LETTERS

# Topical drug rescue strategy and skin protection based on the role of *Mc1r* in UV-induced tanning

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**Ultraviolet-light (UV)-induced tanning is defective in numerous 'fair-skinned' individuals, many of whom contain functional disruption of the melanocortin 1 receptor (MC1R)[1–3]. Although this suggested a critical role for the MC1R ligand melanocyte stimulating hormone (MSH) in this response, a genetically controlled system has been lacking in which to determine the precise role of MSH–MC1R. Here we show that ultraviolet light potently induces expression of MSH in keratinocytes, but fails to stimulate pigmentation in the absence of functional MC1R in red/blonde-haired *Mc1r*$^{e/e}$ mice. However, pigmentation could be rescued by topical application of the cyclic AMP agonist forskolin, without the need for ultraviolet light, demonstrating that the pigmentation machinery is available despite the absence of functional MC1R. This chemically induced pigmentation was protective against ultraviolet-light-induced cutaneous DNA damage and tumorigenesis when tested in the cancer-prone, xeroderma-pigmentosum-complementation-group-C-deficient genetic background. These data emphasize the essential role of intercellular MSH signalling in the tanning response, and suggest a clinical strategy for topical small-molecule manipulation of pigmentation.**

Fair-skinned individuals have an increased incidence of skin cancer and often exhibit weak tanning responses[4]. Although multiple signalling pathways affect melanin production[5,6], 'fair' pigmentation in humans is largely the result of sequence variants in *MC1R*—encoding the receptor for MSH[1,7,8]—that generate weak ligand-induced cAMP responses[2]. Deficient tanning in *MC1R* variant individuals is consistent with a critical role for MSH/cAMP in this response[3], but some studies have indicated that melanocyte-directed DNA damage might mediate UV-induced pigmentation[9,10]. We compared UV tanning responses of wild-type C57BL/6 mice with an intact MSH pathway (*Mc1r*$^{E/E}$) to those of animals possessing an inactivating mutation of the MSH receptor (*Mc1r*$^{e/e}$, formerly known as *extension*), the homologue of a gene implicated in having fair skin in humans. Whereas UV-induced hyperpigmentation was grossly and microscopically observed in the ears of control mice, *Mc1r*$^{e/e}$ (blonde-haired/pheomelanotic) mice lacked detectable pigmentation changes (Fig. 1a) despite comparable melanocyte numbers (Supplementary Fig. 1a). Murine pinnae (external ears) resemble human skin because of the presence of epidermal melanocytes that are lacking in truncal/fur skin.

