

## Proanthocyanidins Inhibit Photocarcinogenesis through Enhancement of DNA Repair and Xeroderma Pigmentosum Group A–Dependent Mechanism

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### Abstract

Dietary grape seed proanthocyanidins (GSP) inhibit photocarcinogenesis in mice; however, the molecular mechanisms underlying this effect have not been fully elucidated. As ultraviolet B (UVB)-induced DNA damage in the form of cyclobutane pyrimidine dimers (CPDs) has been implicated in skin cancer risk, we studied whether dietary GSPs enhance repair of UVB-induced DNA damage and, if so, what is the potential mechanism? Supplementation of GSPs (0.5%, w/w) with AIN76A control diet significantly reduced the levels of CPD<sup>+</sup> cells in UVB-exposed mouse skin; however, GSPs did not significantly reduce UVB-induced CPD<sup>+</sup> cells in the skin of interleukin-12p40 (IL-12) knockout (KO) mice, suggesting that IL-12 is required for the repair of CPDs by GSPs. Using IL-12 KO mice and their wild-type counterparts and standard photocarcinogenesis protocol, we found that supplementation of control diet with GSPs (0.5%, w/w) significantly reduced UVB-induced skin tumor development in wild-type mice, which was associated with the elevated mRNA levels of nucleotide excision repair genes, such as *XPA*, *XPC*, *DDDB2*, and *RPA1*; however, this effect of GSPs was less pronounced in IL-12 KO mice. Cytostaining analysis revealed that GSPs repaired UV-induced CPD<sup>+</sup> cells in xeroderma pigmentosum complementation group A (*XPA*)-proficient fibroblasts from a healthy individual but did not repair in *XPA*-deficient fibroblasts from *XPA* patients. Furthermore, GSPs enhance nuclear translocation of XPA and enhanced its interactions with other DNA repair protein ERCC1. Together, our findings reveal that prevention of photocarcinogenesis by GSPs is mediated through enhanced DNA repair in epidermal cells by IL-12- and XPA-dependent mechanisms.

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### Introduction

Grapes (*Vitis vinifera*) are one of the most widely consumed fruits in the world. Grapes are rich in polyphenols and approximately 60% to 70% of grape polyphenols exist in the grape seeds. The grape seeds are available as byproducts of the industrial production of grape juice and wine. The seeds contain a larger fraction of proanthocyanidins, which are composed of dimers, trimers, tetramers, and oligomers of monomeric catechins or epicatechins (1–3). These grape seed proanthocyanidins (GSP) have been shown to have antioxidant (4, 5), antimutagenic, antiinflammatory, and anticarcinogenic (6, 7) activities. GSPs are cytotoxic to various cancer cell lines of different organs (8–10) with no apparent toxic effects on normal

cells including human keratinocytes (4, 11). We have shown previously that supplementation of the diet with GSPs inhibits the growth and development of ultraviolet (UV) radiation-induced skin tumors in SKH-1 hairless mice (7). However, the molecular mechanism underlying this inhibitory effect of dietary GSPs is not clearly established.

Cutaneous malignancies, including melanoma and non-melanoma skin cancers, are a major burden on the health care system, and the incidence of cutaneous malignancy is equivalent to the incidence of malignancies in all other organs combined (12). Exposure of the skin to solar UV radiation is one of the major environmental hazards that induces oxidative stress, inflammation, suppression of immune system, DNA damage, and gene mutation. Collectively, all these events lead to the development of various skin diseases including the risk of cutaneous malignancies (13, 14).

UV irradiation induces DNA damage predominantly the formation of cyclobutane pyrimidine dimers (CPD). UV-induced CPDs have been recognized as a molecular trigger for the initiation of photocarcinogenesis (15–17). Reduction or repair of CPDs through application of DNA repair enzymes considerably reduces the risk of photocarcinogenesis (17, 18). Among the various UV-induced DNA repair

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mechanisms, nucleotide excision repair (NER) represents the most versatile DNA repair pathway in cells (19). NER recognizes bulky, helix-distorting changes, such as CPD. Xeroderma pigmentosum complementation group A (*XPA*) gene plays an indispensable role in the NER pathway (20). Patients with *XPA*-deficiency have a high incidence of skin cancer at an early age on sun-exposed skin caused by loss of the ability to repair UV-induced DNA damage (21).

Here we conducted the studies to determine whether dietary GSPs could prevent ultraviolet B (UVB)-induced skin tumor development through rapid repair of UVB-induced DNA damage and to assess potential mechanisms of this effect. We hypothesized that the rapid repair of UVB-induced DNA damage by dietary GSPs is mediated through the enhanced levels of NER genes, postulating further that DNA repair mechanism by GSPs is mediated through the enhanced levels of interleukin (IL)-12, which has been shown to have DNA repair ability and antitumor activity. Furthermore, if this is the case, dietary GSPs would be unable to repair UVB-induced DNA damage and photocarcinogenesis in IL-12 knockout (IL-12 KO) mice.

## Materials and Methods

### Animals

Female C3H/HeN mice (6–7 weeks old) were purchased from Charles River Laboratory. The IL-12p40 KO mice on C3H/HeN background were generated and bred in our Animal Resource Facility, as described previously (22). All mice were maintained under standard conditions of a 12-hour dark/12-hour light cycle, a temperature of  $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and relative humidity of  $50\% \pm 10\%$ . The mice were given control AIN76A diet with or without supplementation with GSPs and drinking water *ad libitum* throughout the experiment. The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

### Antibodies and real-time PCR primers

The antibodies specific for CPDs were purchased from Kamiya Biomedical Co. The manufacturer-supplied standardized real-time PCR primers for NER genes (*XPA*, *XPC*, *RPA1*, and *DDB2*), and  $\beta$ -actin were obtained from the SuperArray BioScience Corp.

### Grape seed proanthocyanidins and dietary administration

The GSPs were obtained from the Kikkoman Corporation, and the chemical composition has been described earlier (7). Experimental diets containing GSPs (0.5%, w/w) were commercially prepared in a pellet form in the AIN76A powdered control diet by TestDiet (Richmond) using the GSPs that we provided for this purpose. Dietary administration of GSPs was started at least 2-week before UVB-irradiation of mice and continued until the termination of the experiment.

### UVB irradiation of mice

Mice were UVB-irradiated as described previously (5, 7). Briefly, the clipper-shaved dorsal skin was exposed to UV radiation from a band of four FS20 UVB lamps (Daavlin, UVA/UVB Research Irradiation Unit) equipped with an electronic controller to regulate UV dosage. The UV lamps emit UVB (280–320 nm;  $\approx 80\%$  of total energy) and ultraviolet A (UVA) (320–375 nm;  $\approx 20\%$  of total energy), with ultraviolet C (UVC) emission being significantly less ( $<1\%$ ). The majority of the resulting wavelengths of UV radiation are in the UVB (290–320 nm) range with peak emission at 314 nm.

