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Differentiation-Dependent p53 Regulation of Nucleotide Excision Repair in Keratinocytes

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The role of the tumor suppressor p53 in repair of ultraviolet light (UV)-induced DNA damage was evaluated using a host-cell reactivation (HCR) assay. HCR determines a cell's ability to repair UV-damaged DNA through reactivation of a transfected CAT reporter plasmid. Most UV damage is removed through nucleotide excision repair (NER). Primary murine keratinocytes isolated from p53-deficient and wild-type p53 mice were used in the HCR assay. The NER was reduced in $p53^{-/-}$ keratinocytes as compared with $p53^{+/+}$ keratinocytes. The reduced DNA repair in $p53^{-/-}$ mice was confirmed with a radioimmunoassay comparing cyclobutane dimers (CPDs) and (6-4) photoproducts in $p53^{+/+}$ and $p53^{-/-}$ keratinocytes after the cells were exposed to UV irradiation. Our results demonstrate that wild-type p53 plays a significant role in regulating NER. Furthermore, as there is evidence that p53 protein levels decrease after keratinocytes become differentiated, we sought to determine whether p53 plays a role in NER in differentiated keratinocytes. Differentiation of the keratinocytes by increasing the Ca^{2+} concentration in the culture media resulted in a marked reduction in NER equally in both $p53^{+/+}$ and $p53^{-/-}$ groups. This finding suggests that reduced DNA repair after differentiation is p53 independent. A similar reduction in HCR was confirmed in differentiated human keratinocytes. These data, taken together, indicate that p53 or p53-regulated proteins enhance NER in basal undifferentiated keratinocytes but not in differentiated cells. As nonmelanoma skin cancers originate from the basal keratinocytes, our findings suggest that loss of p53 may contribute to the pathogenesis of this common skin cancer. (Am J Pathol 1997, 150:1457–1464)

The p53 gene is the most frequently mutated gene in human cancers.[1] Mutations of the p53 gene have been observed in carcinomas of the colon, breast, lung, bladder, brain, and bone and in chronic myelogenous leukemia[2] as well as skin cancers.[3,4] In the past few years, many studies suggest that mutations of the p53 tumor suppressor gene are involved in the development of nonmelanoma skin cancer. Mutations of the p53 gene occur in most human squamous cell carcinomas,[3] UVB-induced mouse squamous cell carcinomas,[5,6] human basal cell carcinomas,[7] and skin tumors (including squamous cell carcinoma and basal cell carcinoma) from DNA-repair-deficient xeroderma pigmentosum patients.[8] The mutations are predominantly UV-specific C → T or CC → TT transitions. Mutations of the p53 gene appear to be an early event in the development of nonmelanoma skin cancers, as overexpression of the p53 protein was observed in human actinic keratosis, a precursor lesion of squamous cell carcinoma.[9–11] By sequencing the p53 gene, Ziegler et al[12] have demonstrated that actinic keratoses also contain UV-specific p53 mutations. To confirm the role of p53 in the pathogenesis of UV-induced skin cancer, we have previously exposed the p53 transgenic mice[13] that contain mutant alleles of p53 gene to UV radiation and found that the p53 transgenic mice are predisposed to UV-induced squamous cell carcinomas.[14] These data suggest that p53 inactivation

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leads to a selective advantage, and thus wild-type p53 likely plays an important role in protecting the genome of the keratinocytes against UV damage.

p53 is considered to be the guardian of the genome.[15,16] There are two mechanisms for cells to maintain genetic stability: repairing the DNA damage or inducing apoptosis. There is evidence to suggest that p53 is involved in both DNA repair and apoptosis. After a DNA damage event, such as UV and ionizing radiation, the p53 protein is elevated,[17–19] which in turn induces G1 arrest,[19,20] regulates nucleotide excision repair,[21,22] or promotes apoptosis.[12,23–25] G1 arrest is possibly achieved by transcriptional regulation of $p21^{Cip1}$, which is a potent inhibitor of cyclin-dependent kinase (Cdk).[26–28] It is assumed that p53 induces G1 arrest to provide extra time for the cell to repair DNA damage before the damage is propagated during S phase. However, increasing evidence suggests that p53 may directly regulate DNA repair.

