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## Pigment-independent cAMP-mediated epidermal thickening protects against cutaneous UV injury by keratinocyte proliferation

Timothy L. Scott<sup>1</sup>, P.A. Christian<sup>1</sup>, M. Kesler<sup>2</sup>, Kevin M. Donohue<sup>1</sup>, Brent Shelton<sup>3</sup>, Kazumasa Wakamatsu<sup>4</sup>, Shosuke Ito<sup>4</sup>, and John D'Orazio<sup>1,5</sup>

<sup>1</sup>University of Kentucky College of Medicine, Markey Cancer Center, Department of Pediatrics and the Graduate Center for Toxicology, 800 Rose Street, Lexington, KY, 40536

<sup>2</sup>University of Kentucky College of Medicine, Department of Pathology, 800 Rose Street, Lexington, KY, 40536

<sup>3</sup>University of Kentucky College of Medicine, Division of Cancer Biostatistics, Markey Cancer Center, 800 Rose Street, Lexington, KY, 40536

<sup>4</sup>Fujita Health University School of Health Sciences, Department of Chemistry, Toyoake Aichi, 470-1192, Japan

### Abstract

The epidermis increases pigmentation and epidermal thickness in response to ultraviolet exposure to protect against UV-associated carcinogenesis; however, the contribution of epidermal thickness has been debated. In a humanized skin mouse model that maintains interfollicular epidermal melanocytes, we found that forskolin, a small molecule that directly activates adenylyl cyclase and promotes cAMP generation, up-regulated epidermal eumelanin accumulation in fair-skinned melanocortin-1-receptor (Mc1r)-defective animals. Forskolin-induced pigmentation was associated with a reproducible expansion of epidermal thickness irrespective of melanization or the presence of epidermal melanocytes. Rather, forskolin-enhanced epidermal thickening was mediated through increased keratinocyte proliferation, indirectly through secreted factor(s) from cutaneous fibroblasts. We identified keratinocyte growth factor (Kgf) as a forskolin-induced fibroblast-derived cytokine that promoted keratinocyte proliferation, as forskolin induced Kgf expression both in the skin and in primary fibroblasts. Lastly, we found that even in the absence of pigmentation, forskolin-induced epidermal thickening significantly diminished the amount of UVA and UVB that passed through whole skin and reduced the amount of UVB-associated epidermal sunburn cells. These findings suggest the possibility of pharmacologic-induced epidermal thickening as a novel UV-protective therapeutic intervention, particularly for individuals with defects in pigmentation and adaptive melanization.

### Keywords

Photoprotection; epidermal thickness; topical cAMP; melanin

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<sup>5</sup>Author to whom correspondence should be addressed: John D'Orazio, M.D., Ph.D., Associate Professor of Pediatrics, University of Kentucky College of Medicine, Markey Cancer Center, Combs Research Building 204, 800 Rose Street, Lexington, KY 40536-0096, Phone (859) 323-6238, Fax: (859) 257-8940, jdorazio@uky.edu.

## Introduction

Cutaneous UV responses represent a complex interaction between cell-autonomous events as well as intercellular signaling involving keratinocytes, melanocytes and a variety of supporting cells (1–2). The physiologic and molecular events that occur after exposure of the skin to UV radiation are complex, well-studied and involve components of the neuroendocrine (3–4), immunologic (5–6) and pigmentary systems (1, 7–8). Bioactive molecules such as  $\alpha$ -melanocyte stimulating hormones (9–11) and molecules known to increase melanocyte cAMP levels (12) result in activation of pro-differentiation pathways mediated in part through microphthalmia (Mitf) and CREB and ultimately in induction of melanin synthesis. We previously reported a pharmacologic strategy to induce melanization in *Mcl1*-defective mice using the small molecule forskolin (13). This strategy, based on inducing epidermal synthesis and accumulation of melanin, protected mice against the formation of UV-induced photodimers in the skin as well as development of UV-induced carcinomas (13). However, besides inducing melanization of the epidermis, topical forskolin also caused the epidermis to thicken (14). In this report, we studied the mechanisms of forskolin-induced epidermal keratosis and examined the capacity of epidermal thickening to protect against UV damage in the skin independent of melanization. We found that proliferation of basal keratinocytes contributed to forskolin-induced epidermal thickening and that this proliferation was mediated by fibroblast-derived soluble factors. Further, we found that forskolin-induced epidermal thickening protected against UV penetration and UV-induced epidermal apoptosis even in the absence of melanization, raising the possibility of pharmacologic UV protection based on epidermal thickening in individuals with defective melanin synthesis.