### Experimental design and photocarcinogenesis protocol

The IL-12 KO mice on C3H/HeN background and their wild-type (WT) counterparts (C3H/HeN) were divided into three treatment groups with 20 mice in each group. These groups of mice included a control group (non-UVB and non-GSPs), a group that was exposed to UVB in the absence of treatment with GSPs, and a group treated with dietary GSPs prior to UVB irradiation and continued till the termination of the experiment. The photocarcinogenesis protocol employed has been described previously (7). Briefly, the shaved backs of the mice were irradiated with UVB ( $180 \text{ mJ}/\text{cm}^2$ ) three times per week for a total period of 38 weeks. The backs of the mice were shaved using clippers if hairs grown on the skin during the photocarcinogenesis experiment.

UVB-irradiated back of the mice was examined on a weekly basis to check for the growth of papillomas or tumors. At the termination of the experiment, the dimensions of all the tumors on each mouse were recorded. Tumor volumes were calculated using the hemiellipsoid model formula: tumor volume =  $1/2 (4\pi/3) (l/2) (w/2) h$ , where  $l$  = length,  $w$  = width, and  $h$  = height.

### Immunohistochemical detection of cyclobutane pyrimidine dimers

Immunohistochemical detection of CPD<sup>+</sup> cells in the skin samples was performed using a procedure described previously (23, 25). Briefly, frozen skin sections (5- $\mu\text{m}$  thick) were thawed, and nuclear DNA was denatured, followed by neutralization in 100 mmol/L Tris-HCl (pH 7.5) in 70% ethanol. After blocking nonspecific binding, sections were incubated with antibody specific for CPDs. Bound anti-CPD antibody was detected by incubation with biotinylated goat-anti-mouse IgG1 followed by peroxidase-labeled streptavidin. After washing, sections were incubated with diaminobenzidine and counterstained with either hematoxylin and eosin or methyl green. CPD<sup>+</sup> cells were detected and photographed under an Olympus BX41 microscope fitted with a Q-Color-5 digital camera.

### Dot-blot analysis of CPDs

Epidermal genomic DNA was isolated following the standard procedures, and dot-blot analysis was performed

as detailed previously (23, 24). Briefly, genomic DNA (500 ng) was transferred to a positively charged nitrocellulose membrane by vacuum dot-blotting (Bio-Dot apparatus, Bio-Rad) and fixed by baking the membrane for 30 minutes at 80°C. After blocking the nonspecific binding sites, the membrane was incubated with the antibodies specific to CPDs for 1 hour at room temperature. After washing, the membrane was incubated with HRP-conjugated secondary antibody. The circular bands of CPDs were detected using an enhanced chemiluminescence detection system. The genomic DNA was tested at least from five mice/group independently.

#### **XPA-deficient and XPA-proficient cells, culture conditions, UV irradiation, and cytostaining of CPDs**

XPA-deficient and XPA-proficient human fibroblasts were obtained from the Coriell Institute for Medical Research. The XPA-deficient fibroblasts were obtained from patients suffering from xeroderma pigmentosum group A disease. These fibroblasts were authenticated by the Coriell Institute for Medical Research and supplied for only research purpose. The fibroblasts were cultured in a modified Eagle medium with Earle's salts supplemented with 2 mmol/L L-glutamine, 10% fetal bovine serum, and maintained in an incubator at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells were exposed to the UVB radiation (150 J/m<sup>2</sup>) through PBS with or without pretreatment with GSPs (20 µg/mL) for 3 hours. The UVB-induced CPD<sup>+</sup> cells were detected using a protocol described previously (25). Briefly, after UVB irradiation, cells were harvested and cytospun (≈1 × 10<sup>5</sup> cells/slide). Remaining procedure is similar to tissue staining of CPDs. CPD<sup>+</sup> cells were counted under the Olympus BX41 microscope in 5 to 6 different fields, and the data are presented as the mean of the percentage of CPD<sup>+</sup> cells ± SD from two separate experiments.

#### **RNA extraction and quantitative real-time polymerase chain reactions analyses for NER genes**

The epidermal RNA was extracted from the mouse skin samples using TRIzol reagent (Invitrogen), as described (24). The mRNA expression of NER genes, such as XPA, XPC, RPA1, and DDB2, in skin samples was determined using real-time polymerase chain reactions (PCR). For the mRNA quantification, complementary DNA (cDNA) was synthesized using 3 µg RNA through a reverse transcription reaction (iScript cDNA synthesis kit, BIO-RAD). Using SYBR Green/Fluorescein PCR master mix (SuperArray Bioscience Corporation), cDNA was amplified using real-time PCR with a BioRad MyiQ thermocycler and SYBR green detection system (BioRad). Samples were run in triplicate to ensure amplification integrity. Manufacturer-supplied (SuperArray, Bioscience Corporation) primer pairs were used to measure the expression levels of NER genes. The standard PCR conditions were 95°C for 15 min, then 40 cycles at 95°C, 30 seconds; 55°C, 30 seconds; and 72°C, 30 seconds, as recommended by the manufacturer. The expression levels of genes were

normalized to the expression level of the β-actin mRNA in each sample. The threshold for positivity of real-time PCR was determined based on negative controls. For mRNA analysis, the calculations for determining the relative level of gene expression were made using the cycle threshold method.

#### **Statistical analysis**

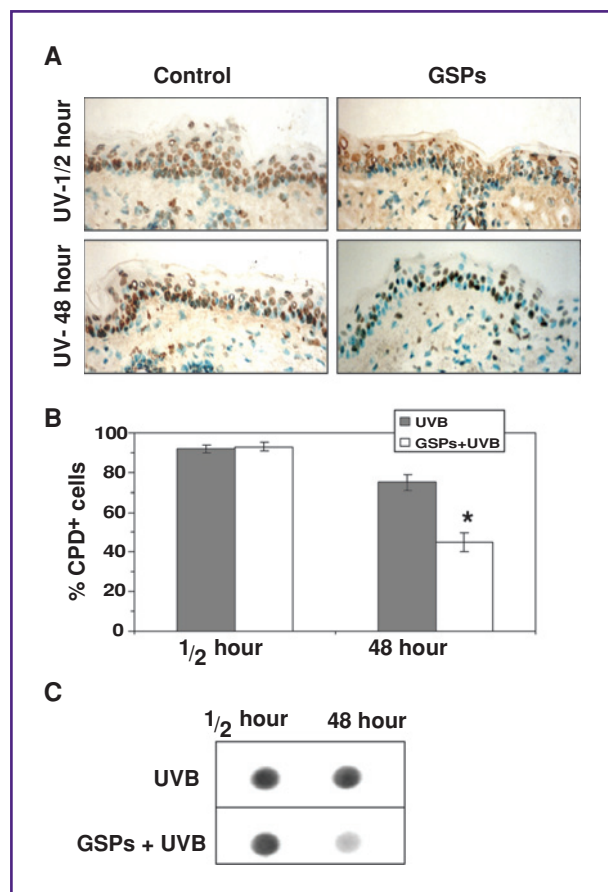
Tumor incidence in the UVB and GSPs and UVB treated groups was compared using the  $\chi^2$  test. Tumor multiplicity data were analyzed using the Wilcoxon rank sum test, and tumor volume was statistically analyzed using ANOVA followed by Tukey's *post hoc* test. A simple ANOVA followed by an appropriate *post hoc* test was used to calculate statistical significance of the data obtained from tumor volume and the number of CPD<sup>+</sup> cells in different treatment groups. A *P* value < 0.05 was considered significant.

