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Fate of UVB-induced *p53* mutations in SKH-hr1 mouse skin after discontinuation of irradiation: relationship to skin cancer development

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Chronic exposure to ultraviolet (UV) radiation causes skin cancer in humans and mice. We have previously shown that in hairless SKH-hr1 mice, UVB-induced *p53* mutations arise very early, well before tumor development. In this study, we investigated whether discontinuation of UVB exposure before the onset of skin tumors results in the disappearance of *p53* mutations in the skin of hairless SKH-hr1 mice. Irradiation of mice at a dose of 2.5 kJ/m² three times a week for 8 weeks induced *p53* mutations in the epidermal keratinocytes of 100% of the mice. UVB irradiation was discontinued after 8 weeks, but *p53* mutations at most hotspot codons were still present even 22 weeks later. During that period, the percent of mice carrying $p53^{V154A|R155C}$, $p53^{H175H|H176Y}$, and $p53^{R275C}$ mutant alleles remained at or near 100%, whereas the percentage of mice with $p53^{R270C}$ mutation decreased by 45%. As expected, discontinuation of UVB after 8 weeks resulted in a delay in tumor development. A 100% of tumors carried $p53^{V154A|R155C}$ mutant alleles, 76% carried $p53^{H175H|H176Y}$ mutants, and 24 and 19% carried $p53^{R270C}$ and $p53^{R275C}$ mutants, respectively. These results suggest that different UVB-induced *p53* mutants may provide different survival advantages to keratinocytes in the absence of further UVB exposure and that skin cancer development can be delayed but not prevented by avoidance of further exposure to UVB radiation.
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Introduction

The incidence of skin cancer exceeds the incidence of all other human cancers combined (Urbach, 1991; Miller and Weinstock, 1994). Epidemiologic, clinical, and biologic studies indicate that solar ultraviolet (UV) radiation is the major etiologic agent in skin cancer development (Urbach, 1991, 1997; Brash *et al*., 1991; Miller and Weinstock, 1994). Wavelengths in the UVB range of the solar spectrum (290–320 nm) are absorbed by the skin, producing erythema, burns, immunosuppression, DNA mutations, and nonmelanoma skin cancers (NMSC) (Gilchrest, 1990; Young, 1990; Brash *et al*., 1991; Kripke, 1991; Kanjilal *et al*., 1993; de Gruijl and Forbes, 1995; Brash *et al*., 1996; Ananthaswamy *et al*., 1997). Several studies have shown that the *p53* tumor suppressor gene is susceptible to UV-induced mutations and plays a critical role in the induction of NMSC (Brash *et al*., 1991, 1996; Kanjilal *et al*., 1993; Ziegler *et al*., 1994; Jonason *et al*., 1996; Ananthaswamy *et al*., 1997). In response to DNA damage, the p53 protein transactivates downstream genes such as $p21^{Waf1/Cip1}$ and *gadd45* that induce cell-cycle arrest at the G_1–S phase to allow DNA repair (Kuerbitz *et al*., 1992; Li *et al*., 1996). If the damage is not repaired, p53-dependent apoptosis, or 'cellular proofreading,' is triggered to eliminate severely damaged cells (Ziegler *et al*., 1994; Brash, 1996; Smith and Fornace, 1997). Upon repeated exposure to UV radiation, DNA lesions in the *p53* gene are transformed into mutations, mainly C→T or CC→TT transitions at dipyrimidine sites, thereby initiating the molecular process of skin carcinogenesis (Leffell and Brash, 1996). Thousands of *p53*-mutant cell clones are found in sun-exposed skin that appears normal (Jonason *et al*., 1996; Ren *et al*., 1996). The frequency of UV-signature mutations in the *p53* gene is high in precancerous lesions and reaches 50–90% in human basal and squamous cell carcinomas (SCCs) and 100% in murine UV-induced skin tumors (Kanjilal *et al*., 1993; Ziegler *et al*., 1994; Brash *et al*., 1996).

UV radiation is a complete carcinogen in that it not only initiates tumorigenesis by inducing mutations in the *p53* tumor suppressor gene but also promotes tumor development (Epstein and Epstein, 1963; Blum, 1969). As a tumor promoter, UV radiation induces cell proliferation by stimulating the production of various growth factors and cytokines as well as the activation of their receptors (De-Metys *et al*., 1995; Rosette and

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Karin, 1996; Bender *et al*., 1997; Kuhn *et al*., 1999; Jost *et al*., 2000; Peus *et al*., 2000; Ullrich, 2000; Walterscheid *et al*., 2002). In addition, cell survival and growth following UV irradiation involves activation of NF-κB, mitogen-activated protein kinase, and phosphoinositide-3′-kinase/Akt pathways (Coffer *et al*., 1995; De-Metys *et al*., 1995; Rosette and Karin, 1996; Bender *et al*., 1997). Repeated exposure of skin to UV radiation results in the clonal expansion of *p53*-mutant cells (Ziegler *et al*., 1994; Berg *et al*., 1996; Rebel *et al*., 2001; Zhang *et al*., 2001). Two mechanisms are believed to contribute to the selective expansion of *p53*-mutant cells: their resistance to UV-induced apoptosis and their proliferative advantage over normal keratinocytes in response to stimulation with UV radiation. While evidence supports the role of mutant p53 in enabling apoptosis resistance in keratinocytes (Ziegler *et al*., 1994; Tron *et al*., 1998; Zhang *et al*., 2001; Mudgil *et al*., 2003), the possibility that mutant p53 provides keratinocyte progenitors with proliferative advantages remains largely unexplored, even though two phenotypes may be related to the same underlying molecular changes (Kuhn *et al*., 1999; van Hogerlinden *et al*., 1999; Peus *et al*., 2000; Marconi *et al*., 2003). Nevertheless, discontinuation of UV irradiation has been shown to result in the fast spontaneous regression of some mutant p53 clones in mouse skin, although the mechanisms involved in this process are unclear (Berg *et al*., 1996; Rebel *et al*., 2001; Remenyik *et al*., 2003).