## Methods

### Animals and primary cells

*K14-Scf* transgenic C57BL/6J mice were generated as described (13, 15–16). Epidermal primary keratinocytes isolated from C57BL/6J wild type mice were grown in keratinocyte-selective media as previously published (17–19) until no pigmented, dendritic cells (i.e. melanocytes) or spindle-shaped cells (fibroblasts) were observed among the cuboidal-appearing keratinocytes (typically after 4–6 weeks in culture). As an additional control, neither fibroblast (vimentin) or melanocyte (*mitf*) mRNA were distinguishable above background after selection (data not shown). Melanins were quantitatively analyzed by HPLC as described (20).

### Topical treatments

Mice were used between 4 and 8 weeks of age and were depilated by trimming with electric shears followed by topical depilatory cream (Nair®). Preparations of topical agents were spread evenly over the entire dorsal skin. Forskolin was prepared as described (13). Solvent (vehicle) control consisted of 70% ethanol/30% propylene glycol. Animals were treated once daily for 5 days a week unless otherwise stated; for forskolin-treated animals, this equated to roughly 80 micromoles of drug applied daily to the skin.

### Skin color measurement

Skin reflective colorimetry was assessed with a CR-400 Colorimeter (Minolta Corporation, Japan) calibrated against a white background. Degree of melanization (darkness) was quantified as the colorimetric measurement on the \*L axis (white-black axis) of the CIE standard color axis (21).

## UV exposure

For most experiments, mice were exposed to a double bank of UVB lamps (UV Products, Upland, CA). Unless stated otherwise, animals were exposed to radiation once daily, five days a week for three weeks (fifteen total doses), with a daily dose of 2 kJ/m<sup>2</sup> UVB and 0.5 kJ/m<sup>2</sup> UVA (therefore 2.5 kJ/m<sup>2</sup> total UV per day and 37.5 kJ/m<sup>2</sup> cumulative total UV dose). 2 kJ/m<sup>2</sup> per day was chosen because it represents a sub-erythemal dose of UV light previously found to induce tanning in our animal model (in an Mc1r-intact background) (13). For the UV penetration experiments, a 1.6 kW Xenon short arc solar simulator UV source (Sciencetech, London, ON Canada) was used with custom filters to eliminated all but UVB or UVA resulting in >90% pure UVB or UVA radiation respectively. UV emittance was measured with a Model IL1400A handheld flash measurement photometer (International Light, Newburyport, MA) equipped with separate UVB (measuring wavelengths from 265–332 nm; peak response at 290 nm) and UVA (measuring wavelengths from 315–390 nm; peak response at 355 nm) corresponding to International Light product numbers TD# 26532 and TD# 27108 respectively. Spectral output of the lamps was determined to be roughly 75% UV-B and 25% UV-A.

## Histology and immunohistochemistry

Skin biopsies were fixed (10% buffered formalin), paraffin-embedded, sectioned (5 μm) and Fontana-Masson stained (American Master\*Tech Scientific, Inc., Lodi, CA) (22). Epidermal thickness was determined by multiple measurements in fields without sectioning artifacts. To quantify nucleated cells, sections were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO) (23), and numbers of nuclei in the epidermis were quantified. Nuclei were quantified along the entire biopsy, with roughly 15–20 high power fields per biopsy, excluding sections that displayed major artifactual disruptions (e.g. torn or folded cutting artifacts) or evidently non-perpendicular lengths. Non-follicular epidermal nuclei were counted along the entire image. For keratin 14 and Ki67 staining, antigen retrieval was performed by heating (45 minutes, 10 mM citrate buffer pH 6.0) in a rice cooker. Immunohistochemistry was performed using rabbit anti-keratin 14 antibody (1:500 dilution of 1 mg/ml stock; Covance, Princeton, NJ), rabbit anti-Ki67 antibody (1:1000 dilution of 2 mg/ml stock; Abcam, Cambridge, MA) and FITC-conjugated goat anti-rabbit secondary antibody (1:1000, Abcam, Cambridge, MA). Images were captured and measurements were made using the QCapture Pro program (QImaging Software).