Two of the major photoproducts caused by UV radiation, cyclobutane pyrimidine dimers (CPDs) and the (6-4) photoproducts ((6-4)PDs), are repaired primarily in a process known as nucleotide excision repair (NER). NER involves a complex series of proteins that orchestrate the identification and removal of damaged DNA, addition of nucleotides, and finally re-ligation of the DNA strand.[29] Recent studies have directly implicated a role of p53 in regulating NER. Wild-type p53 binds to and modulates XPB and XPD, two components of the TFIIH transcription unit, which possesses helicase, ATPase, and kinase activity.[30,31] Another direct link between p53 and NER is replication protein A, a factor that is essential for the first step of DNA replication.[32] p53 is known to bind and inhibit replication protein A, suggesting that p53 may directly interfere with DNA replication.[33] p53 also binds to DNA stand breaks, possibly to recruit repair proteins.[34] Thus, interaction between p53 and components of NER or damaged DNA provide indirect evidence for its role in UV-induced DNA repair.

The role of p53 in NER is further supported by comparing the repair efficiency of cells carrying wild-type p53 and mutant p53. Cells containing mutant p53 protein showed reduced repair capability.[21,22,31,35,36] However, the results from these studies may not reflect the direct role of wild-type p53 in NER for the following reasons: 1) mutant p53 may directly inhibit NER in a gain of function manner, rather than binding and inactivating the wild-type p53 and 2) spontaneous genomic instability may have occurred in cultured cancer cell lines or extensively passaged fibroblasts that carry a mutant or null p53 gene.[37,38] We therefore elected to use primary cultures of keratinocytes from p53-deficient mice[39] to study the involvement of p53 in NER. We report here that the host-cell reactivation of a UV-damaged plasmid DNA as well as the global DNA repair after UV irradiation were reduced in the keratinocytes from p53-deficient mice and that the regulation of NER by p53 is differentiation dependent.

Materials and Methods

Cell Culture

Keratinocytes were isolated from 6- to 8-week-old $p53^{+/+}$ and $p53^{-/-}$ mice (GenPharm, Mountainview, CA) as previously described.[21] Briefly, the tail skin was dissected from the mice and treated with 0.25% dispase (Gibco-BRL, Mississauga, Ontario) for 16 to 18 hours. The epidermis was separated from the dermis with a pair of forceps. The epidermal sheet was then trypsinized for 5 minutes and the cells were seeded into 35-mm petri dishes in keratinocyte serum-free medium (SFM) (Gibco-BRL) containing 100 U/ml penicillin G and 100 μg/ml streptomycin. The medium was changed twice a week.

Normal human keratinocytes were obtained from the tissue bank, Vancouver Hospital and Science Centre, and cultured in keratinocyte-SFM. All of the cells were incubated at 37°C in 5% CO_2 atmosphere.

UV Irradiation

Keratinocytes were exposed to UVB (290 to 320 nm), using a bank of four unfiltered FS40 sunlamps (Westinghouse, Bloomfield, NJ). The intensity of the UV light was measured by an IL 700 radiometer fitted with a WN 320 filter and an A127 quartz diffuser (International Light, Newburyport, MA).

Plasmid $pCMV_{cat}$ DNA (5 kb) was irradiated with UVC (254 nm), and 1 ml of stock plasmid DNA (50 μg/ml) was pipetted into 35-mm plates and irradiated at 0, 700, 1000, 1200, and 1500 J/m^2, using an unfiltered germicidal lamp (15-W Champion 15T8 low pressure mercury lamp).

