Xpc
western
80
40
0
Percent removal
of DNA damage
Wildtype
xpc-/-
0
5
10
15
Time (hrs)

Fig. 1

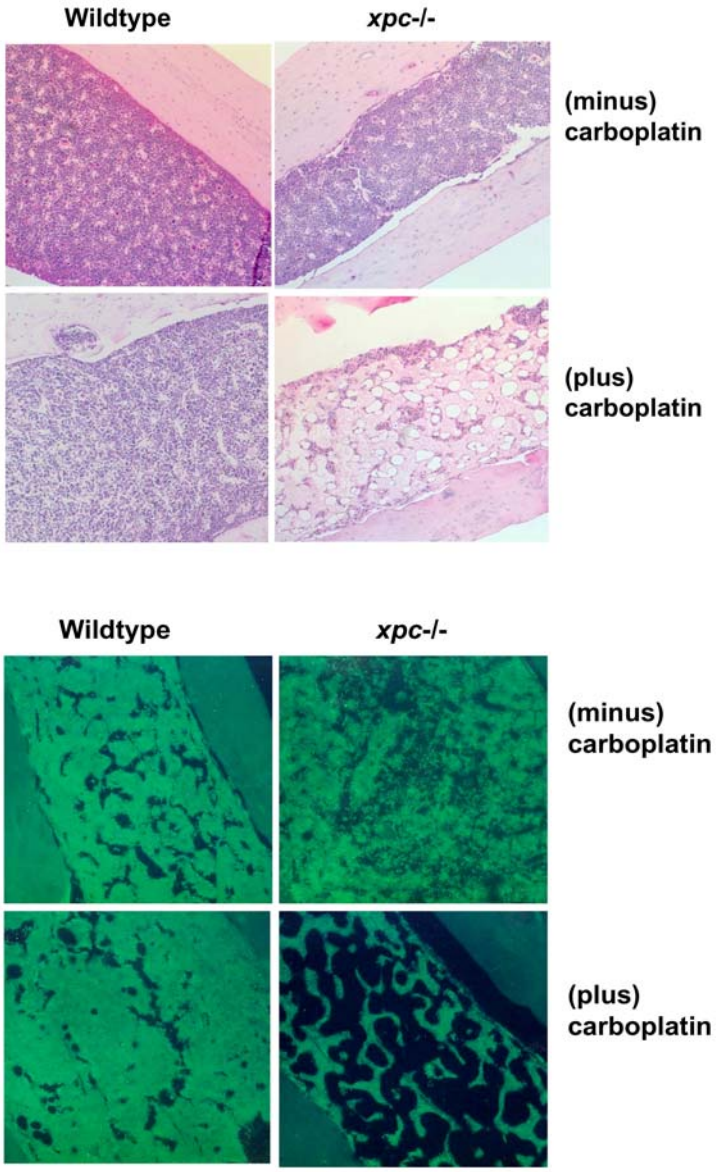

Wildtype
xpc-/-
(minus) carboplatin
(plus) carboplatin
Wildtype
xpc-/-
(minus) carboplatin
(plus) carboplatin