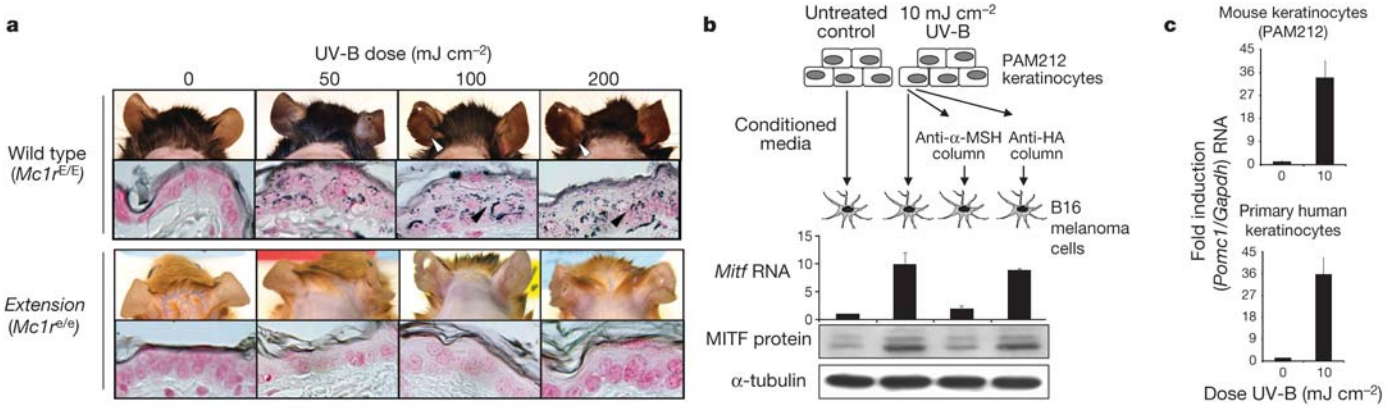


**Figure 1 | UV-induced tanning requires an intact MSH pathway. a**, C57BL/6 mice, either wild-type (*Mc1r*$^{E/E}$) or mutant (*Mc1r*$^{e/e}$) at the MSH receptor, were treated five days per week with daily UV-B delivered by a double bank of UV-B lamps (peak emission at 302 nm) for one month. Upper rows show UV-induced ear skin darkening, with corresponding Fontana–Masson-stained skin sections immediately below to show melanin accumulation (black deposits, ×400 magnification). Note the UV-induced skin darkening (white arrows) and melanin accumulation (black arrows) in *Mc1r*$^{E/E}$, but not in *Mc1r*$^{e/e}$ animals. Although *Mc1r*$^{e/e}$ skin seems unpigmented, *Dct-LacZ*-tagged transgenic analysis reveals that it contains comparable melanocyte numbers (Supplementary Fig. 1a). This experiment was repeated with similar results. **b**, *Mitf* induction by qPCR and by western analysis of B16 melanoma cells incubated (6 h) with 24-h conditioned supernatants from mouse keratinocytes either untreated (first lane) or irradiated with 10 mJ cm$^{-2}$ UV-B (remaining three lanes). Anti-MSH affinity chromatography (but not control anti-HA) abrogated *Mitf* induction by UV-conditioned media. **c**, qPCR-based detection of *Pomc1* mRNA 6 h after UV irradiation of PAM212 mouse keratinocytes or primary human keratinocytes. All PCR data are reported as fold induction by UV, normalized to *Gapdh* (control); samples were done in triplicate, and the standard deviations (s.d.) between samples are shown.

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To examine whether a non-cell-autonomous pathway may mediate the UV pigmentation effect, PAM212 keratinocytes were exposed to UV *in vitro* and culture supernatants were incubated with B16 melanoma cells to test for induction of the microphthalmia transcription factor, MITF[11]. Media from UV-irradiated (versus non-irradiated) keratinocytes induced MITF messenger RNA and protein in melanoma cells (Fig. 1b). Absorption of conditioned media using anti-α-MSH affinity chromatography removed most of the stimulatory activity, which was not removed with anti-(haemagglutinin) HA. Comparable conditioned media from UV-irradiated murine or primary human melanocytes lacked this activity (Supplementary Fig. 1b). Direct measurements revealed >30-fold induction of *Msh* (otherwise known as pro-opiomelanocortin alpha (*Pomc1*)) expression by UV in PAM212 mouse keratinocytes and primary human keratinocytes (Fig. 1c), whereas <4-fold induction was observed in melanocytes (Supplementary Fig. 1c). These observations corroborate previous findings that UV radiation activates expression of *Msh* in keratinocytes[12]. Although melanocytes showed relatively weak UV-induction of *Pomc1* expression, they nonetheless did express some *Pomc1* with or without UV exposure, as previously described[13,14], indicating that autocrine signalling could still contribute to MC1R activity.

The UV-unresponsiveness of *Mc1r* variants could, in principle, arise from an irreversible block to eumelanin synthesis occurring at some developmental stage. If a cAMP agonist could restore pigmentation in this setting, then the inability of UV to induce pigmentation would be more rigorously linked to inactivity of the cAMP pathway within the $Mc1r^{e/e}$ genetic background. To this end, we used congenic C57BL/6 mice harbouring a transgene in which the keratin 14 promoter drives constitutive expression of stem cell factor (*Scf*; also known as *Kitl*) in the epidermis[15]: *K14-Scf* mimics the human pattern of keratinocyte *SCF* expression as well as epidermal melanocyte homing (the presence of melanocytes in the basal layer of the epidermis), independent of MC1R status (Supplementary Fig. 1d). *K14-Scf* transgenic ('humanized') mice, homozygous for $Mc1r^{e/e}$, exhibited fair/pink skin with high pheomelanin and low eumelanin content (Supplementary Fig. 2a–c).

