Epidermal stem and progenitor cells in murine epidermis accumulate UV damage despite NER proficiency

Joanne G.W.Nijhof1,*, Carina van Pelt1, Adriaan A.Mulder1, David L.Mitchell3, Leon H.F.Mullenders2 and Frank R.de Gruijl1

1Departments of Dermatology and 2Toxicogenetics, Leiden University Medical Center, PO Box 9600, 2300 RC, Leiden, The Netherlands and 3M.D. Anderson Cancer Center, Smithville, TX, USA

*To whom correspondence should be addressed. Tel: +31 715269363; Fax: +31 715268286; Email: J.G.W.Nijhof@LUMC.nl

Ultraviolet (UV) radiation induces cyclobutane pyrimidine dimers (CPDs) and (6–4) photoproducts (6–4PPs) in DNA, which through gene mutations (e.g. in P53) may lead to skin carcinogenesis. Upon chronic low-level UV exposure, certain basal cells in mouse epidermis were reported to accumulate CPDs. These observations raised questions on whether these cells were fully DNA-repair deficient, and whether they were stem or progenitor cells, as suggested by their long residence time. We found that CPD-retaining basal cells (CRBCs) in SKH-1 hairless mice were repair proficient as accumulation of (6–4)IP, which is a hallmark for complete nucleotide excision repair-deficiency in rodents, was not observed. Accumulation of 6–4PP as well as CPD did, however, occur in basal cells in the epidermis of DNA repair-deficient Xpc−/− mice. Chronic UV exposure of DDB2 transgenic mice and DDB2 knockout mice revealed that the occurrence of CRBCs was inversely correlated with DDB2-expression, indicating that a boost in DNA repair lowered CPD accumulation. Stem cells are quiescent cells and can be identified as 5-bromo-2′-deoxyuridine-label retaining cells (BrdU-LRCs). Induction of BrdU-LRCs followed by chronic UV irradiation showed that all BrdU-label retaining stem cells were also CPD-retaining cells. As most CRBCs were not BrdU-labeled we surmised that these cells must include BrdU-negative stem cells and early progenitor cells. In confirmation of the latter, we found that CRBCs occurred among MTS24+-hair follicle progenitor cells. These findings provide the first evidence that epidermal stem and progenitor cells are prone to the accumulation of UV-induced DNA-damage and can be a prominent target in skin carcinogenesis.

Introduction

Skin cancer is the most common type of cancer in fair-skinned people; in the past decades, the total number of skin cancer patients has been dramatically increasing. Malignancies of the skin can be divided into two main categories: melanoma and non-melanoma skin cancer (NMSC). NMSC include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Epidemiologic studies have indicated that cutaneous malignant melanoma and BCC are associated with sunburns, particularly during childhood, whereas SCC show association with a history of cumulative lifetime ultraviolet B (UVB)-exposure (1,2).

Absorption of UVB radiation by DNA induces primarily cyclobutane pyrimidine dimers (CPDs) and (6–4) pyrimidine pirimidone photoproducts [(6–4)PPs] (3,4). Evidence suggests that these DNA photolesions may lead among others to C→T and CC→TT transitions at adjacent pyrimidine bases, which are considered ‘UV-signature’ mutations (5,6). Analysis of skin carcinoma have shown UVB-induced mutations in the p53 tumor suppressor gene, which play an important role in skin cancer development (6,7). The frequency of UV-signature mutations in the p53 gene ranges from 50 to 90% in human BCC and SCC, and up to 100% in UV-induced murine skin cancers (6–8).

Cells are equipped with several DNA repair systems to maintain DNA integrity. Nucleotide excision repair (NER) is responsible for the repair of DNA lesions with significant helix distortions, such as CPDs and (6–4)PPs (9,10). NER can be divided into two pathways: global genome-NER (GG-NER), which is responsible for the repair of photolesions throughout the genome, and transcription-coupled repair (TCR), which is specifically dedicated to the repair of transcription-blocking lesions within the transcribed DNA (9). In GG-NER the xeroderma pigmentosum C (XPC) hHR23B heterodimer recognizes the DNA lesion (11,12). TCR is triggered by RNA polymerases that are stalled at DNA lesions in the transcribed DNA strands. Because of the important role of NER in DNA damage recognition and repair, it is not surprising that humans defective in NER, e.g. because of complete absence or a mutation in the XPC protein, exhibit extreme photosensitivity and occurrences of skin cancer in sun-exposed areas of the skin at very early age (13).