## Proliferation assays

Primary keratinocytes were serum starved overnight and treated for 24h as described (24–25). <sup>3</sup>H-thymidine (Perkin-Elmer, Waltham, MA) was added during the last 3h of treatment. DNA was precipitated with 10% trichloroacetic acid (Sigma-Aldrich, St. Louis, MO) and collected with 0.2N NaOH (Sigma-Aldrich, St. Louis, MO); incorporated radiolabeled thymidine was measured in a Beckman LS 6500 (Beckman Coulter, USA).

## mRNA quantification (qPCR)

Total RNA was harvested from cells using the RNeasy extraction kit (Qiagen RNA) and from skin using Trizol (Invitrogen). cDNA was generated (Fermentas first strand synthesis kit; Thermo Scientific, Glen Burnie, MD) and dual color quantitative real-time PCR analysis was performed using a Roche Lightcycler 480 (20 ng cDNA/reaction using mouse β-actin as the reference gene. Primer sets for Kgf/Fgf7 were 5'-TCTCATCAATCTCCAGTTCACAA (left) and 5'-CTTGCGTTGATTGCTACTCCT (right), for Fgf10 were 5'-CGGGACCAAGAATGAAGACT (left) and 5'-GCAACAACCTCCGATTTCCAC (right) and for pro-EGF5'-CATGCCCCACAGGATTTG (left) and 5'-GGGCAGGAAACAAGTTTCG (right).

## Measurement of UVR transmission

Dorsal skins of animals treated as described were removed, spread flat and placed over a UVR sensor. UV transmission was measured and percent transmission was calculated for UVA and UVB.

## Sunburn cells

Five days after the last topical treatment (21d, 5d/wk), animals were irradiated. Skin biopsies were harvested (24h), processed and stained by hematoxylin and eosin. Sunburn cells, defined as epidermal cells with pyknotic condensed nuclei (26), were quantified.

## Statistical analysis

Statistical comparisons between cohorts of control- versus forskolin-treated animals were evaluated by a 1-way ANOVA with Tukey's correction unless otherwise stated. Differences were considered statistically significant if the  $p$  value was  $< 0.05$ .

## Results

*K14-Scf* transgenic C57BL/6 animals (27) were crossed with established C57BL/6J coat color variants to generate genetically-matched animals with "humanized skin". Thus, unlike non-transgenic mice, *K14-Scf* animals maintain interfollicular epidermal melanocytes in the stratum basale and retain the capacity for epidermal pigmentation (13). In this model, "wild type" *K14-Scf*C57BL/6 mice with functional melanocortin 1 receptor (*Mc1r*) and tyrosinase (*Tyr*) exhibited an intensely eumelanotic phenotype in both the fur and the epidermal skin (Fig. S1a), whereas incorporation of the *Mc1r extension* (*Mc1r<sup>e/e</sup>*) mutation essentially switched the pigment phenotype to a red/blonde pheomelanotic complexion. Loss of tyrosinase, the rate limiting enzyme critical to production of all types of melanin, resulted in near complete loss of pigmentation, irrespective of *Mc1r* function (Fig. S1a). HPLC-based quantification of melanin species of whole depilated skin from these transgenic animals confirmed that the skin of wild type (*Mc1r<sup>E/E</sup> Tyr<sup>+/+</sup>*) animals contained abundant eumelanin, skin of *extension* (*Mc1r<sup>e/e</sup> Tyr<sup>+/+</sup>*) animals contained little eumelanin but preferentially contained pheomelanin, and the dorsal skin of tyrosinase-null (*Mc1r<sup>e/e</sup> Tyr<sup>c2j/c2j</sup>* or *Mc1r<sup>E/E</sup> Tyr<sup>-/-</sup>*) adult animals contained insignificant amounts of either melanin species irrespective of *Mc1r* status (Fig. S1b).

Using pheomelanotic *Mc1r*-defective animals that model fair-skinned humans, we found that daily topical applications of the adenylyl cyclase activator forskolin, but neither vehicle nor daily UV exposure, robustly induced skin darkening (Fig. S2a, b) (13). Epidermal darkening was due to accumulation of melanin throughout the epidermis, as determined by Fontana-Masson melanin staining (Fig. 1a). Previously, we determined that topically-applied forskolin may also lead to increased numbers of epidermal melanocytes in our animal model, which may also contribute to epidermal darkening (14). When skin sections of control- vs. forskolin-treated *extension* animals were carefully compared, we noted that in addition to the increased melanization, the epidermis of forskolin-treated animals was thicker than that of vehicle-treated counterparts (Fig 1a, b). Interestingly, UV-exposed *Mc1r*-null animals demonstrated robust epidermal thickening but failed to exhibit melanization (Fig. 1a, b). Thus, pharmacologic cAMP induction lead to both melanization and epidermal thickening, whereas UV exposure of animals with defective *Mc1r* promoted epidermal thickening without melanization, suggesting that the physiologic mechanisms regulating melanization must be distinct from those regulating epidermal thickening. Forskolin-induced thickening was noted as early as day 7 of treatment, reached maximal levels between 14–21 days of therapy and remained fairly constant through three months of daily treatment (data not shown) (14). To control for the confounding effects on epidermal