Just as in non-transgenic $Mc1r^{e/e}$ mice, we observed no measurable UV-induced melanization in *K14-Scf*-transgenic, $Mc1r^{e/e}$ mice (Fig. 2a; Supplementary Fig. 2d). When forskolin, a cell-permeable diterpenoid that activates adenyl cyclase[16], was topically applied to $Mc1r^{e/e}$; *K14-Scf* (pheomelanotic) mice, significant melanization was observed, both when applied throughout (Fig. 2b; Supplementary Fig. 2d) and when applied only to the rump area (Supplementary Fig. 3c). Daily topical forskolin caused progressive and robust darkening (Fig. 2a, Supplementary Fig. 3a), and we conclude that forskolin-induced eumelanization required epidermal melanocytes, because it was not observed in the truncal skin of mice lacking the *K14-Scf* transgene (Supplementary Fig. 3b). Skin darkening by forskolin was associated with dose-dependent accumulation of melanin in the epidermis (Fig. 2c), was progressive over several weeks, and was reversible with a half-life of approximately 2 weeks (Supplementary Fig. 3a). Melanin quantification[17] revealed a >20-fold increase in eumelanin (Fig. 2d) in treated, depilated skin, together with a shift in the eumelanin:pheomelanin ratio from 0.09 ± 0.1 to 0.9 ± 0.5.

Hair/fur colour was not markedly altered during the time courses examined here. Both crude preparations of forskolin (root extract of *Plectranthus barbatus*, otherwise known as *Coleus forskohlii*)[18] and chemically pure forskolin produced significant melanization (Supplementary Fig. 4a). Importantly, forskolin treatment mimicked melanization to the degree of exhibiting 'nuclear capping', in which