TCR acts effectively on CPD and (6–4)PP in both human and murine skin. In human skin, GG-NER also repairs both the CPDs and the (6–4)PPs. However, the (6–4)PPs are repaired much faster in the global genome than the CPDs due to differences in affinity of the XPC-hHR23B damage sensor (10). For CPD repair, the presence of the UV-damaged DNA-binding protein (UV-DDB) is essential. The UV-DDB protein, a heterodimer complex consisting of two subunits, assists the XPC-hHR23B complex in recognition of CPDs, and also (6–4)PPs (14–16). In contrast to human cells, rodent epidermal cells virtually lack GG-NER of CPDs due to a very low expression of DDB2, a subunit of the UV-DDB heterodimer complex (14,15). Rodents cells do, however, adequately repair (6–4)PPs by GG-NER. Expression of human DDB2 in hamster cells restored the activity of UV-DDB and the repair of CPDs by GG-NER and these cells were less...
prone to UV-induced mutagenesis (14). In rodent epidermis, the vast majority of the CPDs will be removed from the epidermis through cell division, epidermal cell turnover or apoptosis. Interestingly, Mitchell et al. (17) reported the accumulation of CPDs in some isolated basal cells in the mouse epidermis after chronic low-level UVB exposure, indicating that in some epidermal cells UVB-induced DNA damage is retained. Such CPD-retaining basal cells (CRBCs) were still observed 40 days after the last UV exposure. These data suggest that CRBCs might be long-residing quiescent cells, possibly stem cells.

Epidermal stem cells, which form the basis of the continuous self-renewal of the epidermis, are present in the interfollicular epidermis, sebaceous glands and hair follicles (18–20). Generally, epidermal stem cells are rarely dividing and quiescent cells, but in periods of skin development or wound healing they are activated and display a strong capacity to proliferate (21–23).

Within the hematopoietic stem cell (HSC) system, the multipotent HSC give rise to new HSC, which retain self-renewal capacity, and distinct populations of progenitor cells, which show a hierarchy in commitment to differentiate (24,25). It seems likely that such a hierarchy also exist in epidermal progenitor cells: upon asymmetric cell division, epidermal stem cells generate quiescent daughter stem cells and more frequently dividing progenitor cells, called transit amplifying cells, that are committed along a differentiation pathway and are subsequently lost through terminal differentiation (22,26).

Epidermal stem cells can be distinguished from transit amplifying cells by their ability to incorporate and retain [3H]thymidine or 5-bromo-2′-deoxyuridine (BrdU) over a long period of time. Therefore, epidermal stem cells can be identified as label-retaining cells (LRCs) (22,23,27–30). Additionally, mouse epidermal stem cells can be characterized by increased expression of markers such as α6-integrin (in combination with low levels of CD71), keratin 15, and CD34 (31–34). Notably, we reported that CD34 expression was not observed in hairless SKH-1 mice, the mouse strain generally used for UV irradiation experiments (35) (C. Trempos reported sparse and infrequent staining in SKH-1 hair follicles. Personal communication). We showed that the cell-surface marker MTS24, previously characterized as a marker for thymic epithelial precursor cells (36,37), was found on hair follicle progenitor cells of various mouse strains, including SKH-1 mice (35).

The aim of the present study was to characterize CRBCs. First, we established whether or not the CRBCs are completely GG-NER-deficient. To this end, we determined whether CRBCs would also retain (6-4)PPs, which is a hallmark for complete NER-deficiency in rodents. Second, we investigated whether the occurrence of CRBCs was negatively correlated with DDB2-expression by using DDB2 transgenic mice and DDB2 knockout mice. Third, we assessed whether CRBCs coincided with epidermal stem or progenitor cells, using BrdU-label retention and MTS24 labeling as markers.

Materials and methods

Experimental mice
SKH-1 hairless mice were purchased at 6–8 weeks of age from Charles River (Maastricht, The Netherlands). Xpc mutant mice (a kind gift of Dr E.C. Friedberg, Dallas, TX) have been generated as described previously (38) and bred into a SKH-1 hairless background. To study the role of DDB2 expression in CPD-repair, we used hairless DDB2 transgenic mice (with the DDB2 transgene under the control of human K14 promoter), in which DDB2 was expressed ectopically at elevated levels, and hairless DDB2 knockout mice (DDB2−/−/C24), in which the expression of the DDB2 gene was disrupted. These mice were generated as described elsewhere (15). Mice were kept in the animal facility of the Leiden University Medical Centre and all experiments were performed in accordance with legislation and approval of the university’s ethical committee.