## **Results**

### **GSPs repair UVB-induced DNA damage rapidly compared with non-GSPs-treated mice**

In earlier studies, dietary supplementation of GSPs at the level of 0.5% (w/w) significantly inhibited UV-induced skin tumor development (7); therefore, we used 0.5% GSPs in diet for all mice experiments for mechanistic studies. The shaved backs of C3H/HeN mice were exposed to UVB (60 mJ/cm<sup>2</sup>) with and without the treatment of mice with GSPs. Mice were sacrificed either immediately (≈30 min) or 48 hours later; skin samples were collected and subjected to immunohistochemical detection of CPD<sup>+</sup> cells. In skin samples obtained immediately after UV exposure, no differences in the staining pattern of CPDs were observed whether or not the mice were treated with GSPs (Fig. 1A). This observation eliminated the speculation that dietary GSPs might have a filtering effect on UV radiation. However, in skin samples collected at 48 hours after UVB exposure, the number of CPD<sup>+</sup> cells was significantly lower (*P* < 0.001) in the GSP-treated mice than the mice that have not received GSPs in diet but were exposed to UVB. A summary of CPD<sup>+</sup> cells in different treatment groups is shown in Fig. 1B. The CPD<sup>+</sup> cells were not detectable in non-UVB-exposed skin, whether or not they were treated with GSPs (data not shown).

The effect of GSPs on UVB-induced DNA damage was further verified using dot-blot analysis of epidermal genomic DNA isolated from the skin samples from UVB-exposed skin with and without treatment of GSPs. As shown in Fig. 1C, there was no significant difference in the dot-blot pattern of CPDs between skin samples obtained immediately after UVB exposure from UVB-exposed mice whether or not they were treated with GSPs. In samples obtained 48 h after UVB exposure, the intensity of the dot-blot was markedly lower in the GSP-treated mice than the non-GSP-treated control mice. The genomic DNA samples obtained from the groups of mice that were not



**Fig. 1.** Dietary GSPs rapidly repair UVB-induced epidermal CPDs compared with untreated mice. **A**, frozen sections (5- $\mu$ m thick) were subjected to immunoperoxidase staining to detect CPD<sup>+</sup> cells. CPD<sup>+</sup> cells are dark brown. CPD<sup>+</sup> cells were not detected in non-UVB-irradiated skin (not shown). Magnification,  $\times 40$ . **B**, summary of CPD<sup>+</sup> cells. The number of CPD<sup>+</sup> cells was counted in five to six different areas of the sections under an Olympus BX41 microscope, and the numbers reported represent the percentage of CPD<sup>+</sup> cells  $\pm$  SD in epidermis ( $n = 5$ , number of mice/group). \*, Significant difference versus UV alone,  $P < 0.005$ . **C**, analysis of CPDs by dot-blot. GSPs treatment enhanced repair of UVB-induced epidermal CPDs. Epidermal genomic DNA from various treatment groups was subjected to dot-blot analysis using an antibody specific to CPDs. Results are shown from a single experiment that is representative of two independent experiments.

exposed to UV were negative in the dot-blot assay (data not shown).

#### Repair of UVB-induced DNA damage by GSPs requires IL-12

As IL-12 has the ability to enhance repair of UVB-induced DNA damage in mouse skin, and we have found that GSPs enhance the levels of IL-12 in UVB-irradiated mice (26), we evaluated whether IL-12 is required for DNA repair by GSPs in UVB-exposed skin. For this purpose, the shaved backs of IL-12 KO and WT mice were exposed to UVB (60 mJ/cm<sup>2</sup>) with and without pretreatment of mice with diet supplemented with GSPs, and mice were sacrificed 48 h after UVB

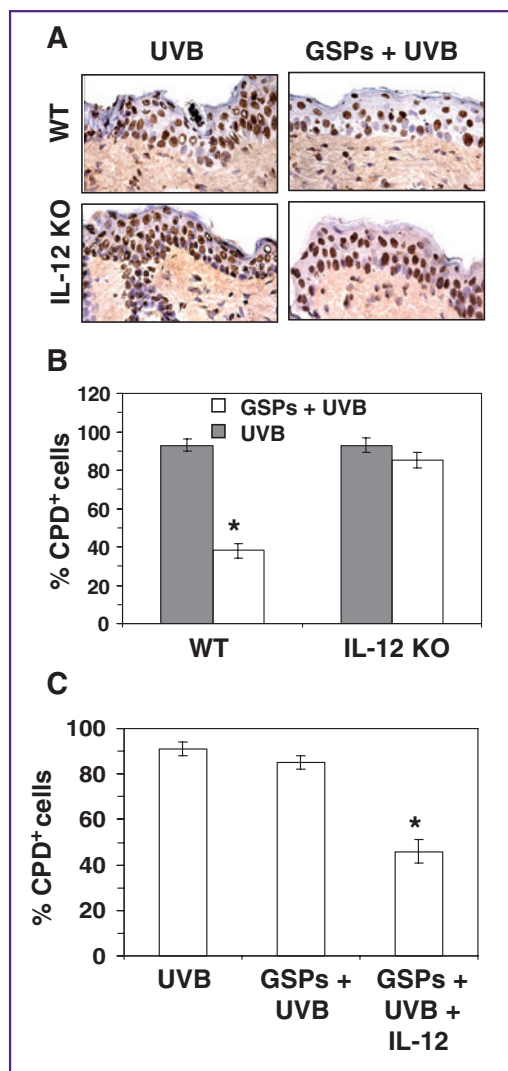
exposure; skin samples were collected and subjected to immunohistochemical analysis of CPD<sup>+</sup> cells. In skin samples obtained at 48 hours after UVB exposure, the numbers of CPD<sup>+</sup> cells were significantly less in the GSP-treated WT mice than the control group that were not treated with dietary GSPs but were exposed to UVB ( $P < 0.001$ ). In contrast, the numbers of UVB-induced CPD<sup>+</sup> cells in the skin of IL-12 KO mice that had been treated with GSPs did not significantly reduced from that in the IL-12 KO mice that had not been treated with GSPs (Fig. 2A and 2B).