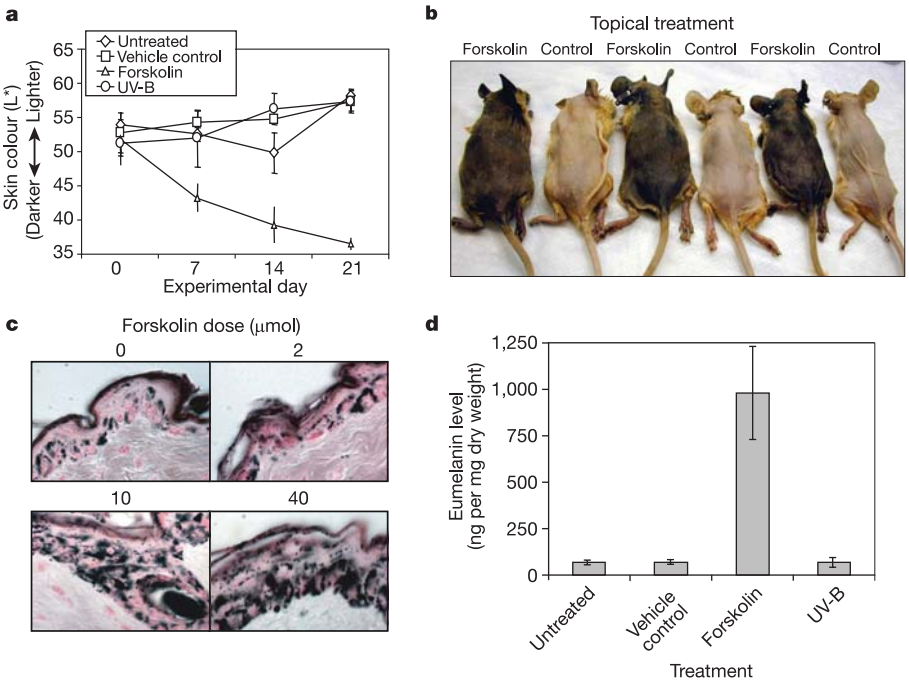



**Figure 2 | Forskolin, but not UV, rescues eumelanin production in mice with defective MSH signalling.** **a**, Reflective colorimetry measurements (CIE L* white–black colour axis)[29] of skin darkening of $Mc1r^{e/e}$; *K14-Scf*-transgenic, depilated C57BL/6 mice either topically treated with vehicle control (70% ethanol, 30% propylene glycol), irradiated with 200 mJ $cm^{-2}$ UV-B, topically treated with 400 μl of *P. barbatus* root extract (80 μmol forskolin) to the dorsal skin surface for 21 days, or untreated. Lower numbers represent darker skin tones. Skin colour measurements were done in triplicate (different anatomic locations within the treated skin field) and s.d. between measurements is shown; this experiment was repeated many times with similar results. **b**, Side-by-side photographs of vehicle-control- or forskolin-treated $Mc1r^{e/e}$; *K14-Scf* animals treated as described in **a**. **c**, Fontana–Masson (eumelanin)-stained skin sections of animals treated for 21 days with the indicated dose of forskolin derived from *P. barbatus* extract (×630 magnification) as described in **a**. **d**, Eumelanin levels (±s.d.) of whole, depilated skin from $Mc1r^{e/e}$; *K14-Scf* mice treated as described in **a** (ref. 17). Melanin quantification measurements were done in triplicate.

melanosomes exported to keratinocytes align in discrete structures that putatively provide solar shielding to keratinocyte nuclei (Fig. 3a, see asterisks)[19]. We conclude that forskolin-induced melanization did not require ectopic SCF because it was also induced on pinnae of non-*K14-Scf*-transgenic (C57BL/6) mice (Supplementary Fig. 4c). Collectively, these data demonstrate strong rescue by forskolin of eumelanin production in MC1R-deficient melanocytes, thus indicating that the inability of UV to trigger eumelanization might not be caused by irreversible inactivation of pigmentation machinery.

We considered that small-molecule-induced pigmentation might be UV-protective, so we examined formation of sunburn cells (apoptotic epidermal keratinocytes[20]) 24 h after a single 200 mJ cm$^{-2}$ dose of UV-B (arrows, Fig. 3a). Whereas vehicle-control-treated *Mc1r*$^{e/e}$ mice were very UV-sensitive, forskolin pre-treatment produced nearly the same degree of UV protection as found in genetically black-skinned (*Mc1r*$^{E/E}$) mice (Fig. 3b, c). Tyrosinase mutants (albino) were not protected by forskolin (Fig. 3b, c), suggesting that forskolin-induced keratinocyte survival was mediated by pigmentation, rather than pigment-independent effects. Fluorescence staining for cyclobutane dimers—the mutagenic and most abundant DNA lesion caused by UV-B—revealed dose-dependent nuclear staining at 20 or 50 mJ cm$^{-2}$ UV-B in *Mc1r*$^{e/e}$ and albino (*Tyr*$^{c2j/c2j}$) skin, but little in *Mc1r*$^{E/E}$ or forskolin-treated *Mc1r*$^{e/e}$ mice (Fig. 3c). Forskolin-induced melanization was nearly as protective as *Mc1r*$^{E/E}$ (genetically black) epidermis.

To test whether forskolin treatment might protect against UV carcinogenesis, xeroderma-pigmentosum-complementation-group-C-deficient mice (*Xpc*$^{-/-}$)[21] were crossed to the fair-skinned *Mc1r*$^{e/e}$; *K14-Scf* (C57BL/6 background) mice and subjected to either forskolin-containing or vehicle-control topical treatments for four weeks, before daily exposure to 250 mJ cm$^{-2}$ UV-B (along with continued topical treatments) for 20 weeks—a UV dose approximating 1–2 h of ambient midday sun exposure at sea level in Florida, during July (http://www.srrb.noaa.gov/UV/). This low-dose chronic UV schedule was chosen for its propensity to induce keratinocyte neoplasms[21]. Vehicle control (non-darkened) mice exhibited gross and histologic evidence of sun damage, including failure to thrive (diminished weight-gain; Supplementary Fig. 5a, b), profound epidermal thickening, and inflammation and scarring (Supplementary Fig. 6a, b). All such damage was significantly prevented by forskolin pretreatment. Following chronic UV, eleven neoplasms developed in the nine vehicle-control, irradiated mice within nine weeks of cessation of UV radiation (Fig. 4a, b), with two of the nine mice developing multiple tumours. As anticipated (by dose and schedule regimen), the majority were squamous cell carcinomas (Fig. 4b). Forskolin pretreatment was significantly protective in delaying onset of UV-induced tumours (median of four weeks in controls versus 25 weeks in forskolin-treated; $P < 0.001$). In all, vehicle-control-treated, irradiated mice developed 11 tumours whereas forskolin-treated, irradiated mice developed six tumours. Given the extreme UV sensitivity of homozygous *Xpc* deficiency, coupled to the high chronic UV doses used, these data suggest a