Host-Cell Reactivation (HCR) Assay

The plasmid vector pCMVcat (a kind gift of Dr. L. Grossman, The Johns Hopkins University, Baltimore, MD) contains a gene encoding for *cat*, under the transcriptional control of the immediate early promoter of the human cytomegalovirus. Transfection of plasmid DNA into the keratinocytes was performed using a liposome-mediated procedure. For a single determination, 5 μg of lipofectin (Gibco-BRL) and

0.5 μg of plasmid DNA were each diluted in 100 μl of keratinocyte-SFM and allowed to sit at room temperature for 30 minutes. Lipofectin and plasmid DNA were then mixed and allowed to sit at room temperature for 10 minutes. The lipofectin/DNA mixture (200 μl) combined with 800 μl of keratinocyte-SFM was immediately added to phosphate-buffered saline (PBS)-washed keratinocytes. After 16 hours, the medium was removed, and 2 ml of fresh keratinocyte-SFM was added to the plates. At 24 hours later, cells were washed with PBS twice, scraped into an Eppendorf tube, and pelleted. The cell pellet was resuspended in a 50-μl solution of 0.25 mol/L Tris-Hcl (pH 8.0) and 5 mmol/L EDTA. Cell-free extracts of the transfected cells were made by three repeated freeze-thawings (liquid nitrogen to freeze, 37°C waterbath to thaw), heated to 65°C for 10 minutes, and centrifuged at 12,000 × *g* for 10 minutes, and the cleared supernatant was used for the CAT assay. The assay reaction mixture contained 7.5 μl of 5 mmol/L chloramphenicol, 50 μl of cell-free extract, 1 μl of 2.5 mmol/L [^{3}H]acetyl-CoA, and 16.5 μl of deionized H_2O. The reaction mixture was incubated at 37°C for 90 minutes. After incubation, 200 μl of ice-cold ethyl acetate was added, and tubes were shaken and centrifuged at 12,000 × *g* for 5 minutes. After quick-freezing the aqueous phase in a dry ice/ethanol bath, the organic phase was removed and extracted with 200 μl of distilled water. The organic phase was dried to completion and radioactivity was determined in a scintillation counter. Determinants were performed in triplicates. Controls included transfection with undamaged plasmid DNA and mock transfection without plasmid DNA.

Radioimmunoassay (RIA)

$p53^{+/+}$ and $p53^{-/-}$ keratinocytes were incubated in keratinocyte-SFM containing 0.025 μCi/ml [^{14}C]thymidine (51.5 mCi/mmol) and allowed to grow for 48 hours. The cells were irradiated with 250 J/m^2 of UVB, whereas for *in vivo* experiments, animals were exposed to 2500 J/m^2 and the RIA was performed as described.[21,40]

Statistical Analysis

Each determination was obtained from a triplicate set of readings that yielded a mean and standard deviation for each UV dose. The percentage of chloramphenicol acetyltransferase (CAT) activity was expressed as a ratio of net cpm damage dose/net cpm undamaged plasmid DNA. The estimation of the ratio of two means with variation was taken as the quotient of two normally distributed, uncorrelated random variables.[41] A significant difference between experimental groups was considered only when the 95% confidence interval for the quotient estimates did not overlap. These differences are significant at $P \leq 0.05$.

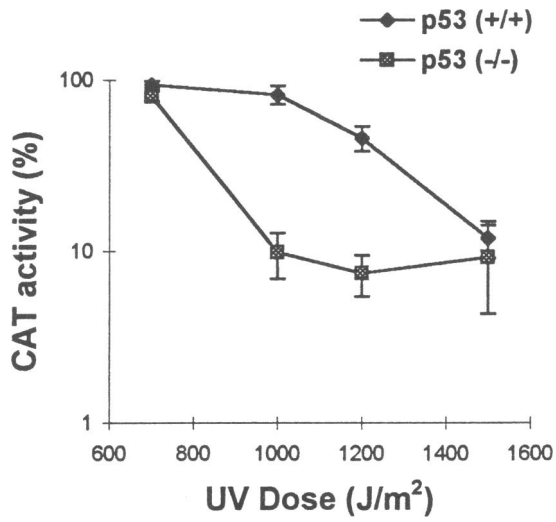

Figure 1. *Host-cell reactivation in keratinocytes isolated from wild-type p53 (+/+) and p53-deficient (−/−) mice. Each point represents the mean of triplicate plates (± SD).*

Results

p53 Regulates Repair of NER in Murine Keratinocytes

To test the effect of p53 on NER, a host-cell reactivation assay was employed.[42] This assay involves the introduction of UV-damaged plasmid DNA harboring a CAT reporter gene into cells by transient transfection. Excision repair of the damaged DNA is then determined as a function of reactivation of CAT enzyme activity. Cells having greater capacity to repair UV damage should demonstrate higher CAT activity.