thickness related to different stages of hair cycling (28);(29), we repeated the three week experiment in 8 week-old C57BL/6 animals, all of which remained in non-anagen phases of the hair cycle throughout the three week experiment. Again, topically-applied forskolin promoted a statistically significant ( $p < 0.05$ ) increase in epidermal thickness, suggesting that this effect was unrelated to hair cycling (Fig. S3). Pharmacologically-mediated epidermal thickening by forskolin appeared to be independent of an inflammatory component, as determined by lack of erythema and by the lack of epidermal or dermal inflammatory cell infiltrate (Fig. S4). Furthermore, we noted no global reproducible effects on keratinocyte differentiation as judged by expression of keratin-1, keratin-14 or involucrin within seven days of treatment (Fig. S5).

Since forskolin promoted robust melanization along with epidermal thickening, we reasoned that forskolin-induced epidermal thickening may be due to melanin accumulation. To test this hypothesis, we determined the effect of topical forskolin on *Mc1r*-defective albino *K14-Scf* animals incapable of making melanin due to a defective tyrosinase gene (*Tyr<sup>c2j/c2j</sup>*). Unlike their fair-skinned *Mc1r*-defective tyrosinase-intact *extension* counterparts, albino animals showed no forskolin-induced skin darkening (Fig. S2c, d). Fontana-Masson-stained skin biopsies confirmed robust epidermal expansion in the absence of melanization in forskolin-treated *K14-Scf* albino *extension* animals (Fig. 1c). In fact, the degree of forskolin-induced epidermal thickening was similar between tyrosinase-intact (melanin-containing) and albino animals (Fig. 1d), indicating that forskolin-induced epidermal thickening was independent of tyrosinase function or melanin deposition. We next tested the contribution of the *K14-Scf* transgene and interfollicular melanocytes on forskolin-induced epidermal thickening by comparing non-transgenic albino animals with their *K14-Scf* counterparts. We found similar levels of forskolin-dependent epidermal thickening in either group of animals (Fig. 1d), suggesting that forskolin-induced epidermal thickening is independent of c-kit signaling or the presence of interfollicular melanocytes.

We reasoned that forskolin-induced epidermal thickening could result either from an increase in the number of epidermal keratinocytes or from hypertrophy of existing cells. To help distinguish between these possibilities, we stained skin sections from control-versus forskolin-treated *K14-Scf extension* animals with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that avidly binds DNA and facilitates enumeration of nucleated cells. We found that topically-applied forskolin, like daily low-dose UV (positive control), promoted an accumulation of nucleated cells in the epidermis (Fig. 2a, b). An increase in epidermal keratinocyte number could result from either increased proliferation of basal keratinocytes or from interference with terminal differentiation wherein maturing keratinocytes lose their nuclei as they migrate beyond the stratum spinosum to form the cornified layer. To distinguish between these possibilities, we determined the effect of topical forskolin on keratin 14 expression, a cytokeratin expressed by proliferating basal keratinocytes (30–31). We found that both UV (positive control) or topical forskolin promoted expansion of keratin 14-expressing cells in the epidermis (Fig. 2c, d). Furthermore, forskolin treatment increased the number of Ki67-stained cells in the stratum basale (Fig. 2e, f), consistent with cAMP-induced enhanced proliferation of basal keratinocytes (32). Curiously, there was no difference in the number of epidermal nuclei between control-treated and forskolin-treated animals at day 3 (Fig. 2g), suggesting that the accumulation of epidermal cells caused by enhanced proliferation took longer than 3 days to occur.