To further verify the role of IL-12 in the GSP-mediated repair of UVB-induced DNA damage, the shaved backs of GSP-treated IL-12 KO mice were exposed to UVB (60 mJ/cm<sup>2</sup>) with one group of mice receiving endotoxin-free rIL-12 (1000 ng/100  $\mu$ L PBS) subcutaneously 3 hours before UVB irradiation. Mice were sacrificed 48 hours later, skin samples collected and subjected to the immunohistochemical analysis of CPD<sup>+</sup> cells. Summary of immunohistochemical analysis revealed that the number of CPD<sup>+</sup> cells in the GSP-treated group (85%  $\pm$  4%) was similar to the numbers observed in the group that did not receive GSPs (91%  $\pm$  4%), as shown in Fig. 2C. However, the number of CPD<sup>+</sup> cells in the skin samples of IL-12 KO mice that had been treated with dietary GSPs and had received rIL-12 before UVB exposure was only 45%  $\pm$  5%, indicating a significant reduction of DNA damage ( $P < 0.001$ ). These data support the concept that GSPs promote the repair of damaged DNA in UVB-exposed skin through a mechanism that requires IL-12 activity.

#### Prevention of UV-carcinogenesis by dietary GSPs requires IL-12

If IL-12 is required for GSP-induced DNA repair in UVB-exposed skin, we then asked whether prevention of photocarcinogenesis in mice by dietary GSPs requires IL-12. To test the hypothesis, we examined the effect of dietary GSPs on photocarcinogenesis in IL-12 KO mice. We confirmed that when WT mice were subjected to a standard photocarcinogenesis protocol, dietary GSPs resulted in a significant reduction in UVB-induced skin tumorigenesis in terms of tumor incidence (40%,  $P < 0.01$ ) and tumor multiplicity (60%,  $P < 0.001$ ) compared with non-GSP-treated WT mice at the termination of the experiment (Fig. 3A, 3B, left panels). Each group had 20 mice. As shown in Fig. 3C, the growth of tumors was significantly reduced (67%;  $P < 0.001$ ) in the group of GSP-treated WT mice than the non-GSP-treated mice when measured and expressed in terms of tumor volume/tumor at the termination of the experiment.

In contrast, dietary GSPs did not significantly inhibit photocarcinogenesis in IL-12 KO mice (Fig. 3; right panels). It appeared that the IL-12 KO mice were on a greater risk of UVB radiation-induced skin tumor growth than their WT counterparts, as is evident from the data concerning tumor multiplicity (Fig. 3, right panels). The size of the tumor volume/tumor was higher in the IL-12 KO mice than their WT counterparts (Fig. 3C). These tumor



**Fig. 2.** IL-12 is required in DNA repair by GSPs in UVB-exposed skin. A, frozen skin sections (5- $\mu$ m thick) were subjected to immunoperoxidase staining to detect CPD<sup>+</sup> cells. CPD<sup>+</sup> cells are shown by dark brown. Magnification,  $\times 40$ . B, summary of CPD<sup>+</sup> cells. The numbers of CPD<sup>+</sup> cells were counted in five to six different areas under a microscope, and the numbers reported represent the percentage of CPD<sup>+</sup> cells  $\pm$  SD in the epidermis. Each group has five mice. Significant difference versus UVB-exposed alone (\*,  $P < 0.001$ ). C, *in vivo* treatment of IL-12 KO mice with rIL-12 reduces or repairs UV-induced DNA damage in the form of CPDs. Frozen skin sections were subjected to immunoperoxidase staining to detect CPD<sup>+</sup> cells. The number of CPD<sup>+</sup> cells was counted in five to six different areas of the sections, and the numbers reported represent the percentage of CPD<sup>+</sup> cells as a mean  $\pm$  SD in the epidermis ( $n = 5$ /group). \*Significant difference versus UV alone and/or GSPs + UV (\*,  $P < 0.001$ ).

data suggest that prevention of photocarcinogenesis by GSPs was more pronounced in WT mice than IL-12 KO mice.

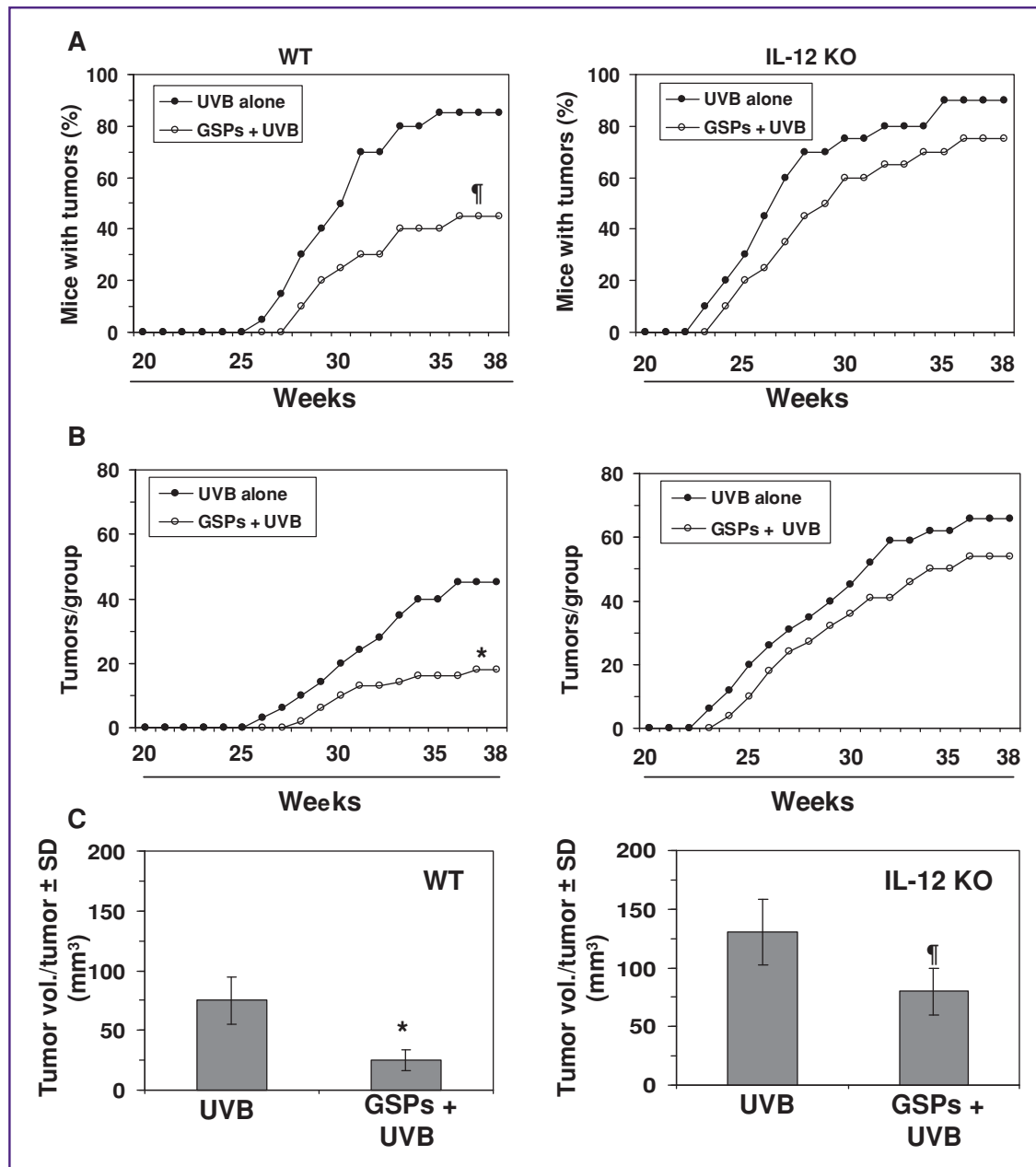
#### GSPs enhance the levels of NER genes in mouse skin