We have previously shown that exposure of SKH-hr1 mice to chronic UV radiation induced SCCs (Ouhtit *et al*., 2000a). UV-signature CC→TT and C→T mutations in *p53* codons 154–155, 175–176, and 270 or 275 were detected in epidermis as early as after 1 week of chronic UV irradiation (Ouhtit *et al*., 2000a). These mutations were detected in the epidermis of 80% of mice irradiated for 4 weeks and 90% of mice irradiated for 8 weeks (Ouhtit *et al*., 2000a). The mice began to develop skin tumors after 8 weeks of chronic irradiation, and all mice developed multiple skin tumors, mostly SCCs, by week 25 of chronic UV irradiation. These results suggested that *p53* mutations arise very early and well before skin tumor development. This finding raised an important question, that is, what would happen to *p53* mutations in the skin if UV irradiation is discontinued after 8 weeks, and how it relates to skin cancer development? We, therefore, investigated the fate of UV-induced *p53* mutations in mouse skin and their relationship to skin cancer development after discontinuation of UV exposure. Our results indicate that while the incidence of some *p53* mutant alleles remains at or near 100% even 22 weeks after UV irradiation is discontinued, other *p53* mutations do get eliminated from skin, evidently as a result of cell differentiation and desquamation. Discontinuation of UV treatment delayed the time required for tumor development, but it did not prevent tumors. All tumors harbored *p53* mutations at one or more of the hotspots, and the dynamics of the retention or loss of these mutations in lesion-free skin after the discontinuation of UV irradiation correlated with their incidence in tumors.

Results

UV-induced p53 *mutations in mouse skin after discontinuation of irradiation*

Representative allele-specific polymerase chain reaction (AS-PCR) gel electrophoresis data shown in Figure 1a indicate that 1 day after discontinuation of UV irradiation, *p53* mutations were detected in the skin of all 10 mice in the group studied. Specifically, $p53^{V154A/R155C}$, $p53^{H175H/H176Y}$, $p53^{R270C}$, and $p53^{R275C}$ mutant alleles were detected in 8, 9, 9, and 10 of the 10 mice, respectively (Figure 1a, Table 1). DNA from unirradiated mouse skin was not amplified by any sets of mutant-specific primers (lane 2 on each gel). In addition, DNA from all 10 (five at 1 day post-UV and five at 22 weeks post-UV time points) unirradiated mouse skin did not amplify at codon 270 (Figure 1d, Table 1) or at any other codons tested (data not shown). The absence of $p53^{R270C}$ mutations in untreated mouse skin versus $p53^{R270C}$ mutation at 1 day post-UV skin was statistically significant ($P = 0.01$). However, genomic DNA from a UV-induced tumor cell line known to contain *p53* mutations at specific codons was amplified (Figure 1a, positive control (+control) lane on each gel). Interestingly, *p53* mutations at all the hotspot codons were still present in the epidermis of mice 22 weeks after

Table 1 Incidence of *p53* mutations in SKH-hr1 mouse skin and tumors after discontinuation of UV irradiation

Time	*No. of mice with mutations in a particular codon/no. of mice or tumors analysed*				
	154–155 CC→TT	*175–176 CC→TT*	*270 C→T*	*275 C→T*	*Total % of mice or tumors with mutations*
1 day post-UV	8/10	9/10	9/10	10/10	100
2 weeks post-UV	10/10	9/10	9/10	8/10	100
6 weeks post-UV	10/10	10/10	9/10	8/10	100
10 weeks post-UV	9/10	10/10	8/10	7/10	100
22 weeks post-UV	9/9	9/9	5/9	8/9	100
No UV skin	0/10	0/10	0/10	0/10	0
Tumors[a]	21/21 (100%)	16/21 (76%)	5/21 (24%)	4/21 (19%)	100

Groups of 10 tumor-free skin specimens collected on day 1 or weeks 2, 6, 10, and 22 after last UV exposure were analysed for mutations in UV-hotspot codons 154–155, 175–176, 270, and 275 of *p53* gene using AS-PCR method. [a]In all, 21 randomly chosen SCCs were analysed for *p53* mutations

discontinuation of UV irradiation. Specifically, at 22 weeks, the incidence of $p53^{V154A/R155C}$, $p53^{H175H/H176Y}$, and $p53^{R275C}$ mutant alleles remained at 90–100% of their incidence 1 day after UV irradiation was discontinued (Figure 1b, Table 1), whereas the incidence of mutation at *p53* codon 270 decreased by 45% (Figure 1c, Table 1), which was not statistically significant ($P = 0.72$). It should be noted that AS-PCR is a highly sensitive method that detects mutations regardless of the size or number of mutant clones. A low amount of mutant *p53* alleles will allow a skin sample to be scored as positive. Consequently, this method does not reflect the actual disappearance of cells with a mutant p53 until it has decreased to the limit of AS-PCR sensitivity. We did not, however, determine the number or size of mutant *p53* clones in our experiments.