Animal housing
For chronic exposure experiments animals were housed individually in Macrolon type 1 cages (Techniplast, Bugguggiate, Italy) under a 12 h light–dark cycle at 23°C/60% humidity. The animal room was illuminated with yellow fluorescent tubes (Philips TL40W/16, Eindhoven, The Netherlands); these lamps did not emit any measurable UV radiation. Standard chow and drinking water were available ad libitum but cage enrichment was absent to prevent shielding of the animal from UV exposure.

Chronic UV exposure experiments and BrdU labeling
For chronic exposure experiments Philips TL-12/40W tubes (Philips, Eindhoven, The Netherlands) 56% output in UVB and 44% output in UVA were used. An emission spectrum of the Philips TL-12 tube is shown in the Supplementary Data. The exact exposure dose was fine-tuned by the use of electric dimmers that controlled the output of the lamps. Under these lamps the minimal erythema dose (MED) of hairless SKH-1 mice, DDB2 transgenic mice, DDB2 knockout mice and Xpc mice was ~500 J/m² (15,39,40). Every day between 12.30–12.45 h hairless SKH-1 mice, DDB2 transgenic mice and Xpc mice were exposed to a total dose of 70 J/m² (which equals a dose of 0.14 MED in these mouse strains) UV radiation for a period of 20 or 40 days. Because of reported promeness of NER-deficient mice to hyperplasia (41), we exposed Xpc mice to a lower daily dose of 50 J/m² (~0.15MED) for 20 days or 25 J/m² (~0.05 MED) UV radiation for a period of 40 days.

To generate BrdU-LRC, we used the protocol as described by Bickenbach and colleagues (28,30). Ten-day-old hairless SKH-1 mice were injected with 5-bromo-2′-deoxyuridine (BrdU) over a period of time (5 days). Respectively, at 1 week or 2 week after the last expression in CPD-repair, we used hairless DDB2 transgenic mice (with the DDB2 transgene under the control of human K14 promoter), in which DDB2 was expressed ectopically at elevated levels, and hairless DDB2 knockout mice (DDB2−/−/C24), in which the expression of the DDB2 gene was disrupted. These mice were generated as described elsewhere (15). Mice were kept in the animal facility of the Leiden University Medical Centre and all experiments were performed in accordance with legislation and approval of the university’s ethical committee.

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Tissue preparation
After CO₂ asphyxiation dorsal skin was directly embedded in tissue-tek O.C.T compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and snap frozen in liquid nitrogen. After CO₂ asphyxiation dorsal skin was directly embedded in tissue-tek O.C.T compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and kept in liquid nitrogen until use. After CO₂ asphyxiation dorsal skin was directly embedded in tissue-tek O.C.T compound (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen until use.

Antibodies
The following primary antibodies were used: mouse anti-CPD (clone KT5M5; Kamiya Biomedical Company, Seattle, USA), mouse anti-(6-4)PP (clone EP43; a kind gift from Dr T. Matsunaga, Kanazawa, Japan), fluorescent isocyanate conjugated (FITC)-conjugated anti-BrdU (DakoCytomation B.V, Heverlee, Belgium) and rat anti-MTS24 (a kind gift from Dr. Boyd, Melbourne, Australia). Cy3-conjugated rabbit anti-mouse (Jackson Immuno-Research Laboratories, Cambridge, UK), Cy3-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories, Cambridge, UK), FITC-conjugated goat anti-mouse (Serotec, Oxford, UK) and horseradish peroxidase-conjugated rabbit anti-mouse (DakoCytomation B.V, Heverlee, Belgium) were used as secondary antibodies. All antibodies were diluted in PBS/1% bovine serum albumin.

Immunohistochemistry
For CPD and (6-4)PP staining tissue sections were fixed in acetone for 10 min at room temperature (RT). Antigen retrieval was performed by boiling the tissue sections in 10 mM citrate buffer (pH 6.0) for 10 min. Upon antigen retrieval tissue sections were cooled down slowly until 37°C after which tissue sections were preincubated for 20 min at RT with 2% normal human serum (NHS, obtained from LUMC blood bank) to block non specific
antibody binding. Tissue sections were either incubated with mouse anti-CPD or with mouse anti-(6-4)PP antibody for 1 h at RT. To optimize the CPD and (6-4)PP staining, we exposed hairless SKH-1 mice to a single UV dose of 0.1, 0.2, 0.5, 1.0, 2.0 or 4 MED and incubated the irradiated tissues with different dilutions of the anti-CPD and the anti-(6-4)PP antibody. Optimal staining was defined as a clear signal without background staining. CPDs were detected at all single UV-doses and 1:500 was the lowest dilution that could be used to detect CPDs without introducing background staining. At a single dose of 0.1–0.2 MED only a few faintly stained cells were detected. In contrast, no (6-4)PP were detected below 0.5 MED, not even at a dilution of 1:500. Because we used an accumulated total UV dose of ~2 MED or more in our UV irradiation experiments, we decided to use the optimal antibody dilutions at this dose level: mouse anti-CPD antibody at a dilution of 1:4000 and mouse anti-(6-4)PP antibody at a dilution of 1:1500. After incubation with either mouse anti-CPD or mouse anti-(6-4)PP, tissues were incubated with Cy3-conjugated rabbit anti-mouse (1:500) for 1 h at RT. Tissue sections were counterstained with DAPI (Molecular Probes, Invitrogen, Breda, The Netherlands) solution (6 μg/ml in deionized water) for 3 min, mounted with VECTASHIELD (Brunswwich chemie, Amsterdam, The Netherlands) and coverslipped.