We reasoned that forskolin could increase keratinocyte proliferation either directly via cAMP stimulation or indirectly through activation of other skin cells. The literature is mixed with respect to the effect of cAMP stimulation on keratinocytes, with some manuscripts reporting enhanced proliferation (33–34) and others describing cAMP-mediated effects on migration and differentiation instead (35–36). In order to clarify between these possibilities,

we purified primary keratinocytes from C57BL/6 animals (19) and tested the ability of purified forskolin to affect their proliferative capacity *in vitro*. Instead of enhancing the proliferative rate of primary keratinocytes, purified forskolin consistently inhibited keratinocyte thymidine incorporation (Fig. 3a), unlike the positive control epidermal growth factor (EGF) (37). Thus, we concluded that cAMP stimulation does not directly increase proliferation of purified primary murine keratinocytes.

Since topically-administered forskolin enhanced keratinocyte proliferation the context of whole skin but not in purified keratinocytes, we hypothesized that forskolin promoted keratinocyte proliferation indirectly via the participation of other skin cell type(s). We tested the role of soluble factors in forskolin-mediated keratinocyte proliferation with the use of Transwell<sup>®</sup> chambers. Into the upper chamber were placed either B16 murine transformed melanocytes, primary C57BL/6 keratinocytes (negative control) or primary C57BL/6 fibroblasts. The proliferative rate of purified primary keratinocytes (in the lower chamber) was measured in the presence of either vehicle or forskolin. Whereas forskolin inhibited keratinocyte proliferation directly, it potentially enhanced keratinocyte proliferation if primary fibroblasts were included in the Transwell<sup>®</sup> chambers (Fig. 3b). Transformed B16 melanocytes had a lesser stimulatory effect (Fig. 3b). We concluded that forskolin stimulated keratinocyte proliferation indirectly through one or more soluble factors produced by other skin cells, particularly fibroblasts. We then sought to determine the identity of the cAMP-induced fibroblast growth factors responsible for forskolin-induced keratinocyte proliferation. We noted marked up-regulation in *Kgf* (*Fgf7*) mRNA in forskolin-treated primary fibroblasts with a modest increase in *Fgf10* mRNA and no change in *Egf* expression (Fig. 3c). We found a similar increase in *Kgf* mRNA in forskolin-exposed skin (Fig. 3d). To determine if *Kgf* was able to overcome the anti-proliferative effect of forskolin treatment seen *in vitro*, we repeated the <sup>3</sup>H-thymidine incorporation assay with primary keratinocytes pretreated with forskolin for 6 hours. We found that recombinant *Kgf* (20 ng/ml) partially rescued forskolin-mediated keratinocyte proliferation (Fig. 3e). Together, these data suggested that forskolin-induced keratinocyte proliferation may be mediated indirectly through induction of fibroblast-derived *Kgf*.

Finally, we sought to determine whether forskolin-induced epidermal thickening could protect skin against UV penetration. We compared cutaneous responses between tyrosinase-intact, melanin-inducible animals (*K14-Scf Mc1r<sup>e/e</sup>, Tyr<sup>+/+</sup>*) with those of their tyrosinase-defective genetically-matched counterparts (*K14-Scf Mc1r<sup>e/e</sup>, Tyr<sup>c2j/c2j</sup>*). We assessed UV protection by measuring the amount of UV radiation that would pass through dorsal skin treated topically (3 wk) with either forskolin or vehicle. Consistent with previous observations, daily application of forskolin to *K14-Scf Mc1r*-defective animals promoted skin thickening irrespective of tyrosinase function and melanin deposition (Fig. S6a), however, only in the tyrosinase-intact background did significant melanization occur (Fig. S6b). Control-treated albino *extension* skin allowed 36.3% and 8.8% of UVA or UVB photons to pass through respectively (Fig. 4a, b). In contrast, albino *extension* skin that had been pretreated with forskolin allowed only 7.1% (UVA) and 1.3% (UVB) to pass through (Fig. 4a, b). Therefore, melanin-independent forskolin-induced epidermal thickening was associated with 80.4% (UVA) and 85.2% (UVB) reductions in the amount of UV penetration through the skin as measured in this manner. If melanization was added to the system by using tyrosinase-intact *K14-Scf extension* (*Mc1r<sup>e/e</sup>, Tyr<sup>+/+</sup>*) animals, then forskolin pretreatment was associated with transmission of 0.7% UVA and 0.1% UVB, representing reductions of 92.5% and 90.0% respectively over control-treated counterparts (Fig. 4a, b). The importance of eumelanin to UV photoprotection was also demonstrated by noting the UV-blocking capacity of untreated *K14-Scf Mc1r*-intact (fully eumelanotic) wild type animals. In these mice, thought to be maximally pigmented yet with a thin, pharmacologically-unstimulated epidermis, only 5.1% UVA and 0.73% UVB passed

through the skin (Fig. 4a, b). Overall, these data showed that epidermal thickening blocked penetration of UVA and UVB into the skin even in the complete absence of pigmentation.