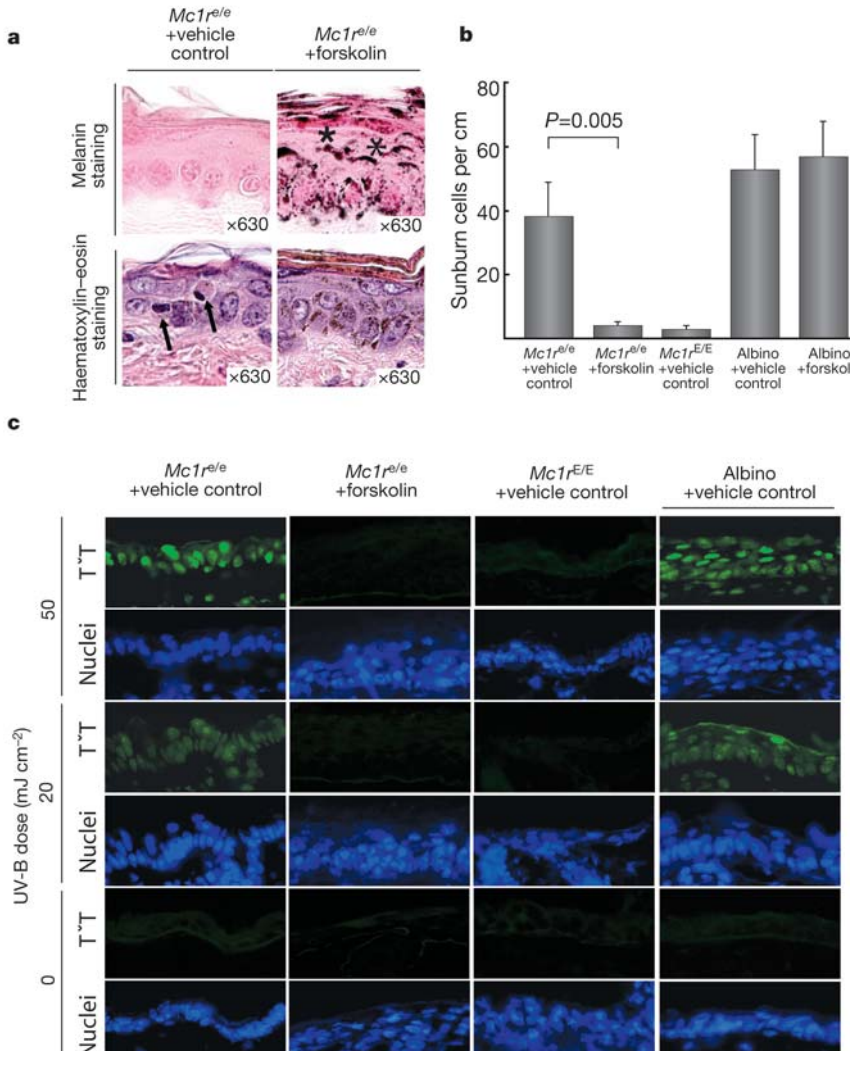


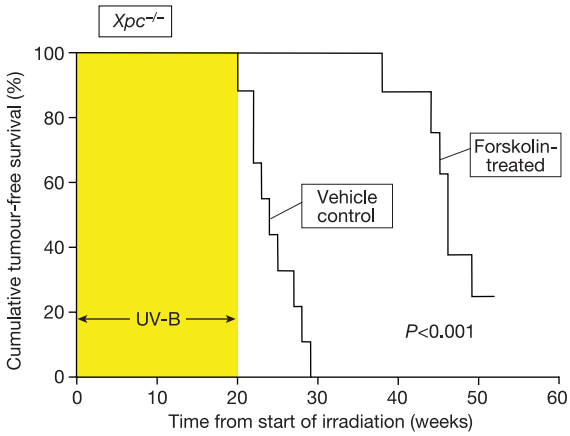

**Figure 3 | Forskolin-induced melanin deposition protects against UV-mediated cutaneous damage.** *Mc1r*$^{e/e}$; *K14-Scf*, depilated animals were pretreated for 15 days with either vehicle control or *P. barbatus* root extract (80 μmol forskolin) before exposure to 200 mJ cm$^{-2}$ UV-B. **a**, Melanin distribution in the skin 24 h after UV exposure as determined by Fontana–Masson (upper panels) or haematoxylin–eosin staining (lower panels). We note the greatly enhanced deposition of epidermal melanin (black staining) in forskolin-treated animals in a keratinocyte nucleus-capping pattern (see asterisks) and the presence of 'sunburn cells', which represent apoptotic keratinocytes in UV-irradiated animals not pre-treated with forskolin (arrows). **b**, Sunburn cell quantification in UV-exposed depilated animals treated as described in **a**. Shown are means ±s.d. of at least three samples per condition. Forskolin pre-treatment of fair-skinned animals yielded as much protection as *Mc1r*$^{E/E}$ (eumelanotic) animals. Amelanotic (albino) animals had high levels of UV-induced apoptosis regardless of whether they were treated with forskolin before UV exposure, suggesting that forskolin's protective effect is pigmentation-dependent. **c**, UV-induced thymine dimer formation in depilated skin harvested ten minutes after UV irradiation from *Mc1r*$^{e/e}$, *Mc1r*$^{E/E}$ or *Tyr*$^{c2j/c2j}$ (albino); *K14-Scf* mice pretreated with either vehicle control or *P. barbatus* root extract (80 μmol forskolin). DAPI stain highlights epidermal nuclei (blue), as does thymine-dimer-directed immunofluorescence (T˜T, green) in DNA-damaged skin in a dose-dependent fashion. Non-fluorescent (immunohistochemical) staining for pyrimidine dimers revealed the same protection, ruling out potential fluorescence quenching by melanin (Supplementary Fig. 4b). This experiment was repeated at least once with similar results.

significant protection afforded by topical rescue of MC1R functional deficiency.