As we have observed that dietary GSPs enhance the repair of UVB-induced DNA damage in the skin, and it

seems that DNA repair by GSPs is IL-12 dependent, the next question was whether GSPs repair DNA damage through the stimulation of NER genes and whether IL-12 is involved in it? For this purpose, shaved backs of the IL-12 KO and their WT counterparts were exposed to UVB (60 mJ/cm<sup>2</sup>) with and without the treatment of GSPs in diet. Mice were sacrificed 1 hour after UVB exposure, skin samples were collected and epidermal RNA isolated and subjected to the analysis of mRNA expression of some selected NER genes (i.e., *XPA*, *XPC*, *RPA1*, and *DDB2*) using real-time PCR. The acute exposure of the mouse skin with UVB mildly enhances the levels of NER genes (not significant) compared with non-UVB-exposed skin. As shown in Fig. 4, the mRNA levels of NER genes were significantly enhanced ( $P < 0.01$ – $0.001$ ) in the skin of WT mice treated with dietary GSPs compared with non-GSPs-treated UVB-exposed WT mouse skin. In contrast, the levels of mRNA expression of *XPA*, *XPC*, *RPA1*, and *DDB2* were not significantly elevated in the UVB-exposed skin of IL-12 KO mice, which were given GSPs in the diet. These data suggest the role of IL-12 in the NER gene expression.

#### GSPs enhance repair of UVB-induced DNA damage in XPA-positive human fibroblasts but do not repair in XPA-deficient human fibroblasts

As it has been shown that *XPA* gene plays an indispensable role in the NER pathway (20), we further examined whether *XPA* gene is required in GSP-mediated DNA repair. For this purpose, *XPA*-deficient fibroblasts from an *XPA*-patient and *XPA*-proficient fibroblasts from a healthy person were exposed to UVB with or without prior treatment with GSPs (20  $\mu$ g/mL). Cells were harvested either immediately or 24 hours after UVB irradiation and subjected to cytochemical staining using CPD-specific antibody. CPD<sup>+</sup> cells were not detectable in non-UVB-irradiated cells whether or not they were treated with GSPs (Fig. 5A). When the cells were analyzed for CPDs immediately after UVB-exposure, no differences were observed in the cells treated with or without GSPs in terms of the number of CPD<sup>+</sup> cells. This finding suggests that GSPs do not prevent immediate formation of CPDs after UVB exposure and excludes a UVB radiation filtering effect. When the cells were analyzed 24 hours after UVB irradiation, the number of CPD<sup>+</sup> cells was significantly decreased (79%,  $P < 0.001$ ) in the *XPA*-proficient cells (Fig. 5A and B) compared with non-GSPs-treated UVB-irradiated cells, suggesting that GSPs might accelerate the repair of UVB-induced CPDs through an *XPA* mechanism. A significant reduced number of CPD<sup>+</sup> cells were also observed in non-GSPs-treated *XPA*-proficient cells 24 hours after UVB irradiation compared with UVB-irradiated cells immediately after UVB exposure; suggesting that some endogenous defense mechanisms are involved in DNA repair. However, this DNA-repairing effect of GSPs was not observed in the *XPA*-deficient human fibroblasts 24 hours after UVB irradiation. It may be due to the absence of *XPA* gene in these cells.

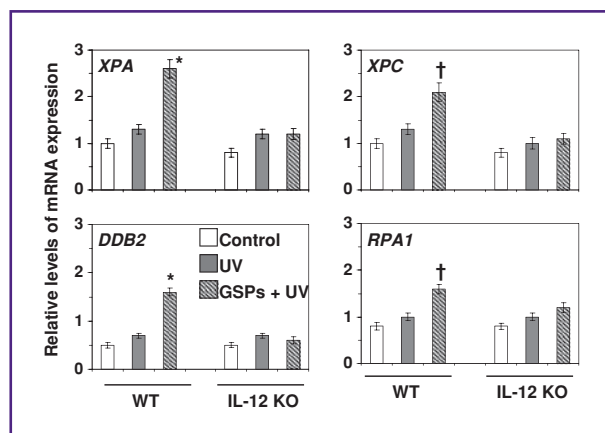


**Fig. 3.** Dietary GSPs significantly inhibit UVB-induced skin tumor development in WT mice but does not inhibit in IL-12 KO mice. The experimental protocol is described in Materials and Methods. The percentage of mice with tumors (A) and a total number of tumors per group (B) are plotted as a function of the number of weeks on treatment. Each treatment group had 20 mice. A significant reduction in tumors versus UVB alone at the termination of the experiment (\*,  $P < 0.01$ ; \*,  $P < 0.001$ ). C, treatment of GSPs inhibits the growth of UVB-induced tumors in WT mice while less pronounced inhibitory effect was observed in IL-12 KO mice. The tumor volume in each treatment group was recorded at the termination of the photocarcinogenesis experiment. Significant inhibition versus UVB alone (\*,  $P < 0.001$ ; \*,  $P < 0.01$ ).