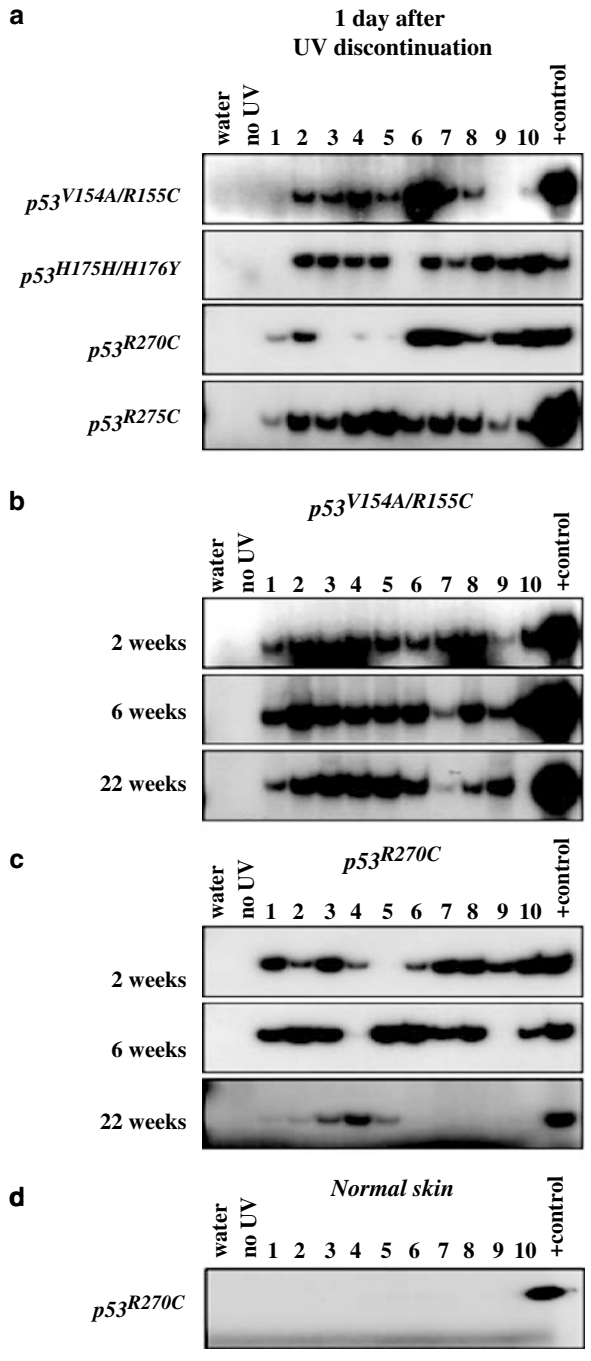

Figure 1 AS-PCR detection of *p53* mutations in mouse skin. (**a**) At 1 day after discontinuation of chronic UV irradiation (3× per week for 8 weeks). (**b, c**) Different time points after UV discontinuation. Lanes marked from 1 to 10 represent genomic DNA from treated skin samples. Positive control (+control) is genomic DNA from four different established mouse skin tumor cell lines containing *p53* mutations at codons of interest. DNA from an unirradiated mouse skin (lane marked No UV) did not amplify at any of the codons of interest. (**d**) DNA from 10 unirradiated mice (five at 1 day post-UV time point, lanes 1–5 and five at 22 weeks post-UV time point, lanes 6–10) did not amplify at codon 270 or at other codons (data not shown)

Histologic changes in skin after discontinuation of UV

Epidermal hyperplasia To determine whether discontinuation of irradiation affects epidermal hyperplasia induced by chronic UV, we examined hematoxylin and eosin (H&E)-stained skin sections of mice at various time points after discontinuation of UV exposure. As expected, chronic UV irradiation for 8 weeks induced epidermal hyperplasia (Figure 2). However, termination of UV treatment led to a noticeable decrease in epidermal hyperplasia as early as 7 days later (Figure 2). At 14 days post-UV irradiation and thereafter, the epidermis looked quite normal and resembled the skin of unirradiated mice. Analogous to the gross H&E data, immunohistochemical analysis also indicated the presence of numerous (59.5 ± 7.4 per 100 nucleated cells) proliferating cell nuclear antigen (PCNA)-positive cells throughout the epidermis of mouse skin irradiated with UV light for 8 weeks (Figure 2, 1 day post-UV). Correspondingly, PCNA-positive cells were fewer in

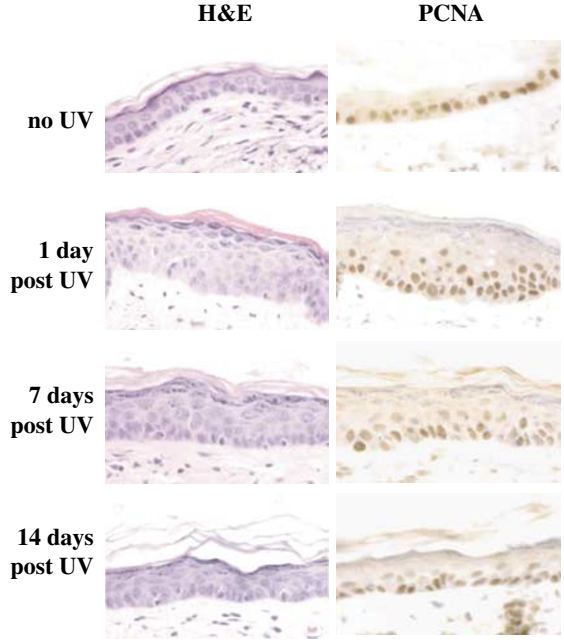

Figure 2 H&E stains and immunohistochemical analysis for PCNA protein expression in nonirradiated skin and in skin taken 1, 7, and 14 days after discontinuation of UV treatment