For BrdU-CPD double-labeling tissue sections were fixed in methanol/acetic acid (1:1) for 10 min. Antigen retrieval was now performed by incubating tissue sections with 10 mM NaOH for 3 min and subsequently, sections were treated with 0.02% pepsin (Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in 2 N HCl for 30 min. The reaction was stopped by adding 1 M TRIS solution for 5 min. We had to resort to this antigen retrieval technique because boiling in citrate buffer did not work in the BrdU/CPD double-labeling. This alternative, however, slightly reduced the brightness of the CPD-staining. Upon fixation and antigen retrieval tissue sections were preincubated with 5% NHS for 30 min to block non-specific antibody binding. Tissue sections were incubated with mouse anti-CPD (1:4000) for 1 h at RT, followed by incubation with rabbit anti-mouse Cy3 (1:750) for 1 h. Subsequently, tissue sections were incubated with FITC-conjugated anti-BrdU (1:50) overnight at 4°C, and counterstained.

For co-immunolabeling of MTS24 and CPD, tissues were fixed in acetone for 10 min and preincubated for 30 min with 5% NHS. Tissues were incubated with rat anti-mouse MTS24 (1:50) and followed by incubation with donkey anti-rat Cy3 (1:125). Antigen retrieval was then performed by incubating tissue sections with 0.1% pepsine (dissolved in 2 N HCl) and subsequently with 0.1 N sodium borate (dissolved in deionized water). Sections were then preincubated with 5% normal goat serum followed by overnight incubation with mouse anti-CPD (1:4000) at 4°C. The next day, sections were incubated with goat anti-mouse FITC (1:200) and counterstained.

Quantification and statistical analysis
For quantification of CPD-, (6-4)PP-, BrdU- and BrdU/CPD-retaining cells, five microscopic frames of epidermis (spanning 2.5 mm of epidermis or ~150 basal cells) were examined per section, and quantification was performed on three separate epidermal sections per mouse. The occurrence of CPD-, (6-4)PP, BrdU and BrdU/CPD-retaining cells were expressed as a percentage of the total number of basal cells within the examined stretch of the epidermis. Mean and standard error of the mean (SEM) were determined to calculate differences between groups. Statistical significance was determined using SPSS 12 (SPSS, Chicago, IL, USA) with Student’s t-tests for parametric testing of independent samples, or with Jonckheere’s k-sample test for non-parametric testing of independent samples.

Results
CPD accumulate in SKH-1 mice
Using immunofluorescence microscopy we determined the occurrence of CPD- and (6-4)PP-retaining cells in epidermis of NER-proficient SKH-1 mice after an exposure to a daily UV dose of 70 J/m² (0.14 MED) for a period of 20 or 40 days. The accumulation of CPD in SKH-1 mouse skin biopsied 24 h after a UV exposure period of 40 days (total accumulated UV dose of 2.8 kJ/m²) is shown in Figure 1A. Also, after UV exposure for a period of 20 days (total accumulated UV dose of 1.4 kJ/m²) CPD-retaining cells were observed (data not shown). Within the epidermis we distinguished cells that showed high levels of CPD lesions (referred as CPD-bright cells in immunofluorescence; Figure 1A, arrowheads) and cells that showed lower but detectable levels of CPD lesions (referred as CPD-dim cells; Figure 1A, arrow). Most of the suprabasal CPD-bright cells were located just above the basal layer, and these cells could also be part of the basal layer but positioned just out of the cross-sectional plane of the deepest basal cells. Also within the hair follicle CPD-retaining cells were found (Figure 1A, asterisk). Additionally, CPD-retaining cells were observed within the dermis (Figure 1A, crosshatch), indicating that fibroblasts did not get rid of the DNA damage through cell