A requirement for MSH/MC1R signalling in UV-induced pigmentation is plausible, given previous studies of pheomelanin production[2,3], agouti signalling protein (a natural MC1R antagonist)[22], and UV induction of *Msh* expression in keratinocytes[12]. UV also upregulated *MSH* in melanocytes (as previously reported[13,14]), but it did so to a much smaller degree than in keratinocytes. These studies therefore cannot firmly discriminate the relative roles of keratinocytes versus melanocyte MSH (paracrine versus autocrine) in the observed UV response, and indeed it is possible that both are contributory. Furthermore, the observed essential role of MC1R signalling in the tanning response does not diminish the potential contributions of other pathways. Thus, although forskolin was singularly capable of restoring dramatic melanization, other signalling factors (endothelin-1, β-FGF, NO, SCF, p38, USF and others) have been implicated in the UV adaptive tanning response[23–28] and it will be valuable to determine their relative contributions.

It will also be important to understand the pathway through which UV induces *Pomc1*/*Msh* expression in keratinocytes, because it is possible that alterations in *Msh* induction by UV could contribute to cancer risk (for example, in dark-haired, fair-skinned people). Previous work with topical cAMP agonists in other animal models showed that forskolin did not darken light-skinned swine, but did promote histologically identifiable melanin accumulation in the epidermis[18] (although this was not studied in a UV-resistant *Mc1r* variant background). The greater robustness of the forskolin response in mice may involve a thinner epidermis or genetic features that are ill-defined in swine. Clearly, small-molecule delivery would need to be optimized before pigmentation in the absence of sun could be successful in humans.