number (42.4±12.6 per 100 cells, $P<0.1$ by Student's *t*-test) at 7 days post-UV exposure. At day 14 after UV radiation discontinuation, the number of PCNA-positive cells was significantly lower when compared to day 1 after UV radiation discontinuation (32.5±6.8 per 100 cells, $P<0.5$), and was equal to that seen in unirradiated skin (32.0±3.5 per 100 cells, $P>0.1$). In addition, most of the PCNA-positive cells were already localized in the basal layer of the epidermis 14 days after termination of UV exposure, a pattern seen in unirradiated mouse skin (Figure 2). Thus, both H&E and PCNA expression data indicate that after discontinuation of UV irradiation, epidermal hyperplasia subsides, at least in part, because of a gradual decrease in the number of proliferating keratinocytes.

Apoptosis and terminal differentiation To determine whether the decreased epidermal hyperplasia seen in the mouse skin after discontinuation of UV irradiation was mediated by apoptotic death or terminal differentiation followed by keratinocyte desquamation, we analysed the skin of mice at various time points after discontinuation of UV exposure for the presence of TUNEL-positive cells and expression of keratin 10 and loricrin. TUNEL assay data indicated the absence of TUNEL-positive cells in mouse skin irradiated with UV light for 8 weeks. This was expected because we showed previously that chronic UV irradiation results in apoptosis resistance (Ouhtit *et al*., 2000a). However, TUNEL-positive cells were also absent in the epidermis of mice at all time points after UV irradiation discontinuation (data not shown), suggesting that decreased epidermal hyperplasia was not due to apoptosis. Further, expression patterns of the keratinocyte terminal differentiation markers keratin 10 and loricrin were similar in nonirradiated skin and in irradiated skin after discontinuation of UV irradiation. Keratin 10 expression was observed throughout the epidermis but not in the basal cell layer (Figure 3). The expression of loricrin was localized in the upper layer of the epidermis (Figure 3). Denucleated cells in the upper epidermis expressed the highest levels of keratin 10 and loricrin, which is quite similar to the pattern seen in unirradiated epidermis (Figure 3). Finally, we observed an increase in the thickness of the stratum corneum layer, which was particularly evident at weeks 2 (Figures 2 and 3) and 4 (data not shown) after UV radiation discontinuation. Together with the PCNA expression data, these results suggest that epidermal hyperplasia induced by chronic UV irradiation occurs owing to an increase in the number of proliferating cells rather than because of a severe inhibition of terminal differentiation. Discontinuation of UV exposure causes a decrease in the number of proliferating cells, a gradual elimination of excess keratinocytes owing to desquamation, and the re-establishment of normal epidermal thickness.

Expression of p53 after discontinuation of UV irradiation

We showed previously that UV-irradiated mouse skin expresses high levels of p53 protein (Ouhtit *et al*., 2000a). To determine whether discontinuation of UV irradiation affects expression of p53 protein, we conducted the immunohistochemical analysis of mice skin for p53 expression at various time points after discontinuation of UV radiation. Strong nuclear immunoreactivity with the CM5 anti-p53 antibody, attributed to the expression of mutant p53 protein, was observed in the epidermis of the mice at all time points. In particular, 1 day after discontinuation of UV irradiation, keratinocytes in both the upper and basal layers of the epidermis expressed high levels of p53 (Figure 4). In addition, even though there was a decrease in epidermal hyperplasia at day 14 after UV discontinuation and thereafter, p53-positive cells were present in all layers of epidermis, including basal cells, transient proliferating

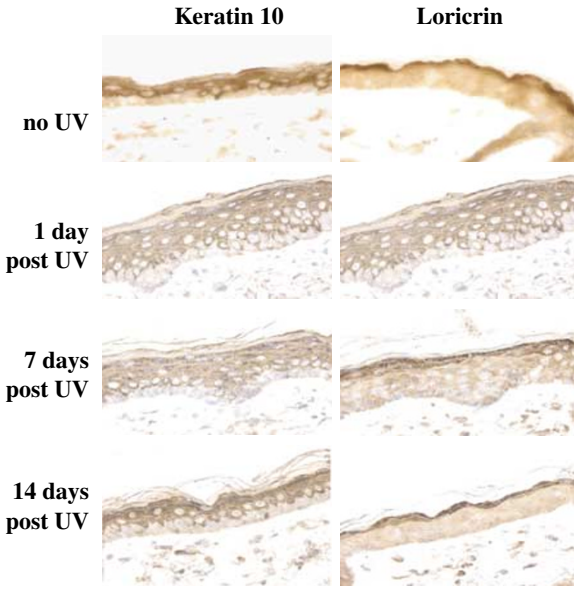

Figure 3 Immunohistochemical analysis for keratin 10 and loricrin protein expression in nonirradiated skin and in skin taken 1, 7, and 14 days after discontinuation of UV treatment