Fig. 1. CPD accumulate in NER-proficient SKH-1 mice after chronic UV exposure. SKH-1 hairless mice were exposed to a daily dose of 70 J/m² (0.14 MED) for 20 or 40 days. Skin sections were labeled with mouse anti-CPD as described under Materials and methods. (A) SKH-1 mouse dorsal skin irradiated with 70 J/m² for 40 days showing CPD-bright cells (arrowheads), CPD-dim cells (arrow) and CPD-retaining cells within the hair follicle (asterisk). Also in the dermis CPD-retaining cells were observed (crosshatch). In this figure, as well as in the next figures, red staining of the stratum corneum is due to autofluorescence. Quantification of CPD-bright (black bars) and CPD-dim cells (open bars) in the epidermal basal layer (B) and epidermal suprabasal layer (C). Data are shown as mean ± SEM, n = 4 *p ≤ 0.05, **p ≤ 0.001. Scale bar: 25 μm.
No accumulation of (6-4)PP could be detected in wild-type SKH1 mice.

(6-4)PP only accumulate in NER-deficient Xpc mice
As we observed CPD-retaining cells in the epidermis of NER-proficient SKH-1 mice, but no accumulation of (6-4)PPs, we studied the accumulation of these DNA lesions in NER-deficient homozygous Xpc knockout (−/−) mice, as well as in their heterozygous Xpc (+/−) and wild-type Xpc(+/+) NER proficient littermates. To induce CPD-retaining cells, we exposed Xpc mice to a daily dose of 50 J/m² (~0.1 MED) for a period of 20 days, or to 25 J/m² (~0.05 MED) for a period of 40 days (total accumulated UV dose of 1.0 kJ/m²). At 24 h after the last UV dose, we observed accumulation of CPD in NER-proficient Xpc +/+ mice (Figure 2A) and Xpc +/+ mice (Figure 2B), and also in NER-deficient Xpc −/+ mice (Figure 2C). Similarly to SKH-1 mice, we distinguished CPD-bright cells (Figure 2, arrowheads) and CPD-dim cells (Figure 2, arrows) in all three genotypes. Again, in Xpc +/+ , +/− and −/− mice we observed CPD-retaining cells within the dermis (Figure 2, crosshatches) as well as in the hair follicle (Figure 2, asterisks, for Xpc −/− mice data not shown). The average frequency of CPD-bright cells in the basal layer of Xpc +/+ mice after exposure to a total dose 1.0 kJ/m² was 12% (SEM = 1.8, n = 4), which was similar to the frequency we observed in the basal layer of SKH-1 mice (Figure 1B). Interestingly, in Xpc +/+ and in Xpc −/+ mice we observed a reduced level of CPD-bright basal cells; i.e. 8.7% (SEM = 1.4, n = 4) and 4.7% (SEM = 1.0, n = 4), respectively, compared to Xpc +/+ mice. Although the UV dose was much below the MED of Xpc mice, we still observed significant hyperplasia in Xpc −/+ mice compared to Xpc +/+ mice (3.2 versus 2.6 cell layers, P < 0.05).

Similar to what was found in NER-proficient SKH-1 mice, no accumulation of UV-induced (6-4)PP was observed in NER-proficient Xpc +/+ mice and Xpc +/+ mice (data not shown). However, we did observe accumulation of (6-4)PP in NER-deficient Xpc −/+ mice: i.e. ~1.8% (SEM = 0.8, n = 4) of epidermal basal cells were (6-4)PP-bright cells (Figure 3A; arrowhead and Figure 3B). Within the suprabasal layer no accumulation of (6-4)PP was observed. The percentage of (6-4)PP-bright cells was the same for a UV irradiation regimen of 20 × 0.1 MED or 40 × 0.05 MED (Figure 3B). Similar to what was observed with CPD, the number of (6-4)PP-dim cells in the basal layer of Xpc −/+ mice was higher in the 20 day irradiation group compared to the 40 day irradiation group.

CPD accumulation is inversely correlated with DDB2 expression
To assess whether CRBCs correlate with DDB2 expression, we used DDB2 transgenic mice (DDB2 TG) in which the DDB2 transgene was under the control of human K14 promoter resulting in ectopically elevated expression of DDB2 in basal cells, and DDB2 knockout mice (DDB2 −/−), in which the expression of the DDB2 gene was disrupted (15). We compared DDB2 TG, DDB2 WT, DDB2 +/+ and DDB2 −/− mice after daily irradiation with a UV dose of 70 J/m² (0.14 MED) for a period of 40 days. Twenty-four hours after the last irradiation dorsal skin was obtained. As shown in Figure 4, an increase in the average frequency of accumulation was observed.
CPD-retaining bright cells correlated significantly \((P < 0.05)\) with a decrease in \(DDB2\) expression. No hyperplasia was observed (data not shown).