Fig. 2 A, B

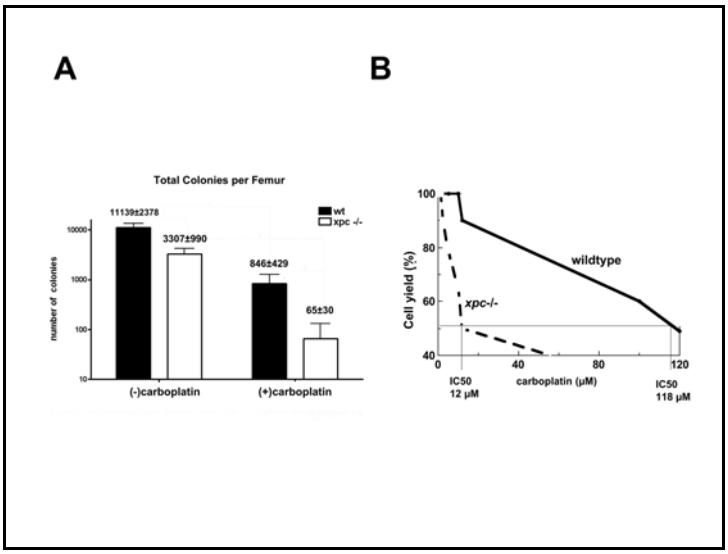

A
B
Total Colonies per Femur
wt
xpc -/-
11139±2378
3307±990
846±429
65±30
number of colonies
10000
1000
100
10
(-)carboplatin
(+)carboplatin
100
80
60
40
Cell yield (%)
wildtype
xpc-/-
0
40
80
120
IC50
12 µM
carboplatin (µM)
IC50
118 µM

Fig. 3

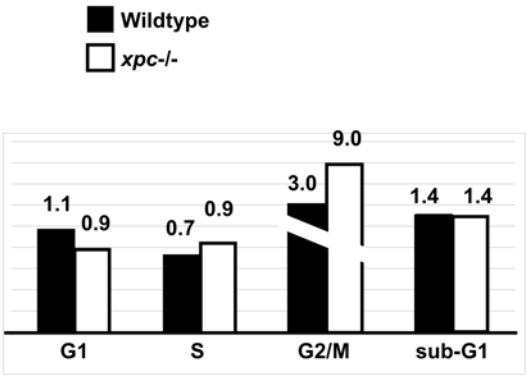


Fig. 4

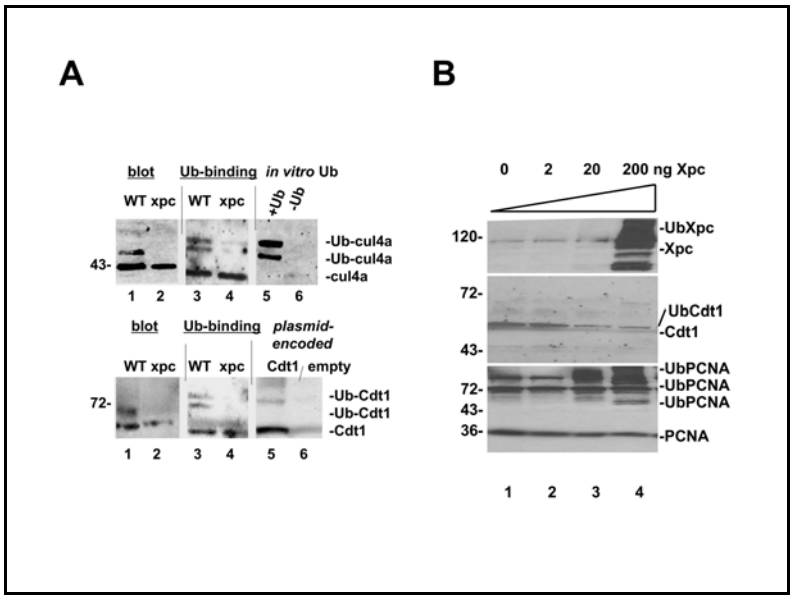

A
B
blot
Ub-binding
in vitro Ub
WT xpc
WT xpc
+Ub
-Ub
-Ub-cul4a
-Ub-cul4a
-cul4a
43-
1 2 3 4 5 6
blot
Ub-binding
plasmid-encoded
WT xpc
WT xpc
Cdt1
empty
-Ub-Cdt1
-Ub-Cdt1
-Cdt1
72-
1 2 3 4 5 6
0 2 20 200 ng Xpc
120-
-UbXpc
-Xpc
72-
UbCdt1
-Cdt1
43-
-UbPCNA
72-
-UbPCNA
43-
-UbPCNA
36-
-PCNA
1 2 3 4

Fig. 5