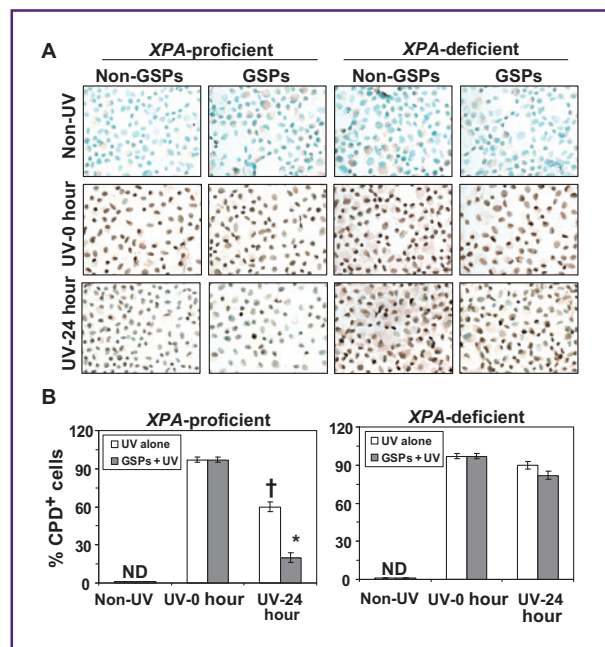
### GSPs induce the nuclear translocation of XPA protein

As XPA is a critical component of the NER pathway, we then asked how GSPs affect XPA activity to enhance DNA repair upon UVB irradiation. It has been shown that after UVB irradiation, XPA undergoes translocation from cytoplasm to nucleus, and once inside the nucleus, XPA is a "docking" protein for components of the NER system (27, 28). For this purpose, the shaved C3H/HeN mice were

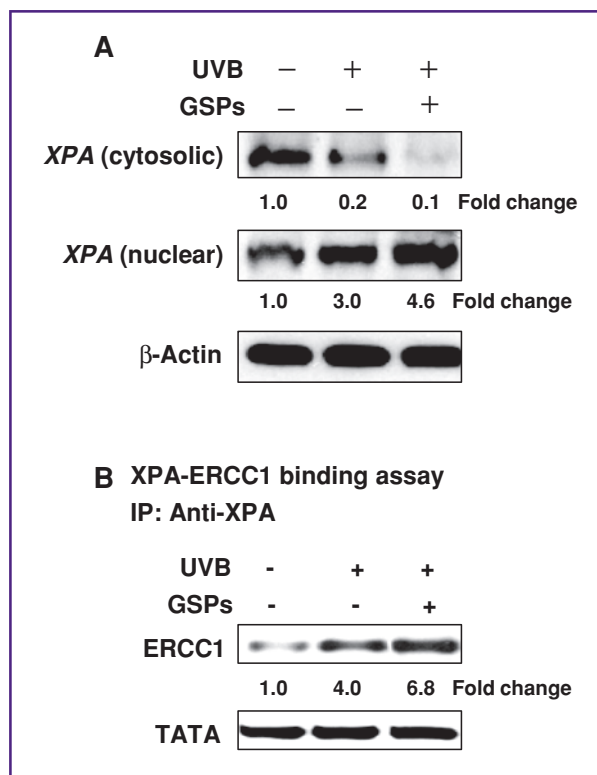
exposed to UVB (60 mJ/cm<sup>2</sup>) with and without the treatment of dietary GSPs. Mice were sacrificed 1 hour later. Non-UVB-exposed mice served as a control. Cytosolic and nuclear fractions were prepared and subjected to Western blot analysis using XPA-specific antibody. As shown in Fig. 6A, we observed a reduction in the XPA protein level in cytosol after UVB exposure with or without the treatment with GSPs. Furthermore, there was an



**Fig. 4.** Dietary GSPs enhance the levels of mRNA expression of NER genes in the UVB-exposed skin of WT mice but do not enhance the levels of NER genes in the UVB-irradiated skin of IL-12 KO mice. The levels of epidermal mRNA expression were determined using real-time PCR. The data of mRNA expression levels of various NER genes are expressed as mean  $\pm$  SD. Five mice were used per group and the experiment was repeated once. Statistically significant versus UVB alone (\*,  $P < 0.001$ ; †,  $P < 0.01$ ).



**Fig. 5.** GSPs significantly repair UVB-induced DNA damage in XPA-proficient cells but do not repair in XPA-deficient cells. A, XPA-proficient and XPA-deficient human fibroblasts were exposed to UVB (150 J/m<sup>2</sup>) with or without the treatment of GSPs (20  $\mu$ g/mL), and cells were harvested immediately or 24 hours later, cytospun, and subjected to cytofluorescence to detect CPD<sup>+</sup> cells. CPD-positive cells are dark brown. Magnification,  $\times 40$ . B, the number of UVB-induced CPD<sup>+</sup> cells in XPA-proficient and XPA-deficient cells is expressed in terms of the percentage of CPD<sup>+</sup> cells as a mean  $\pm$ SD of the results of two independent experiments. A significant decrease in the number of CPD<sup>+</sup> cells versus UVB alone-irradiated cells harvested immediately after UVB exposure in XPA-proficient cells (†,  $P < 0.01$ ; \*,  $P < 0.001$ ). CPD<sup>+</sup> cells were not detectable in non-UVB-exposed cells. ND, CPD<sup>+</sup> cells were not detectable.



**Fig. 6.** GSPs enhance nuclear localization of XPA protein and its binding to ERCC1. A, cytosolic and nuclear fractions of skin samples were subjected to Western blot analysis using antibodies specific to XPA. B, in binding assay, XPA nuclear protein was immunoprecipitated using XPA-specific antibody from the skin samples exposed to UVB and treated with or without GSPs 1 hour after UVB exposure. Non-UVB-exposed mouse skin served as a control. Protein samples were subjected to Western blot analysis, using antibodies against ERCC1, as detailed in Materials and Methods. The densitometric data (arbitrary) of each band of the blot is shown under the immunoblots. IP, immunoprecipitation.

accumulation of XPA nuclear protein in the UVB-irradiated skin. Dietary GSPs enhanced the accumulation of nuclear XPA protein compared with the non-GSPs-treated UVB-exposed mice. We also determined whether GSPs could enhance the binding of other repair factors to XPA to augment the repair of damaged DNA. Specifically, we assessed the interactions between XPA and ERCC1 upon UVB irradiation with and without treatment with GSPs with XPA immunoprecipitation followed by Western blotting. As shown in Fig. 6B, we found an increase in XPA and ERCC1 association in the GSPs and UVB-irradiated group of mice compared with the UVB-alone group of mice. These data suggest that GSPs promote nuclear translocation of XPA and enhance its interactions with other DNA repair protein, such as ERCC1.

## Discussion

In the present study, we demonstrate a novel mechanism by which GSPs prevent photocarcinogenesis in mice.