In order to determine whether forskolin-induced epidermal thickening was associated with functional UV protection, we quantified “sunburn cells” in the epidermis at varying doses of UVB radiation. Sunburn cells are thought to represent keratinocytes dying apoptotically after UV exposure (26). By this measure, we found that animals pre-treated with forskolin had fewer sunburn cells in the epidermis 24h after exposure to 1 kJ/m<sup>2</sup> UVB (Fig. 4c). This protection was due to increased epidermal thickness alone since these experiments were carried out in albino *extension* animals incapable of melanizing their epidermis (Fig. 4d). This photoprotection could be overcome by increasing the dose of UV administered (2 kJ/m<sup>2</sup>; Fig. 4c). These findings are novel because of the ability of this animal model to disconnect epidermal thickening from pigmentation in a genetically defined system. Together, these data show that pharmacologic-induced epidermal thickening is protective against UV even in the absence of epidermal melanization.

## Discussion

Using a genetically-defined animal model with “humanized skin”, we found that topical cAMP up-regulation promoted epidermal thickening through enhanced keratinocyte proliferation. This pharmacologically-induced epidermal thickening was UV-protective independently of pigmentation. Since most reports of UV-induced epidermal thickening involved pigmented human or porcine skin (38–39) or non-transgenic murine models wherein the epidermis lacks interfollicular melanocytes, the role of melanocytes and their melanin products in UV-induced epidermal thickening has been unclear. We found that epidermal thickening occurred even in tyrosinase-null albino animals implying that UV-induced epidermal thickening was not simply due to an accumulation of melanin in epidermal keratinocytes. Rather, forskolin-induced epidermal thickening was associated with an increase in the proliferative rate of basal keratinocytes and a subsequent accumulation of nucleated cells in the epidermis, findings which might have implications for wound healing and other cutaneous processes reliant on keratinocyte proliferation.

Our studies suggest that direct cAMP stimulation inhibits proliferation of primary keratinocytes, in agreement with previous findings by others (40). Instead, our data suggest that one or more soluble cAMP-induced factors derived from fibroblasts, likely Kgf or Fgf10, mediates keratinocyte proliferation. Both of these cytokines are known to be produced by fibroblasts (41–42) and to stimulate keratinocyte proliferation (43–44). In our system, Kgf was the growth factor whose levels increased the most upon cAMP stimulation. Though Kgf is known to promote keratinocyte proliferation in the context of wound healing (45–47) and in certain pathologic conditions (e.g. psoriasis) (48), its contribution to UV-induced epidermal keratosis is unclear. Nonetheless, Kgf seems to exert UV-protective effects on epidermal skin cells, specifically mediating resistance to UV-mediated oxidative injury (49), epidermal apoptosis (50) and increased uptake of melanosomes (51). Though forskolin clearly induced *Kgf* mRNA directly in purified fibroblasts, our data do not rule out the contribution of other mediators or cytokines (e.g. keratinocyte-derived IL-1 (52)) in affecting fibroblast Kgf production or epidermal thickening. It is important to point out that Kgf was identified by mRNA expression alone. We were technically unable to detect Kgf protein by conventional means (Western analysis, ELISA), presumably because anti-Kgf commercial reagents that were used were designed to measure human Kgf rather than murine Kgf. Therefore, it will be important in future studies to verify the involvement of Kgf at the protein level in forskolin-induced epidermal thickening.

Pharmacologically-enhanced epidermal thickening significantly contributed to UV photoprotection even in amelanotic skin. Indeed, the degree of photoprotection afforded by forskolin-induced epidermal thickening in an amelanotic (albino) background approached that observed in untreated pheomelanotic (fair-skinned) animals (Fig. 4). This observation may be clinically significant, as amelanotic persons who suffer from albinism tend to be much more UV sensitive than otherwise fair-skinned persons whose epidermis contains pheomelanin and little eumelanin. Though it might offer the potential benefit of enhanced UV-protection, pharmacologically-induced epidermal thickening might also be associated with potential negative effects as well, including potentiating conditions of hyperkeratosis including psoriasis and atopic dermatitis. Clearly, preclinical studies will have to be done before this approach can safely be considered for clinical practice.