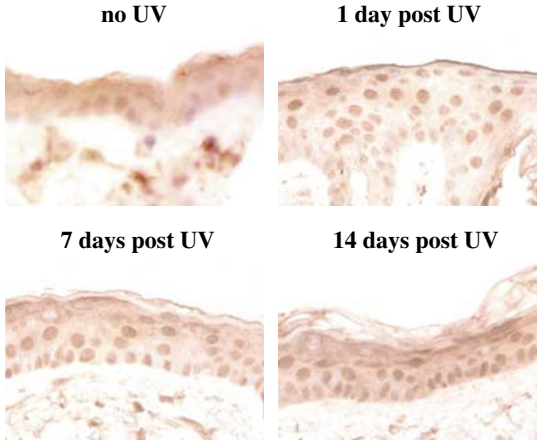

Figure 4 Immunohistochemical analysis for p53 protein expression in unirradiated skin and in skin taken 1, 7, and 14 days after discontinuation of UV treatment

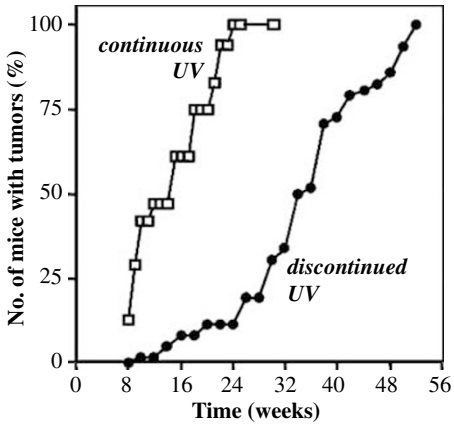

Figure 5 Time course of tumor development in SKH1-hr mice exposed to continuous UV radiation (open symbols) or discontinuous UV radiation (closed symbols). For continuous UV treatment, each point represents data from 20 mice; for discontinuous UV treatment, each point represents data from 50 mice. Lesions >3.0 mm were counted as established tumors in both the continuous UV irradiation experiment (data reproduced from Ouhtit *et al*., 2000a) and the discontinuous UV experiment

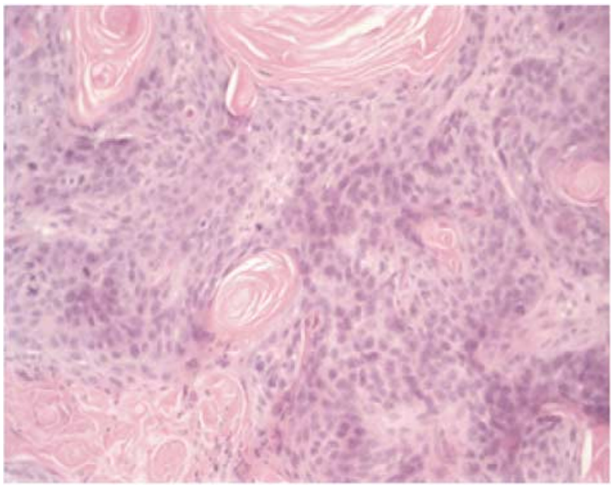

Figure 6 H&E staining of a typical SCC, which developed in an SKH-hr1 mouse after discontinuation of UV treatment

cells, and cells undergoing terminal differentiation (Figure 4), suggesting that some keratinocytes with mutant p53 can still undergo terminal differentiation. In contrast, no p53 expression was observed in unirradiated mouse skin (Figure 4).

Development of skin tumors after discontinuation of UV treatment

To determine whether discontinuation of UV irradiation after 8 weeks of exposure abrogates or reduces the incidence of skin tumors, we monitored 50 SKH-hr1 mice for tumor development. Even though UV irradiation was discontinued, all the mice developed skin tumors; however, the time to 100% tumor incidence was significantly delayed. A small lesion (<2 mm in diameter) was first observed in a mouse at 2 weeks after discontinuation of UV irradiation, and by week 52, all mice developed skin tumors (Figure 5). In contrast, none of the 10 nonirradiated mice developed skin tumors. The combined tumorigenicity data for continuous (Ouhtit *et al*., 2000a) and discontinuous UV irradiation experiments show differences in the kinetics of tumor development (Figure 5). It should be noted that the tumorigenicity data were obtained several years apart as part of different studies, and a direct comparison is only shown for reference. Nevertheless, the time needed for 50% of mice to develop tumors under the continuous irradiation protocol was about 14 weeks, compared to 34 weeks under the UV radiation discontinuation protocol. In addition, while 100% of mice continually irradiated with UV light developed skin tumors of at least 3 mm in diameter by week 24, it took significantly longer (52 weeks) for tumors to develop in the UV radiation discontinuation experiment. In both protocols, the mean number of 3-mm or larger tumors was approximately equal: 3.0 ± 1.1 and 3.3 ± 0.4 per mouse for continuous and discontinuous UV protocols, respectively. Finally, analogous to the continuous UV irradiation protocol, most of the tumors that developed after UV radiation discontinuation were diagnosed as SCC (Figure 6). In addition, analysis of 21 SCCs that developed in mice after discontinuation of UV irradiation revealed that 100% of the tumors carried $p53^{V154A/R155C}$ mutant alleles, 76% carried $p53^{H175H/H176Y}$ mutants, and 24 and 19% carried $p53^{R270C}$ and $p53^{R275C}$ mutants, respectively (Table 1).