Keratinocytes were isolated from $p53^{+/+}$ and $p53^{-/-}$ mice and then transfected with the plasmid $pCMV_{cat}$ DNA, which had been exposed to UV at varying doses. As shown in Figure 1, at UV doses of 1000 and 1200 J/m^2 the CAT activity was reduced by six- to eightfold in the p53-deficient keratinocytes ($P < 0.05$). This experiment has been repeated twice with similar results. At a lower dose of 700 J/m^2 or a higher dose of 1500 J/m^2 there were no statistical difference in CAT activity between $p53^{+/+}$ and $p53^{-/-}$ keratinocytes.

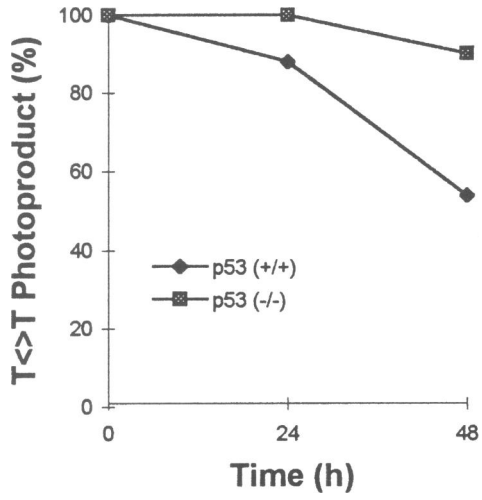

Figure 2. *RIA for the CPD photoproducts in UV-irradiated keratinocytes isolated from wild-type p53 (+/+) and p53-deficient (−/−) mice. The RIA was performed in duplicate.*

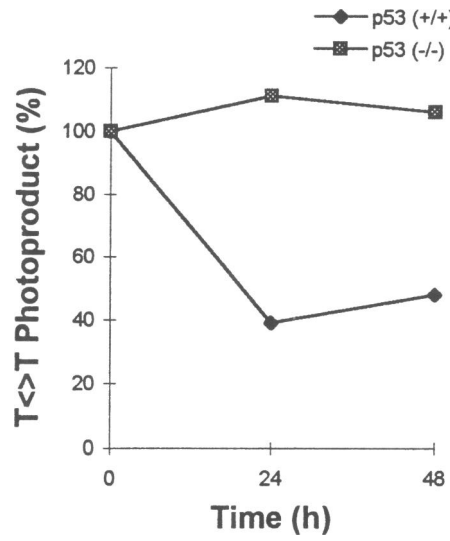

Figure 3. *RIA for the CPD photoproducts in the epidermis of UV-irradiated wild-type p53 (+/+) and p53-deficient (−/−) mice. The RIA was performed in duplicate.*

Previously we demonstrated that the global repair of CPDs was reduced in the keratinocytes of p53 transgenic mice that carry mutant alleles of the p53 gene.[21] To further confirm the role of p53 in NER, we used RIA to examine the global repair of CPDs and (6-4)PDs in p53-deficient keratinocytes after UV irradiation. Keratinocytes were isolated from $p53^{+/+}$ and $p53^{-/-}$ mice and exposed to 250 J/m^2 of UVB. This dose produces approximately 375 (6-4)PDs/megabase, which is easily detected using this assay in these cells.[21] As seen in Figure 2, $p53^{+/+}$ keratinocytes removed 50% of the CPDs by 48 hours, whereas 90% of the CPDs still remained in $p53^{-/-}$ cells. These findings were confirmed *in vivo*; $p53^{-/-}$ mice demonstrated reduced removal of CPDs (Figure 3). Finally, $p53^{+/+}$ keratinocytes removed virtually all detectable (6-4)PDs by 24 hours (Figure 4), whereas more than 30% of (6-4)PDs remained in p53-deficient cells.