We realize that forskolin, a potent and promiscuous activator of adenylyl cyclases, may not be an optimal translational product for inducing UV photoprotection in humans. Rather, we view these data as proof-of-concept studies to show the potential benefit of topical cAMP-mediated epidermal thickening in UV resistance. The use of topical agents to increase epidermal cAMP may also protect the skin in other ways, most notably by enhancing removal of UV-induced DNA damage as was shown by Hearing and coworkers in a reconstructed skin model (53). Nonetheless, a more targeted approach may have to be identified before translating these findings into clinical application. Topical application of clinically available phosphodiesterase inhibitors, for example, also increase cellular levels of cAMP and exhibited activities similar to forskolin in the murine model that we used in our studies (54). Recent work by Hwang and colleagues, for example, showed that 5,7-dimethoxyflavone up-regulated pigment synthesis in melanocytes through cAMP-dependent signaling (12), suggesting other potential agents for topical cAMP manipulation. Overall, these data suggest that pharmacologic manipulation of epidermal thickness may represent a novel means to enhance the photoprotective properties of the skin.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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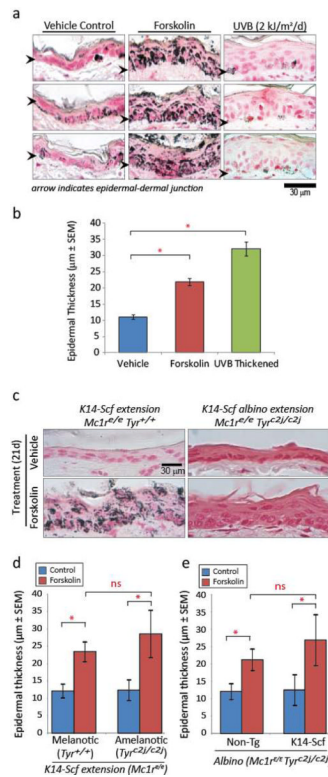
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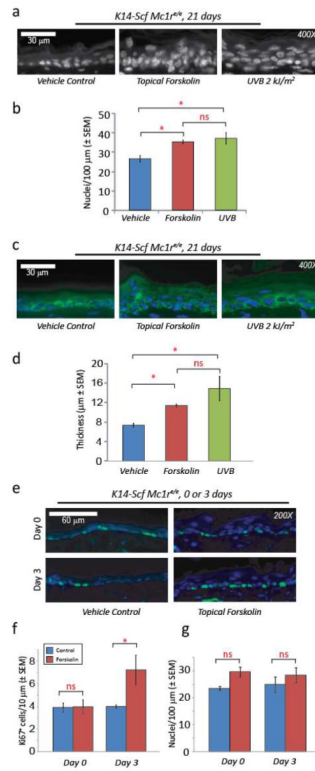
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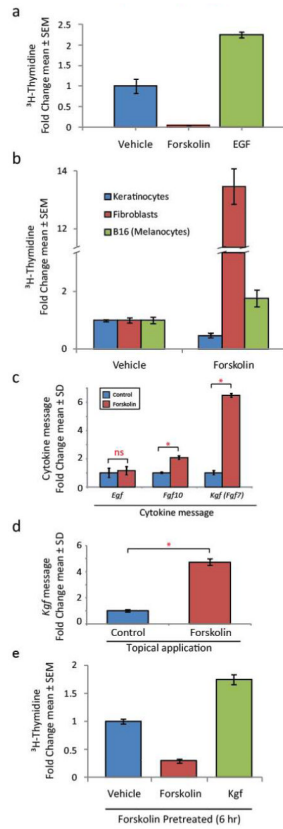
**Figure 1.**

Forskolin treatment increases epidermal thickness. (a, b) Representative images and epidermal thickness measurements of Fontana-Masson stained dorsal skin biopsies taken from *K14-Scf Mc1r<sup>e/e</sup> Tyr<sup>+/+</sup>* (*extension*) animals treated (3wk, 5d/wk) with vehicle (n=4), forskolin (n=4), or UVB (2kJ/m<sup>2</sup>) (n=3). (c, d) Representative images (c) and epidermal thickness measurements (d) of Fontana-Masson stained skin sections from *K14-Scf Mc1r<sup>e/e</sup> Tyr<sup>+/+</sup>* (*extension*) (n=4) or amelanotic *K14-Scf Mc1r<sup>e/e</sup> Tyr<sup>c2j/c2j</sup>* (*albino extension*) (n=3) animals treated as indicated above. (e) Epidermal thickness measurements of amelanotic *K14-Scf* transgenic (n=3) vs. non-transgenic (n=2) *Mc1r<sup>e/e</sup> Tyr<sup>c2j/c2j</sup>* *albino extension* animals treated (3wk, 5d/wk) with vehicle or forskolin. Scale bar=303m, \* p < 0.05. Note that forskolin-induced epidermal thickening is independent of either melanin (c, d) or stem cell factor (e).