Discussion

We showed previously that UV-induced *p53* mutations arise very early in mice, well before the onset of skin cancer (Ananthaswamy *et al*., 1997; Ouhtit *et al*., 2000a), which suggests that this event may initiate the process of multistage carcinogenesis. In hairless mouse skin, UV-induced *p53* mutations could be detected by AS-PCR as early as 1 week after the first UV radiation exposure, with 80–90% of animals incurring *p53* mutations after 8 weeks of UV treatment (Ouhtit *et al*., 2000a). Clones of keratinocytes carrying mutant *p53* have also been detected in mouse skin within 2–3 weeks after UV treatment (Berg *et al*., 1996; Rebel *et al*., 2001; Remenyik *et al*., 2003). In this study, we investigated the fate of *p53* mutations and the development of skin cancer after discontinuation of UV treatment. We found that discontinuation of UV irradiation before the onset of skin tumor development did not drastically decrease the overall frequency of *p53* mutations. While $p53^{V154A/R155C}$ and $p53^{H175H/H176Y}$ mutations persisted in keratinocytes at or near 100% of the 8-week incidence for as long as 22 weeks after UV radiation discontinuation, the incidence of $p53^{R270C}$ mutations decreased by 45% at 22 weeks post-UV irradiation (Table 1), which was not considered statis-

tically significant ($P = 0.72$). Previous studies have shown that the number and size of p53-positive cell clones in mouse epidermis decrease within 2 weeks after the discontinuation of chronic UV treatment (Berg *et al*., 1996; Remenyik *et al*., 2003). This acute regression phase may be followed by a significantly slower second phase (Berg *et al*., 1996). Berg *et al*. (1996) found that more than 50% of p53-positive patches induced in Skh-hr1 mouse skin by exposure to 39 kJ/m^2 of total UV radiation over a period of 30 days disappeared within the first 2 weeks after discontinuation of UV irradiation. The rate of disappearance of p53-positive patches was significantly higher (85%) when the total dose of UV radiation was decreased to 22 kJ/m^2 (Berg *et al*., 1996). However, in both UV regimens, p53-positive clones were still detectable for as long as 8 weeks after the last UV radiation exposure (Berg *et al*., 1996). Similarly, Remenyik *et al*. (2003) found that approximately 50% of p53-positive keratinocyte clones in C57Bl/6 mouse skin disappeared within 2 weeks after discontinuation of UV treatment (37 or 47 kJ/m^2 total UV). In our experiments, there was a slight decrease in the incidence of $p53^{R270C}$ mutations at 22 weeks after UV radiation discontinuation, but it was not statistically significant. However, our data do not dispute the existence of the acute regression phase because the highly sensitive AS-PCR method we used to detect mutations reveals the presence of *p53* mutations regardless of the size or number of mutant clones. Thus, it is possible that even though *p53* mutations were present in 100% of UV-irradiated mice at 22 weeks after discontinuation of UV irradiation, the number of *p53* mutant clones or colonies may have decreased during the same period. We did not, however, determine the number or size of mutant *p53* clones in our experiments.

Remenyik *et al*. (2003) demonstrated that regression of precancerous p53-positive clones occurs owing to mechanisms other than antigen-specific immunity, since it proceeds with similar kinetics in the skin of immune-deficient $Rag1^{-/-}$ mice and their wild-type counterparts. In addition, they found that precancerous p53-positive cells have normal morphology and that patches of these cells are not infiltrated with lymphocytes or any other immune cells (Remenyik *et al*., 2003). Two other possible mechanisms for elimination of initiated keratinocytes are apoptotic death and normal cell turnover. Our histologic and immunohistochemical studies indicate that chronic UV irradiation for 8 weeks induced epidermal hyperplasia and that discontinuation of UV irradiation resulted in a decrease in epidermal hyperplasia. This decrease in epidermal hyperplasia after UV irradiation discontinuation could have occurred through keratinocyte differentiation and desquamation rather than apoptotic elimination. This conclusion is supported by the fact that (1) the stratum corneum increased in thickness during the first 14 days after discontinuation of UV irradiation (Figures 2 and 3); (2) TUNEL-positive keratinocytes were absent after UV irradiation discontinuation; and (3) some p53-positive terminally differentiated keratinocytes were localized in the upper layers of epidermis (Figure 4).

Despite discontinuation of UV irradiation, all mice developed skin tumors, but the kinetics of tumor development in the current study were quite different from those in the continuous UV irradiation experiment. For example, the time required for 50% of mice to contract tumors (t_{50}) was 34 weeks in the present discontinuous UV protocol versus 14 weeks in the continuous UV study. Secondly, the time required for tumor development in 100% of mice increased from 24 weeks in the continuous UV irradiation experiment to 52 weeks in the UV discontinuation experiment. Analogous to this finding, de Gruijl and van der Leun (1991) showed that limiting the duration of UV treatment from continuous to 35 (53 kJ/m^2 total UV dose) or 19 days (29 kJ/m^2 total UV dose) delayed t_{50} from 20 weeks to 40 and 92 weeks, respectively. de Gruijl and van der Leun (1991) have further adapted a mathematical model that relates tumor occurrence to the daily dose of UV radiation and the time needed for animals to develop tumors, thus separating UV- and time-dependent phases of skin carcinogenesis. The 60-kJ/m^2 total UV dose administered to mice over a period of 8 weeks in our experiments yielded a t_{50} value of 34 weeks, which correlates with the results of de Gruijl and van der Leun (1991).