We show that the dietary GSPs stimulate repair of UVB-induced DNA damage in the form of CPDs in the mouse skin. In addition to the formation of CPDs in UVB-exposed skin, UVA in particular is also responsible for oxidative DNA damage and that can be repaired by a base excision repair mechanism. As UVB-induced CPDs have been recognized as a molecular trigger for the induction of immunosuppression as well as an initiator of skin carcinogenesis, we focused our attention on the repair mechanism of CPDs by dietary GSPs. Moreover, UVB radiations are comparatively more immunosuppressive, mutagenic, and carcinogenic in nature than UVA. The DNA repair process by GSPs is mediated through the induction of IL-12. IL-12 has the ability to repair UVB-induced CPDs (23, 25, 29). The subcutaneous injection of IL-12 at the site of UVB irradiation enhanced the repair of UVB-induced CPDs in GSP-treated IL-12 KO mice. These data support the concept that GSPs stimulate the repair of CPDs from the UVB-exposed skin through a mechanism that involves IL-12 activity. To further prove this concept, we have conducted photocarcinogenesis experiments with dietary GSPs using the IL-12 KO mouse model. The chemopreventive effect of dietary GSPs on photocarcinogenesis was less pronounced in IL-12 KO mice, but a significant inhibitory effect was observed in their WT mice, suggesting that the prevention of photocarcinogenesis by GSPs is mediated through IL-12. The treatment of IL-12 KO mice with GSPs did result in some inhibition of UVB-induced tumor development, although these effects were not statistically significant in these experiments. Our data that suggest a role for GSP-mediated induction of IL-12 in their anticarcinogenic activity are consistent with the demonstrated antitumor activity of IL-12 against cutaneous malignancies and experimental metastases (23, 30–33). As we observed that treatment of WT mice with GSPs increased the levels of IL-12 in UV-exposed mice (26), it is possible that this enhancement of the levels of IL-12 is involved in the inhibition of growth of tumors. IL-12 is a key component of numerous immune functions and has been found to cure or improve the survival of tumor-bearing mice by enhancing *in vivo* antitumor activity in a number of different tumor models (30–33). The comparison of available information from other tumor models with the present study conducted in the IL-12 KO mouse model indicates that IL-12 has an inhibitory effect on the UV-induced skin tumor development, and its deficiency renders the mice susceptible to photocarcinogenesis.

The NER is a major mechanism of DNA repair in mammalian cells. Since the treatment of GSPs enhances the repair of UVB-induced DNA damage, we further examined whether the repair of UV-induced CPDs by GSPs is mediated via induction of NER genes. Our real-time PCR data reveal that treatment of mice with GSPs increases the levels of NER genes (e.g., *XPA*, *XPC*, *RPA1*, and *DDB2*) in UVB-exposed skin sites of WT mice compared with non-GSPs-fed mice and that may have con-

tributed in the rapid repair of damaged DNA in the mouse skin. In contrast, this effect of GSPs was not observed in the UVB-exposed skin of IL-12 KO mice. The role of NER was further confirmed by assessing the effect of GSPs on UVB-induced DNA damage in human *XPA*-deficient cells obtained from human patients suffering from xeroderma pigmentosum disease and *XPA*-proficient cells from normal healthy persons. Cells derived from patients suffering from xeroderma pigmentosum either lack or have reduced DNA repair capacity due to genetic mutations in several components of the NER. The *XPA* complementation-type represents the most severe phenotype, because the *XPA* gene is the most crucial component in the repair process and, thus, cells lacking the *XPA* gene are completely deficient in NER (29). Our immunostaining data indicated that GSPs were able to repair UV-induced CPDs in *XPA*-proficient cells but was not able to repair in *XPA*-deficient cells. These observations indicate that repair of UV-induced DNA damage by GSPs is mediated through the *XPA*-dependent mechanism. *XPA* is part of the core incision complex of the NER system (34). Here we also demonstrated that dietary GSPs induces or augments *XPA* nuclear translocation and then activates the core incision complex in the NER system. Similar observations related with the role of *XPA* in DNA repair in UVB-exposed skin cells were also found by the treatment of  $\alpha$ -melanocyte-stimulating hormone (35). Stoner et al. have studied the effect of diet containing freeze-dried black raspberries on chemical carcinogen *N*-nitrosomethylbenzylamine-induced dysregulated genes in rat esophagus (36). They have found that 462 of the 2,261 *N*-nitrosomethylbenzylamine-dysregulated genes were restored to near-normal levels of expression by black raspberries. RNA microarray analysis revealed that dietary black raspberries upregulated the expression of 24 genes whereas downregulated the expression of 12 genes. The upregulated genes were associated with transcription regulation, apoptosis, metabolism, and signal transduction cascades (37). Our study further explains the mechanism that GSPs stimulate nuclear translocation of *XPA* and binds to other DNA repair protein and thus activates the system. These findings have important implications for the chemoprevention of skin cancer by GSPs and identify a new mechanism by which GSPs prevent UV-induced skin tumor development.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## References