p53 Does Not Regulate NER in Differentiated Murine Keratinocytes

Within the epidermis, basal keratinocytes are the stem cells that have proliferating ability. A subpopulation of basal keratinocytes undergo differentiation as they migrate to the surface of the skin. It has been previously shown that differentiated murine keratinocytes have reduced levels of p53 as compared to basal, proliferating keratinocytes.[43] Therefore, NER may be altered in differentiated keratinocytes. We sought to determine whether p53 continues to regulate NER in differentiated keratinocytes. To do so, we elevated the calcium concentration to 1.0 mmol/L for 48 hours, which had previously been shown to induce differentiation-specific markers in murine keratinocytes.[43] The cells were then transfected with the damaged plasmid $pCMV_{cat}$ DNA, and CAT activity was examined. Figure 5 demonstrates that the $p53^{+/+}$ keratinocytes grown at 0.09 mmol/L Ca^{2+} had significantly higher CAT activity than $p53^{-/-}$ cells ($P < 0.05$), confirming the data in Figure 1. However, after exposure to 1 mmol/L Ca^{2+} for 48 hours, a striking reduction in DNA repair was observed in both $p53^{+/+}$ and $p53^{-/-}$ keratinocytes. The reduction in NER was not the result of reduced transfection efficiency of differentiated keratinocytes, as the CAT activity of undamaged control plasmid

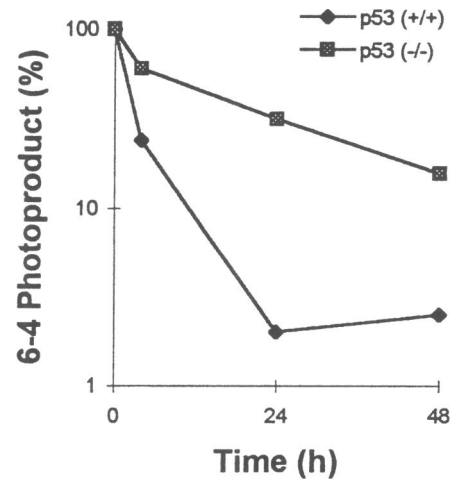

Figure 4. *RIA for the (6-4) photoproducts in UV-irradiated keratinocytes isolated from wild-type p53 (+/+) and p53-deficient (−/−) mice. The RIA was performed in duplicate.*

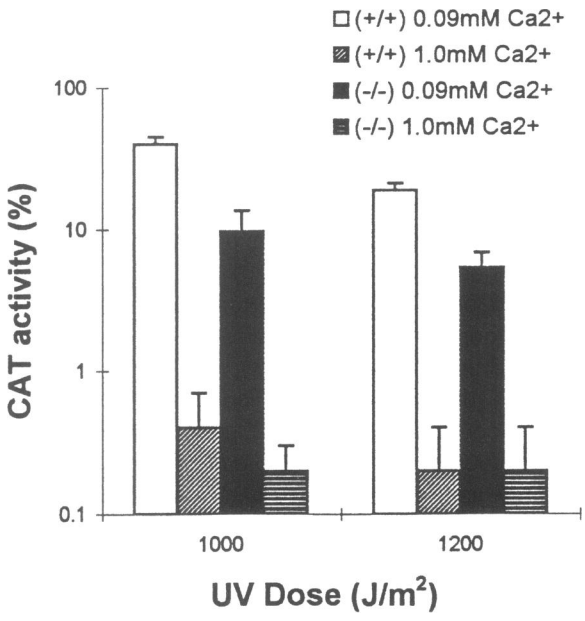

Figure 5. *Host-cell reactivation in proliferating (0.09 mmol/L Ca^{2+}) and differentiated keratinocytes (1.0 mmol/L Ca^{2+}) isolated from wild-type p53 (+/+) and p53-deficient (−/−) mice. Each point represents the mean of triplicate plates (± SD).*