There is evidence to indicate that acute UV exposure induces the expansion of *p53*-mutant cells by stimulating their proliferation while inducing apoptosis in normal keratinocytes (Ziegler *et al*., 1994; Zhang *et al*., 2001; Mudgil *et al*., 2003). However, under continuous UV irradiation, the selective proliferative advantage of mutant cells over normal cells could contribute to the clonal expansion of *p53*-mutant cells because continuous UV treatment quickly induces apoptosis resistance and stimulates hyperproliferation as an adaptive response (Ouhtit *et al*., 2000a). The selective retention of some mutant *p53* alleles seen in our experiments suggests that when a constant supply of UV-induced proliferative stimuli is interrupted by UV irradiation discontinuation, a population of keratinocytes containing specific *p53* mutations still undergoes terminal differentiation, while other keratinocytes with different *p53* mutations survive and proliferate. This proliferative advantage may be due to the increased expression of certain growth factors or growth-related cytokines or their cognate receptors. Interestingly, different human tumor-associated p53 mutants have been shown to exhibit oncogenic features, such as promoting cell growth and tumorigenicity, when compared to *p53*-null phenotypes (Dittmer *et al*., 1993; Sigal and Rotter, 2000). Some p53 mutants have also been shown to transactivate promoters of epidermal growth factor receptor and basic fibroblast growth factor genes and potentiate the expression of vascular endothelial growth factor (Kieser *et al*., 1994; Ueba *et al*., 1994; Ludes-Meyers *et al*., 1996). The suggestion that hyperproliferative and apoptosis-resistant phenotypes may both result from mutant p53's 'gain of function' is supported by the finding that resistance to drug-induced apoptosis in the presence of various tumor-associated p53 mutants relies on their transcriptional capacity and on potentiation of the c-*myc*

expression (Matas *et al*., 2001). Although it remains to be proven whether UV-induced p53 mutant proteins produce similar 'gain of function' phenotypes, our previous studies have shown that mutant p53 protein is localized in the cell nucleus and phosphorylated at critical N- and C-terminal residues in all the UV-induced mouse skin tumors (Melnikova *et al*., 2003), potentially fulfilling some of the requirements for being transcriptionally active 'gain of function' mutants (Lin *et al*., 1995; Lanyi *et al*., 1998; Matas *et al*., 2001).

Even though skin tumors are clonal in origin, most of them harbor multiple mutations in the *p53* gene (Kanjilal *et al*., 1993). The presence of multiple *p53* mutations has been reported in human and UV-induced mouse skin cancers and head and neck cancers (Chung *et al*., 1993; Kanjilal *et al*., 1993, 1995). In addition, we demonstrated previously that UV-irradiated mouse skin also harbors multiple *p53* mutations (Ananthaswamy *et al*., 1997; Ouhtit *et al*., 2000a). It is possible that a cell containing one *p53* mutation acquire secondary and tertiary mutations in the same or different allele due to repeated exposure to UVB. Nonetheless, the data presented herein suggest that different p53 mutants confer different degrees of survival and/or proliferative advantage to keratinocyte progenitors in the absence of further UV exposure. Supporting this assumption, the retention of *p53* mutations in the skin after discontinuation of UV irradiation correlated with their high incidence in skin tumors (Table 1). Conversely, $p53^{R275C}$ mutants were detected in eight of nine (90%) skin specimens at 22 weeks after UV radiation discontinuation, but only four of 21 (19%) tumors carried $p53^{R275C}$ mutant alleles, which was considered statistically significant ($P = 0.04$). However, the approximate mathematical rate constant for the process of mutation disappearance is similarly high for $p53^{R275C}$ and $p53^{R270C}$ mutants, suggesting that a decrease in $p53^{R275C}$ incidence in lesion-free skin could be observed if monitored for a longer period of time.

In summary, our results indicate that mice exposed to chronic UV radiation retain a population of epidermal keratinocytes containing *p53*-mutant alleles long after discontinuation of UV treatment. Correlation between the dynamics of retention of mutant *p53* alleles in the skin after discontinuation of UV radiation and their frequency in tumors suggests that different UV-induced *p53* mutants may provide keratinocyte progenitors with different degrees of survival and/or proliferation advantages in the absence of further UV exposure. Finally, our results show that despite discontinuation after 8 weeks, UV irradiation results in 100% skin tumor incidence, although the kinetics of tumor occurrence is greatly delayed. In terms of human relevance, our results suggest that early life exposure to UV may introduce *p53* gene mutations in epidermal keratinocytes as well as keratinocyte progenitors. While some *p53*-mutated keratinocytes may be eliminated via differentiation and epidermal desquamation, others, perhaps the initiated progenitor cells, may still persist and eventually give rise to skin tumors even in the absence of further UV exposure. However, this process will be greatly promoted by UV exposure. Thus, the cancer development can be delayed but not abrogated upon further avoidance of exposure to UV radiation.

Materials and methods

Mice

Female, 8-week-old SKH-hr1 mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in cages in a room with controlled temperature and humidity and an alternating 12-h light and dark cycle. The room was lit with yellow fluorescent lamps in ceiling fixtures with plastic diffusers to eliminate all ambient UV radiation. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with current regulations and standards of the National Institutes of Health. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee. The mice were fed *ad libitum* with a commercial diet and water.

UV irradiation

UV irradiation was performed as described previously (Ouhtit *et al*., 2000a, b). Briefly, five mice at a time were placed in a standard cage with Plexiglas dividers and exposed three times per week to 2.5 kJ/m^2 of UVB radiation (290–320 nm) from a bank of six Kodacel-filtered FS40 lamps (Westinghouse Electric Corp., Bloomfield, NJ, USA) for 8 weeks. The Kodacel filter (127-μm thick TA422 cellulose triacetate film; Eastman Kodak, Rochester, NY, USA) removed all UV wavelengths below 290 nm. The fluence rate was monitored weekly, and any decrease in fluence was compensated for by a corresponding increase in irradiation time.