**BrdU-label retaining and MTS24-positive cells show CPD retention**

To investigate whether CRBCs have epidermal stem cell characteristics we injected ten day-old hairless SKH-1 mice repeatedly with BrdU and after a chase period of 4 weeks BrdU-labeled mice were exposed to \(40 \times 0.1\) MED/d or \(5 \times 0.3\) MED/d of UV radiation. After one week following \(40 \times 0.1\) MED or 2 weeks following \(5 \times 0.3\) MED (to remove CPDs in epidermal turnover and thus enrich for persistent CPD-bright cells), dorsal skin was obtained and the co-localization of BrdU and CPD was determined using immunofluorescence staining. At day 1 after the last injection with BrdU the common part of epidermal basal cells were labeled with BrdU (Figure 5A), indicating that the majority of the epidermal cell population replicated during the pulse-period. After a chase-period of 4 weeks, only a few cells (2–3% of the total number of basal cells) were BrdU-label retaining (Figure 5B), indicating that these cells had rarely divided. All the other cells that were labeled during the pulse period were lost or had lost their BrdU-label through frequent cell division as occurs in transit amplifying cells. After low-dose UV irradiation, we did not observe any signs of phototoxicity in the BrdU-labeled mice (data not shown). Both the \(5 \times 0.3\) MED (Figure 5C–E) and the \(40 \times 0.1\) MED (Figure 5F) UV exposure regimen resulted in BrdU-LRC/CPD double-positive cells (Figure 5F, arrowheads) and CPD single-positive cells (Figure 5F, arrows). No BrdU single-positive cells were found, i.e. all early labeled BrdU-positive cells showed CPD-accumulation. Quantification revealed that 8% (SEM = 1%, \(n = 6\)) of the total number of epidermal CPD-retaining cells were also BrdU-label retaining cells (LRCs). Considering our finding that \(~\)12% of the epidermal basal cells were CPD-retaining cells, this implies that around 1% of the basal cells were BrdU label-retaining stem cells, which is the same frequency as found in unirradiated BrdU-labeled controls (data not shown).

Recently, we found that the cell-surface marker MTS24, in combination with the basal cell marker alpha-6 integrin, identified a new population of murine hair follicle progenitor Fig. 3. (6-4)PP only accumulate in \(Xpc\) knockout mice after chronic UV exposure. \(Xpc\)-/– mice were exposed to a daily dose of \(50 \text{J/m}^2\) (~0.1 MED) for 20 days or \(25 \text{J/m}^2\) (~0.05 MED) for 40 days and 24 h after the last UV exposure skin was obtained. (6-4PP)-labeling was performed as described under Materials and methods. (A) Only in NER-deficient \(Xpc\)-/– mice accumulation of UV-induced (6-4)PP was observed in the epidermal basal layer [arrowhead showing (6-4)PP-bright cell]. (B) Quantification of (6-4)PP-bright (black bars) and (6-4)PP-dim (open bars) cells in the epidermal basal of \(Xpc\)-/– mice after chronic UV exposure. Data are shown as mean ± SEM, \(n = 4\). Scale bar: 25 µm.

Fig. 4. CPD accumulate in \(DDB2\) transgenic and in \(DDB2\) knockout mice after chronic UV exposure. Quantification of CPD-bright (black bars) and CPD-dim (open bars) cells after UV exposure of \(70 \text{J/m}^2\) for 40 days. CPD-labeling was performed as described under Materials and methods. We compared \(DDB2\) TG, \(DDB2\) WT, \(DDB2\) +/- and \(DDB2\) -/- mice to study whether the number of CPD-retaining cells was inversely correlated with the \(DDB2\)-allelic dose. Added trend line indicates that an increase in ectopic \(DDB2\)-expression (TG>WT>+/– > –/–) correlated significantly \((P < 0.05)\) with a decrease in the number of CPD-retaining bright cells. In \(DDB2\) TG mice CPD-accumulation is not completely prevented, indicating that \(DDB2\) expression is not the limiting factor in CPD repair in mice. Data are shown as mean ± SEM, \(n = 6\).
cells (35). Co-staining of MTS24 and CPDs revealed CPD-retaining cells present in the outer root sheath of the murine hair follicle (Figure 5G–H, arrowheads).