- Shi J, Yu J, Pohorly JE, Kakuda Y. Polyphenolics in grape seeds-biochemistry and functionality. *J Med Food* 2003;6:291-9.
- Prieur C, Rigaud J, Cheyrier V, Moutounet M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* 1994;36:781-9.
- Silva RC, Rigaud J, Cheyrier V, Chemina A. Procyanidin dimers and trimers from grape seeds. *Phytochemistry* 1991;30:1259-64.
- Mantena SK, Katiyar SK. Grape seed proanthocyanidins inhibit UV radiation-induced oxidative stress and activation of MAPK and NF- $\kappa$ B signaling in human epidermal keratinocytes. *Free Rad Biol Med* 2006;40:1603-14.
- Sharma SD, Meeran SM, Katiyar SK. Dietary grape seed proanthocyanidins inhibit UVB-induced oxidative stress and activation of mitogen-activated protein kinases and nuclear factor- $\kappa$ B signaling in vivo SKH-1 hairless mice. *Mol Cancer Ther* 2007;6:995-1005.
- Nandakumar V, Singh T, Katiyar SK. Multi-targeted prevention and therapy of cancer by proanthocyanidins. *Cancer Letts* 2008; 269:378-87.
- Mittal A, Elmets CA, Katiyar SK. Dietary feeding of proanthocyanidins from grape seeds prevents photocarcinogenesis in SKH-1 hairless mice: relationship to decreased fat and lipid peroxidation. *Carcinogenesis* 2003;24:1379-88.
- Mantena SK, Baliga MS, Katiyar SK. Grape seed proanthocyanidins induce apoptosis and inhibit metastasis of highly metastatic breast carcinoma cells. *Carcinogenesis* 2006;27:1682-91.
- Vayalil PK, Mittal A, Katiyar SK. Proanthocyanidins from grape seeds inhibit expression of matrix metalloproteinases in human prostate carcinoma cells, which is associated with the inhibition of activation of MAPK and NF- $\kappa$ B. *Carcinogenesis* 2004;25:987-95.
- Meeran SM, Katiyar SK. Grape seed proanthocyanidins promote apoptosis in human epidermoid carcinoma A431 cells through alterations in Cdk1-Cdk-cyclin cascade, loss of mitochondrial membrane potential, caspase-3 activation. *Exp Dermatol* 2007; 16:405-15.
- Bagchi D, Bagchi M, Stohs S, Ray SD, Sen CK, Preuss HG. Cellular protection with proanthocyanidins derived from grape seeds. *Ann NY Acad Sci* 2002;957:260-70.
- Housman TS, Feldman SR, Williford PM, Fleischer AB Jr, Goldman ND, Acostamadiedo JM, Chen GJ. Skin cancer is among the most costly of all cancers to treat for the Medicare population. *J Am Acad Dermatol* 2003;48:425-9.
- Katiyar SK, Matsui MS, Mukhtar H. Kinetics of UV light-induced cyclobutane pyrimidine dimers in human skin in vivo: an immunohistochemical analysis of both epidermis and dermis. *Photochem Photobiol* 2000;72:788-93.
- Nichols JA, Katiyar SK. Skin photoprotection by natural polyphenols: anti-inflammatory, antioxidant and DNA repair mechanisms. *Arch Dermatol Res* 2010;302:71-83.
- Kripke ML, Cox PA, Alas LG, Yarosh DB. Pyrimidine dimers in DNA initiated systemic immunosuppression in UV-irradiated mice. *Proc Natl Acad Sci USA* 1992;89:7516-20.
- Yarosh D, Alas LG, Yee V, et al. Pyrimidine dimer removal enhanced by DNA repair liposomes reduces the incidence of UV skin cancer in mice. *Cancer Res* 1992;52:4227-31.
- Yarosh D, Klein J, O'Connor A, Hawk J, Rafal E, Wolf P. Effect of topically applied T4 endonuclease V in liposomes on skin cancer in xeroderma pigmentosum: a randomised study. *Xeroderma Pigmentosum Study Group. Lancet* 2001;357:926-9.
- de Vries A, van Oostrom CT, Hofhuis FM, et al. Increased susceptibility to ultraviolet-B and carcinogens of mice lacking the DNA excision repair gene XPA. *Nature* 1995;377:169-73.
- Cline SD, Hanawalt PC. Who's on first in the cellular response to DNA damage? *Nat Rev Mol Cell Biol* 2003;4:361-72.
- Sancar A, Lindsey-Boltz LA, Unsal-Kaçmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem* 2004;73:39-85.
- Bootsma D, Kraemer KH, Cleaver JE, Hoeijmakers JHJ. In: *The Genetic Basis of Human Cancer*. Vogelstein B, Kinzler KW, editors. New York: McGraw Hill;1998:245-74.
- Meeran SM, Mantena SK, Meleth S, Elmets CA, Katiyar SK. Interleukin-12-deficient mice are at greater risk of ultraviolet radiation-induced skin tumors and malignant transformation of papillomas to carcinomas. *Mol Cancer Ther* 2006;5:825-32.
- Meeran SM, Akhtar S, Katiyar SK. Inhibition of UVB-induced skin tumor development by drinking green tea polyphenols is mediated through DNA repair and subsequent inhibition of inflammation. *J Invest Dermatol* 2009;129:1258-70.
- Katiyar SK, Vaid M, van Steeg H, Meeran SM. Green tea polyphenols prevent UV-induced immunosuppression by rapid repair of DNA damage and enhancement of nucleotide excision repair genes. *Cancer Prev Res* 2010;3:179-89.
- Meeran SM, Mantena SK, Katiyar SK. Prevention of ultraviolet radiation-induced immunosuppression by (-)-epigallocatechin-3-gallate in mice is mediated through interleukin 12-dependent DNA repair. *Clin Cancer Res* 2006;12:2272-80.
- Sharma SD, Katiyar SK. Dietary grape-seed proanthocyanidin inhibition of ultraviolet b-induced immune suppression is associated with induction of IL-12. *Carcinogenesis* 2006;27:95-102.
- Nitta M, Saijo M, Kodo N, Matsuda T, Nakatsu Y, Tamai H, Tanaka K. A novel cytoplasmic GTPase XAB1 interacts with DNA repair protein XPA. *Nucleic Acids Res* 2000;28:4212-8.
- Wu X, Shell SM, Liu Y, Zou Y. ATR-dependent checkpoint modulates XPA nuclear import in response to UV irradiation. *Oncogene* 2007;26:757-64.
- Schwarz A, Stander S, Berneburg M, et al. Interleukin-12 suppresses ultraviolet radiation-induced apoptosis by inducing DNA repair. *Nat Cell Biol* 2002;4:26-31.
- Colombo MP, Vagliani M, Spreafico F, et al. Amount of interleukin 12 available at the tumor site is critical for tumor regression. *Cancer Res* 1996;56:2531-34.
- Brunda MJ, Luistro L, Warriar RR, et al. Antitumor and antimetastatic activity of interleukin-12 against murine tumors. *J Exp Med* 1993; 178:1223-30.
- Nastala CL, Edington HD, McKinney TG, et al. Recombinant IL-12 administration induces tumor-regression in association with IFN- $\gamma$  production. *J Immunol* 1994;153:1697-706.
- Wigginton JM, Komschlies KL, Back TC, Franco JL, Brunda MJ, Wiltrout RH. Administration of interleukin 12 with pulse interleukin 2 and the rapid and complete eradication of murine renal carcinoma. *J Natl Cancer Inst* 1996;88:38-43.
- Camenisch U, Dip R, Schumacher SB, Schuler B, Naegeli H. Recognition of helical kinks by xeroderma pigmentosum group A protein triggers DNA excision repair. *Nat Struct Mol Biol* 2006;13:278-84.
- Dong L, Wen J, Pier E, et al. Melanocyte-stimulating hormone directly enhances UV-Induced DNA repair in keratinocytes by a xeroderma pigmentosum group A-dependent mechanism. *Cancer Res* 2010;70: 3547-56.
- Stoner GD, Dombkowski AA, Reen RK, et al. Carcinogen-altered genes in rat esophagus positively modulated to normal levels of expression by both black raspberries and phenylethyl isothiocyanate. *Cancer Res* 2008;68:6460-7.
- Lechner JF, Reen RK, Dombkowski AA, et al. Effects of a black raspberry diet on gene expression in the rat esophagus. *Nutr Cancer* 2008;60:61-9.