Post-UV procedures and observations

UV irradiation was discontinued after 8 weeks of treatment, and the mice were randomly divided into two groups. The first group of 50 mice was monitored for skin tumor development. The second group of 80 mice was divided into eight subgroups, and the 10 mice in each subgroup were killed at 1, 4, 7, 10, and 14 days and 6, 10, and 22 weeks after discontinuation of UV exposure. The skin samples were collected and analysed for *p53* mutations. As a control, a group of 10 unirradiated mice were monitored for tumor development. In addition, two groups of five unirradiated mice were each killed at 1 day and 22 weeks post-UV exposure, and their skin was used as controls in *p53* mutation and other assays.

Isolation of skin samples

A 2×2 cm area of dorsal skin was excised from each mouse that was killed and cut into two pieces. One piece was immediately fixed in 4% buffered formaldehyde for paraffin-embedded sectioning. The other piece was floated dermis-side down in buffered 0.5 M ethylenediaminetetraacetic acid solution, pH 7.4, for 1 h at 37°C to separate the epidermis from the dermis. The epidermal tissues were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Tumors and pathologic analysis

All 50 mice in the group being monitored for skin tumor development were visually examined three times per week. Lesions larger than 3 mm in diameter that persisted for more

than 2 weeks were considered skin tumors and recorded for each mouse. When the tumors reached approximately 10 mm in diameter, the mice were euthanized with CO_2 gas, and the tumors were excised and fixed in 4% formalin and then embedded in paraffin. Tissue sections were cut, stained with H&E, and examined for histopathologic characteristics by a certified veterinary pathologist.

Allele-specific polymerase chain reaction

Epidermal or tumor DNA was isolated using the phenol–chloroform method and analysed using AS-PCR for CC→TT mutations at codons 154/155 and 175/176 in exon 5 and for C→T mutations at codons 270 and 275 in exon 8 of the *p53* gene, as described previously (Ananthaswamy *et al*., 1997; Hill *et al*., 1999; Ouhtit *et al*., 2000a). The mutant-specific forward primers used were 5′-CCTCCAGCTGGGAGCCGTGCTT-3′ and 5′-TCGTGAGACGCTGCCCCCATT-3′ for mutations at codons 154/155 and 175/176, respectively. The reverse primer used for amplification of codons 154/155 and 175/176 was 5′-GCCTGCGTACCTCTCTTTGC-3′. C→T mutations at codons 270 and 275 were detected using forward mutant-specific primers 5′-GGACGGGACAGCTTTGAGGTTT-3′ and 5′-GTGTTTGTGCCTGCCT-3′, respectively. The reverse primer used to detect both mutations was 5′-GCCTGCGTA CCTCTCTTTGC-3′. PCR reactions were performed with 360 ng of genomic DNA, 2 μM of each primer labeled with 3000 Ci/nmol [γ-^{32}P]ATP, 2.5 μM dNTPs, 0.2 U *Taq* DNA polymerase (Promega) in a buffer containing 1 mM $MgCl_2$, in a final volume of 25 μl. Following initial denaturation step (94°C, 4 min), 35 cycles (denaturation at 94°C, 1 min; annealing for 1 min at 58°C (codons 154–155 mutations), 57°C (codons 175–176), 69°C (codon 270) or 65°C (codon 275); and extension (72°C, 1 min)) were carried on a DNA thermal cycler (Perkin-Elmer/Cetus). An aliquot (7 μl) of the PCR product was mixed with sequencing stop solution (3 μl) and electrophoresed on 8% (codons 154 and 175) or 6% (codons 270 and 275) polyacrylamide gel at 150 V for 30 min. The gel was dried and visualized on autoradiographic film.

Immunohistochemistry

Sections (5 μm) of paraffin-embedded tissues were analysed for the expression of p53, PCNA (a marker of hyperplasia), keratin 10, and loricrin (markers of differentiation for epidermal keratinocytes) using immunohistochemical analysis, as described previously (Hill *et al*., 1999; Ouhtit *et al*., 2000a, b). After deparaffinization, the sections were treated with target retrieval solution (DAKO, Carpinteria, CA, USA), washed three times with phosphate-buffered saline (PBS), and incubated in H_2O_2/methanol/PBS solution (1 : 50 : 50) for 15 min to block endogenous peroxidase activity. After three washes in PBS with 0.5% Tween-20, the sections were preincubated for 10 min in 10% normal goat serum in PBS and then incubated overnight at 4°C with rabbit polyclonal anti-mouse p53 antibody (cat. # NCLp53-CM5; NovoCastra Laboratories Ltd, Newcastle upon Tyne, UK; dilution factor 1 : 200), rabbit polyclonal PCNA antibody (clone FL-261, cat. # sc-7907; Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution factor 1 : 100), or rabbit polyclonal antibodies against mouse keratin 10 or loricrin (cat. # PRB-159P and PRB-145P, respectively; Covance, Berkley, CA, USA; dilution factor 1 : 500). After three washes in PBS plus 0.5% Tween-20, the sections were incubated for 1 h at room temperature in corresponding horseradish peroxidase-linked secondary antibody solution (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). After a wash in PBS, the staining was performed using the Vectastain Elite ABC kit with diaminobenzidine as the chromagen, as recommended by the manufacturer. Counterstaining was performed with hematoxylin. As a negative control, tissue sections were stained with secondary antibody only.

Statistical analysis

Immunohistochemical data were analysed with unpaired Student's *t*-test and *p53* mutation data were analysed by two-sided Fisher's Exact test. *P*-values of <0.05 were considered to be statistically significant.

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