**Discussion**

As described by Mitchell *et al.* (17), and confirmed by data shown in this paper, CRBCs are found in SKH-1 mouse skin after chronic low-level UV exposure. In this study, we found that these CRBCs in SKH-1 mice are NER-proficient. Accumulation of CPDs was observed in SKH-1 mice, in *Xpc* mice (in *Xpc* +/+, +/− and −/− mice) and in DDB2 mice (in TG, WT +/- and −/−) upon chronic low-level UV exposure. However, the percentage of CRBCs varied between different mouse strains, ranging from ~12% in SKH-1 and *Xpc* +/- mice to ~9 and 5% in *Xpc* +/- and *Xpc* −/− mice, respectively. This reduction in number of CPD-retaining cells observed in *Xpc* +/- and *Xpc* −/− mice paralleled an increase in epidermal thickness. This increased hyperplasia may explain the reduced level of CPD-bright cells as the cell proliferation will lower the accumulation of CPDs.
The basal cells that accumulated CPDs over the longest period of time were apparently detected as ‘bright’ cells in immunofluorescent microscopy, but we also detected ‘dim’ cells. The latter cells had probably attained the damage as a daughter from a more damaged cell, or had not lived long enough to accumulate CPDs up to the level of a bright cell; in either case the dim cell was either a transit amplifying or differentiated cell. This was confirmed by the observation that the CPD-bright cells were clearly very persistent whereas the CPD-dim cells were more rapidly lost in epidermal turnover after discontinuation of UV exposure.

Upon chronic UV irradiation, we observed that ~5% of the epidermal basal cells were CPD-bright whereas only ~2% of the epidermal basal cells accumulated (6-4)PP in Xpc−/− mice. In theory, one would expect equal percentages as basal cells that retain CPD should also retain (6-4)PP. It has been reported that UVA radiation induces photosomerization of (6-4)PP into their Dewar isomers (43). As the anti-(6-4)PP antibody (clone 64M-2) is specific for (6-4)PPs and does not recognize Dewar isomers (44), photosomerization of (6-4)PP by UVA may explain the reduced number of (6-4)PP retaining basal cells reported in Xpc−/− mice. However, photosomerization of (6-4)PP into Dewar isomers only occurs substantially at high UVA-doses ranging from 5 up to 30 kJ/m2 (43). Because we have used a very low dose of UV (maximum dose of 2.8 kJ/m²), it is unlikely that photosomerization of (6-4)PP explains the lower number of (6-4)PP-bright cells compared to CPD-bright cells in Xpc−/− mice. It is more likely that the difference in CPD- and (6-4)PP-positive cells is mainly due to differences in sensitivity of detection of the two types of DNA lesions. We determined the percentage of CPD- and (6-4)PP-bright basal cells after a series of single UV doses in SKH-1 mouse skin. Using optimal antibody dilutions, we found that CPD-bright cells after a single UV dose of 2 MED account for ~65% of the epidermal basal cells whereas only 15% of the basal cells were (6-4)PP-bright. With higher levels of DNA damage (higher UV doses) the percentage of CRBCs did not increase, whereas the number of (6-4)PP-retaining cells did, indicating a saturation in the CPD detection. This discrepancy between CPD and (6-4)PP detection after a single UV dose is similar to what we found in Xpc−/− mice upon chronic UV irradiation, and is largely attributable to DNA damage levels and differences in sensitivity of the corresponding antibodies.

The poor recognition and repair of CPD in rodents is attributed to a low expression of DDB2. Our experiments with DDB2 transgenic and knockout mice demonstrated that an increase in DDB2 expression (TG>WT>+/−>−/−) correlated significantly with a decrease in the number of CPD-retaining bright cells. In line with Alekseev et al. (15) who found a moderate improvement of CPD repair in DDB2 TG mice, we found a notable effect but no complete prevention of CPD-accumulation. This result indicates that DDB2 expression is not the main limiting factor in CPD repair in mice.

Under normal conditions, the epidermal turnover of murine skin requires ~8–10 days (45). The finding that CPD-retaining bright cells remained present in epidermis that has been exposed to a low-dose of UV radiation for a long period of time, and therefore has been renewed several times, indicates that CPD retaining bright cells are likely quiescent and not prone to apoptosis.