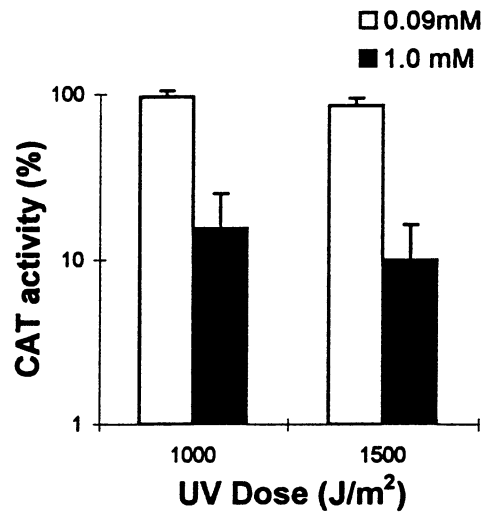

Figure 6. *Host-cell reactivation in proliferating (0.09 mmol/L Ca^{2+}) and differentiated (1.0 mmol/L Ca^{2+}) human keratinocytes. Each point represents the mean of triplicate plates (± SD).*

DNA in differentiated keratinocytes was only slighter lower (70%) than that in undifferentiated keratinocytes (data not shown). The very low CAT activity in both $p53^{+/+}$ and $p53^{-/-}$ keratinocytes after exposure to high Ca^{2+} suggests that UV-damaged DNA is inefficiently repaired in differentiated keratinocytes. The CAT activity of differentiated $p53^{+/+}$ keratinocytes was similar to that of $p53^{-/-}$ keratinocytes, suggesting that the role of p53 in NER of differentiated keratinocytes is negligible.

NER is Reduced in Differentiated Human Keratinocytes

To further determine whether reduction of NER is associated with differentiation of keratinocytes, we compared the rate of NER in human keratinocytes cultured in low Ca^{2+} medium or in high Ca^{2+} medium using HCR. Human keratinocytes were exposed to 1 mmol/L Ca^{2+} for 48 hours, which induces a differentiated phenotype,[44] and then transfected with $pCMV_{cat}$ DNA. Figure 6 demonstrates that differentiated human keratinocytes, similar to murine keratinocytes, have a 7- to 10-fold reduction in NER compared with undifferentiated cells ($P < 0.05$).

Discussion

We investigated the role of p53 in the repair of UV-damaged DNA using primary culture of keratinocytes from p53-deficient mice. p53 knockout keratinocytes were chosen because keratinocytes are natural targets for UV radiation and the p53 tumor suppressor gene is frequently mutated in keratinocyte-derived skin cancers, ie, basal cell and squamous cell carcinomas.[3,7] We employed HCR assay and RIA to study the role of p53 in NER. One major advantage of using HCR is to eliminate the possibility of cell cycle changes that may occur in cells directly exposed to UV. Our data conclusively demonstrate that HCR of a damaged reporter gene, as well as cellular global DNA repair after a UV-damaging event, were reduced in $p53^{-/-}$ keratinocytes compared with that of $p53^{+/+}$ cells (Figures 1 to 4). However, upon the induction of differentiation, the ability to repair UV-damaged DNA is drastically reduced in both $p53^{+/+}$ and $p53^{-/-}$ keratinocytes; no difference in the repair efficiency was observed between these two groups (Figure 5). These results indicate that proliferating basal keratinocytes but not differentiated keratinocytes efficiently repair UV-damaged DNA, which is regulated by p53 or p53-regulated proteins.

Repair of damaged DNA is crucial for maintaining genetic stability after genotoxic stress. The p53 tumor suppressor has been shown to play a significant role in this process. We have previously reported that the global repair of CPDs after UV irradiation is reduced both *in vivo* and *in vitro* in keratinocytes from p53 transgenic mice that carry mutant alleles of the p53 gene.[21] Smith et al[22] showed that colon carcinoma cell lines that carry mutant p53 or HPV E6, which binds and inactivates wild-type p53, exhibited reduced ability to repair UV-damaged DNA using