Whereas rescue of eumelanization was observed with topical forskolin, the cAMP agonist effect was not explicitly targeted to melanocytes within the skin. *In vitro* studies showed no measurable melanocytic stimulation from cultured supernatants of forskolin-treated keratinocytes (data not shown). Still, it is theoretically possible that part of the rescue may have involved non-cAMP or pigment-independent effects, or even potential impurities within the small-molecule preparation, although forskolin could not rescue keratinocyte apoptosis in UV-irradiated albinos. The topical rescue of eumelanization confirms the 'availability' of the pigmentation machinery in adults, given appropriate signals. It remains to be seen whether topical melanization will be achievable in man, and whether it would afford measurable protection against UV skin damage and cancer.




**b**

Tumorigenesis in UV-treated animals ($n$ = 9 mice for each treatment)

| | *Mc1r* $^{e/e}$ control | *Mc1r* $^{e/e}$ forskolin |
|---|---|---|
| Squamous cell carcinoma | 8 | 6 |
| Other tumours | 3 | 0 |
| Multiple tumours | 2 | 0 |
| Total tumours | 11 | 6 |

**Figure 4 | Protective effect of topical forskolin against chronic UV damage.** *K14-Scf*-transgenic, *Mc1r*$^{e/e}$, depilated animals homozygously deficient for the *Xpc* gene[21] were pretreated with either vehicle control or topical forskolin and exposed to daily low-dose UV-B for 16–20 weeks. **a**, Kaplan–Meier analysis of tumour incidence in irradiated, vehicle-control-treated versus forskolin-treated, irradiated *Mc1r*$^{e/e}$; *K14-Scf*; *Xpc*$^{-/-}$ mice. The yellow bar indicates duration of daily UV exposure. **b**, Tumour subtypes and incidence in vehicle-control-treated versus forskolin-treated, irradiated *Mc1r*$^{e/e}$; *K14-Scf*; *Xpc*$^{-/-}$ animals. Some vehicle-control-treated, irradiated animals developed multiple distinct tumours, whereas none of the forskolin-treated animals developed more than one tumour.

## METHODS

See Supplementary Information for detailed methods.

**Mice.** C57BL/6J *Mc1r*$^{E/E}$; *Tyr*$^{+/+}$ mice, C57BL/6JJ *Mc1r*$^{e/e}$ mice and C57BL/6JJ *Mc1r*$^{E/E}$; *Tyr*$^{c2j/c2j}$ mice were crossed with *K14-Scf* transgenic mice[15], dopachrome tautomerase (*Dct*)-*LacZ* transgenic mice or *Xpc*-null animals.

**Melanization experiments.** Depilated animals were exposed to either UV-B with UV-B lamps (UV Products), topical forskolin or vehicle control. Skin reflective colorimetry measurements were assessed with a CR-400 Colorimeter (Minolta) and the degree of melanization was calculated[29]. Skin biopsies were harvested and processed for Fontana–Masson (eumelanin) staining and melanin quantification[17].

**Histology and immunohistochemistry.** Skin biopsies were fixed and paraffin-sectioned (haematoxylin–eosin or Fontana–Masson staining) or frozen-sectioned (immunohistochemistry and β-galactosidase staining)[30]. Immunohistochemistry used an anti-thymine-dimer, monoclonal antibody (Kamiya Biomedical) followed by Alexa-Fluor-488-conjugated, anti-mouse IgG donkey antibody (Molecular Probes).

**Quantitative polymerase chain reaction and western blotting.** Melanocyte or keratinocyte cells were exposed to UV radiation, anti-MSH or treated cell supernatants as indicated. MSH was neutralized from cell supernatants using anti-MSH (Sigma). MITF detection by western blotting employed the C5 monoclonal antibody[11]. RNA was extracted and *Mitf* mRNA expression was quantified by quantitative Taqman polymerase chain reaction (qPCR) using QuantiTect Probe reverse transcription (RT)–PCR kits (Qiagen).

**Tumour formation and chronic UV protection experiments.** C57BL/6 *Mc1r*$^{e/e}$; *K14-Scf*; *Xpc*$^{-/-}$ animals were irradiated (250 mJ cm$^{-2}$ day$^{-1}$) beginning at seven weeks of age over the course of 20 weeks, after one month's topical pretreatment with either forskolin or vehicle control. Animals were weighed and skin samples were harvested after 16 weeks of irradiation. Animals were then monitored for tumour surveillance over the next 52 weeks. Lesions that were grossly identified as tumours were biopsied and examined.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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