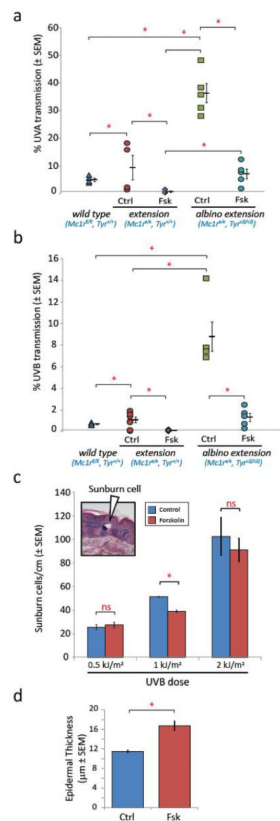
**Figure 2.**

Forskolin promotes epidermal cell accumulation and basal keratinocyte proliferation *in vivo*. (a, b) Representative skin sections (a) and quantification (b) of DAPI-stained epidermal nuclei in *K14-Scf Mc1r<sup>+/e</sup> Tyr<sup>+/+</sup>* (*extension*) animals treated (3wk, 5d/wk) as described in Fig. 1. Scale bar=30μm. (c, d) Representative skin sections stained for keratin 14 (c) and keratin 14 thickness measurements (d) from animals treated as described in “a”. Scale bar=30μm. (e, f, g) Representative Ki67-stained skin sections (e) Ki67 quantification (f) and DAPI-stained nuclei (g) from *K14-Scf Mc1r<sup>+/e</sup> Tyr<sup>+/+</sup>* (*extension*) animals treated (0 or 3 days) with vehicle (n=3) or forskolin (n=4). Scale bar=603m, \* p < 0.05.



**Figure 3.**

Forskolin promotes primary keratinocyte proliferation in the presence of fibroblasts. (a)  $^3\text{H}$ -thymidine incorporation (expressed as fold change over vehicle control) of purified primary C57BL/6 keratinocytes treated with vehicle (1% DMSO; n=3), 100  $\mu\text{M}$  forskolin (n=3) or 20 ng/ml EGF (n=3). (b)  $^3\text{H}$ -thymidine incorporation of purified primary C57BL/6 keratinocytes co-cultured in the presence of either primary C57BL/6 keratinocytes, primary C57BL/6 fibroblasts, or transformed B16 melanocytes in Transwell<sup>®</sup> inserts and treated with either vehicle or forskolin as described above. (c) Expression of *Kgf*, *Fgf10*, or *Egf* mRNA in primary C57BL/6 murine fibroblasts treated (6h) with vehicle or forskolin as described above. (d) *Kgf* mRNA expression at 6 hours in whole skin of C57BL/6 animals treated topically with either vehicle or forskolin as previously described. *Kgf* mRNA data are expressed as fold induction over vehicle control and normalized to  $\beta$ -actin expression. (e)  $^3\text{H}$ -thymidine incorporation of primary C57BL/6 keratinocytes pretreated with forskolin (100  $\mu\text{M}$ ; 6h) and then incubated with vehicle (1% DMSO), forskolin (100  $\mu\text{M}$ ), or Kgf (20 ng/ml) for an additional 24h. \* p < 0.05.



**Figure 4.** Forskolin-mediated epidermal thickening is photoprotective. (a, b) Transmission of (a) UVA (315–390 nm) or (b) UVB (265–332 nm) through whole depilated dorsal skins of age-matched *K14-Scf* animals treated (3wk, 5d/wk) as indicated (n=5 animals per group). Untreated *K14-Scf Mc1r<sup>E/E</sup> Tyr<sup>+/+</sup>* (wild type) animals are included as “maximally pigmented” eumelanotic controls. (c) Quantification of sunburn cells 24h after UV exposure in albino extension animals pre-treated (3wk, 5d/wk) with either vehicle (n=4) or forskolin (n=5) and exposed to the indicated dose of UVB. The inset shows an example of a sunburn cell dense and (d) describes mean epidermal thickness of cohorts of animals used in the sunburn cell assay. \* p < 0.05.