HCR and an *in vitro* repair assay. Repair of CPDs is reduced in human skin fibroblasts from Li-Fraumeni syndrome patients who are homozygous for mutations in both alleles of the p53 gene ($p53^{mut/mut}$)[35] or heterozygous for p53 mutations ($p53^{wt/mut}$)[31] compared with normal cells. These studies used cells carrying mutant p53 genes. It is generally assumed that the mutant p53 binds to wild-type p53 and inactivates the normal alleles in a dominant negative manner. However, this assumption may not be true, because a gain of function has recently been described in animals created by mating p53 transgenic animals carrying mutant p53 and p53-deficient mice. The mice that carried a mutant allele exhibited accelerated tumor development over the p53-deficient (−/−) mice.[45] Therefore, mutant p53 may directly act to down-regulate NER. In our experimental system, cells do not carry mutant p53, and therefore our results truly reflect the function of wild-type p53 in DNA repair after UV damage.

Our data further support the findings of other studies on the role of p53 in DNA repair using p53-deficient mice. The fibroblasts derived from embryos of p53 knockout mice had greater chromatid-type aberrations in G2 phase after exposure to bleomycin, a genotoxic agent.[46] Ishizaki and co-workers[47] also reported that UV irradiation of embryonic fibroblasts resulted in an increase in sister chromatid exchanges in p53 knockout mice compared with wild-type cells. However, they did not find a difference in repair of CPDs and (6-4)PDs between $p53^{+/+}$ and $p53^{-/-}$ cells. The reason for the discrepancy in DNA repair between $p53^{-/-}$ keratinocytes and $p53^{-/-}$ fibroblasts is unclear. It could be that regulation of p53 in DNA repair is tissue and cell type specific. Alternatively, genomic instability, which occurs in passaged mouse fibroblasts,[38] may contribute to the negative results by Ishizaki.[47]

The exact molecular mechanism as to how p53 regulates DNA repair still remains to be elucidated. Several lines of evidence suggest that p53 may directly participate in NER through a protein-protein interaction with repair components or by binding to sites of DNA damage. p53 has been shown to bind *in vitro* to the promoter region of the human mismatch repair gene hMSH2,[48] which plays in important role in the process of lesion recognition in NER, a step that might be rate limiting.[49] p53 has also been shown to bind to single-stranded DNA ends and catalyze DNA renaturation and strand transfer.[34] In addition, p53 has also been found to bind to several transcription repair factors, XPD and XPB, as well as CSB *via* its carboxyl-terminal domain.[30,31,50] Wild-type p53 but not mutant p53 protein inhibits XPD and XPB DNA helicase activities.[31,50] The role of p53 in DNA repair is further supported by the observation that the level of p53 is elevated in the DNA repair gene ERCC-1-deficient mice.[51] The elevated p53 may serve as a monitor of DNA damage or partially compensate for the ERCC1 deficiency.

Wild-type p53 is considered to be an important factor in maintaining genomic stability.[15,16,52] Upon a DNA damaging event, such as UV radiation, the p53 protein is increased.[17] Increased p53 regulates the downstream target genes to promote cell cycle arrest,[19,20] participate in DNA repair,[21,22,31,35,36] or induce apoptosis.[12,23–25] Our finding that p53 regulates DNA repair in proliferating but not in differentiated keratinocytes suggests that proliferating basal keratinocytes and differentiated suprabasal cells may have different mechanisms to protect their genome. From a physiological perspective it may be crucial for the proliferating basal keratinocytes to repair UV-induced damage, whereas for the nonproliferating differentiated suprabasal keratinocytes, the ability of triggering apoptosis may be more important.[53]

It should be noted that p53-mediated DNA repair after a genotoxic event depends on the severity of the DNA damage. After exposure to a high dose of UV radiation, neither $p53^{+/+}$ nor $p53^{-/-}$ keratinocytes efficiently repair DNA damage. p53-dependent DNA repair of the basal cell population may protect the organism from damaging the epidermis, whereas continuous exposure to UV may eventually be detrimental. We conclude that p53 or p53-regulated proteins enhance NER and that loss of this crucial protein may promote genomic instability and in turn lead to tumor development.

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