The finding that CPD-retaining cells appeared to escape apoptosis, implies that CPD-retaining cells are TCR-proficient. TCR prevents a damaged cell from going into apoptosis, and enables resumption of normal cell function and cell cycle progression (46). As Xpc−/− mice are TCR proficient, the CPD- and (6-4)PP-accumulating cells apparently escape apoptosis, despite their genotoxic load. Evidently, these DNA damage-retaining cells are potential ‘hotspots’ of subsequent mutagenesis and carcinogenesis as they remain viable and capable to replicate (17).

Epidermal stem cells are characterized as rarely-dividing quiescent cells and they can, therefore, be identified as 3H]thymidine- or BrdU-LRC (22,23,27,28,30,47). The finding that all BrdU-LRC are CPD-retaining cells confirms the hypothesis that CPD-retaining cells encompass the rarely-dividing stem cells. We observed that ~1% of the basal cells were BrdU label-retaining stem cells. This percentage is in line with numbers reported by others (48,49). However, the majority of CPD-retaining cells were not BrdU-label retaining. As described by others (50,51) label-retaining cells probably encompass only a subset of the total epidermal stem cell population. Together with our finding that most, but not all, of the epidermal basal cells were BrdU-labeled at 1 day after the last injection with BrdU, we may not have labeled all epidermal stem cells. Therefore, the CRBCs we observed may well include non BrdU-labeled stem cells and early progenitor cells. Recently, we reported that the cell-surface marker MTS24, in combination with the basal cell marker alpha-6 integrin, characterized hair follicle progenitor cells in various mouse strains, including SKH-1 hairless mice (35). CPD-retaining cells appeared to be present within this population of MTS24+ cells, confirming the supposition that progenitor cells are among the CPD-retaining cells.

The first direct evidence that epidermal stem cells are important in skin carcinogenesis was provided by Morris et al. (48). Using a double isotope-emulsion autoradiography method with 3H]thymidine, to induce LRC, and [14C]BaP, to induce DNA-adducts, they showed that 3H]thymidine-retaining cells retained the largest number of carcinogen-DNA adducts. In the present study, we employed a similar approach to analyze UV-induced DNA damage, the putative major factor in human skin carcinogenesis. We used BrdU to induce LRC and UV radiation to induce DNA damage. Similar to what was found by Morris et al. (48) we found that BrdU-LRC also retained DNA damage, i.e. CPD. Hence, our data provide the first direct evidence that BrdU-label retaining stem cells retain UV-induced DNA damage, indicating that epidermal stem cells may play a prominent role in UV-induced skin carcinogenesis. As UV irradiation does not penetrate very deeply into the skin, keratinocytes in the deeper parts of the hair follicle. Hence, UV-induced tumor precursor cells are more likely derived from the interfollicular (stem) cells than from (stem) cells in hair follicles. In contrast, chemical carcinogenesis also targets deep-seated follicular keratinocytes. The finding by Morris et al. (48) that carcinogen-retaining cells were observed in low frequency (2%) among interfollicular basal cells but in higher frequency (5%) among follicular basal cells appears to underline this idea. Whether these CPD-retaining epidermal stem cells are indeed causally related to skin tumor formation needs to be further elucidated.
Mitchell et al. (17) showed that upon application of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate, CPD-retaining cells were forced to divide and p53-positive patches, which could be foci of potentially early oncogenic alterations, were formed.

As we found CRBCs to be reduced in number with increasing CPD repair capacity, by increasing DDB2 expression, we would not expect these cells to occur in human skin. Nevertheless, these cells have been observed in human epidermis obtained from patients with BCC or malignant melanoma (17). The apparent failure in CPD repair in these human epidermal basal cells, and subsequent CPD accumulation, clearly needs to be elucidated. The occurrence of CPD-retaining cells in human does indicate that these cells may also be relevant to human skin carcinogenesis.

In summary, our findings demonstrate that CRBCs in SKH-1 mice are NER-proficient in that they show no accumulation of (6-4)PPs. The latter photolysis does, however, accumulate in GG-NER deficient Xpc−/− mice. All BrdU-LRCs accumulate the UV-induced DNA damage, and stem cells therefore appear to be prominent targets in chronic low-dose UV carcinogenesis, as these cells remain viable and do not lose the ability to divide.

Supplementary data
Supplementary data are available at Carcinogenesis Online.

Acknowledgements
The authors would like to thank Dr T. Matsunaga, Kanazawa University, Kanazawa, Japan for providing the anti-(6-4)PP antibody and Dr R. Boyd, Monash University, Melbourne, Australia for providing the MTS24 antibody. This work was supported by a grant from the Dutch Cancer Society (JN, RUL 2002-2737).

Conflict of Interest Statement: None declared.

References

Received September 4, 2006; revised October 17, 2006; accepted October 24, 2006