significantly increased melanins in the epidermis (Fig. 1d). An image analysis showed a increased pigmented area/epidermal area ratio in the PAR-2 AP-treated skin compared with the PAR-2 CP-treated skin (0.354 ± 0.078 vs 0.711 ± 0.028, $P < 0.05$).

## Conclusions

PAR-2 is involved in a broad spectrum of physiological processes in the expressing tissues, and in skin, PAR-2 plays numerous pathological and physiological roles (9). With respect to skin pigmentation, PAR-2 is well known to regulate melanosome transfer via phagocytosis of KCs (4). However, the effect of direct PAR-2 activation in KCs on melanogenesis via KC-derived factors such as SCF has not yet been studied. Therefore, we tried to focus on the effect of SCF expression by direct PAR-2 activation with PAR-2 AP, because SCF is regarded as the main paracrine mediator among several KC-derived factors involved in skin pigmentation (2).

In this study, we found that PAR-2 AP, known PAR-2 activators, increased SCF expression at both the protein and mRNA level in primary KCs. The increased expression of SCF was proven to be mediated via direct PAR-2 activation on KCs by experiments using both a PAR-2 antagonist and PAR-2 siRNA. Moreover, PAR-2 activation in KCs upregulated MMP-9 enzyme activities, which in turn is thought to release soluble form of SCF through proteolytic cleavage of membrane-bound SCF increased by PAR-2 activation. Indeed, increased soluble SCF from KCs by PAR-2 activation subsequently induced melanogenesis of MCs through c-kit/ERK pathway in a paracrine manner in the coculture model of KCs and MCs as well as *ex vivo* organ culture. Therefore, our results imply that PAR-2 is not only involved in melanosome transfer, but also plays a direct role in melanogenesis by increasing SCF secretion from KCs.

## Acknowledgements

JYK, HS and EJL performed the experiments. DSK, HS and SHO designed the study. JYK, DSK, HS and SHO analysed the data. JYK, DSK, HS and SHO wrote thearticle. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0021799).

## Conflict of interest

The authors have declared no conflicting interests.

## Supporting Information

Additional supporting data may be found in the supplementary information of this article.

**Data S1.** Materials and methods
**Data S2.** Figure Legends
**Figure S1.** SCF expression following PAR-2 activation with PAR-2 AP.
**Figure S2.** Increased soluble SCF secretion in HaCaT cells treated with PAR-2 AP.
**Figure S3.** SCF expression after PAR-2 activation with KLK5.
**Figure S4.** Confirmation of PAR-2 silencing by PAR-2 siRNA transfection
**Figure S5.** Melanocytes viability was assessed by MTT assay.

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Letter to the Editor

# The NADPH oxidase inhibitor apocynin inhibits UVB-induced skin carcinogenesis

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## Background

Non-melanoma skin cancer (NMSC) is the most common form of skin cancer and its increasing incidence worldwide represents a major public health problem (1–4). NMSC is primarily caused by excessive exposure to ultraviolet (UV) radiation. UV radiation (particularly the UVB component; 290–320 nm) is a strong carcinogen that increases skin ageing, oxidative stress, inflammation, DNA damage and cancer risk (5,6).

UV radiation to the skin activates various signal transduction pathways involved in skin carcinogenesis. Previous reports have demonstrated that in response to UV, p38, JNK, ERK and Akt pathways are induced and subsequently trigger the activation of transcription factors including nuclear factor κB (NF-κB), activator protein-1 (AP-1) and cyclic AMP response element-binding protein, which regulate gene expression that has been implicated in malignant transformation, inflammation and proliferation (7–9). In addition, key factors involved in inflammation such as cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α) and ornithine decarboxylase (ODC) are known to be overexpressed and promote skin cancer development downstream of the MAPK and Akt pathways (S2–S4). Therefore, targeting these UV-mediated signalling pathways represents an effective strategy for skin cancer prevention.

NADPH oxidase is a key enzyme that generates the superoxide anion and other reactive oxygen species (ROS). Apocynin is an active compound found in the roots of *Picrorhiza kurroa* and functions as an inhibitor of the NADPH oxidase complex by blocking translocation of cytosolic components to the cell membrane (S5, S6). The therapeutic applications of apocynin have been widely studied in various diseases including asthma, arthritis and cancer, as well as neurodegenerative, lung and cardiovascular diseases (S5–S7). Although previous studies have demonstrated a potential role for NADPH oxidase in several types of cancer (S8–S11), if and how this enzyme is involved in the development of UV-induced skin cancer has not previously been investigated. Due to apocynin's attractive features of low toxicity and high selectivity (S5, S6), we sought to determine whether its potent inhibition of NADPH oxidase has chemopreventive effects in UV-mediated skin carcinogenesis.

## Questions addressed

To examine whether the inhibition of NADPH oxidase activity has therapeutic implications in UV-mediated skin carcinogenesis, we investigated the effect of the NADPH oxidase inhibitor, apocynin against UVB-induced skin cancer development and UVB-mediated signalling pathways.

## Experimental design

We analysed the effect of apocynin on UVB-mediated carcinogenic signalling in JB6 P+ cells and whether apocynin treatment can prevent UVB-induced skin cancer development *in vivo* using SKH-1 hairless mice.

## Results

Treatment with apocynin dose dependently suppressed UVB-induced NADPH oxidase activity in JB6 P+ cells (Fig. 1b). As UVB has been reported to activate MAPK and Akt signalling pathways to promote skin carcinogenesis and inflammation (7,8) (S1), we examined the effect of apocynin on these UVB-induced signalling pathways. Apocynin inhibited UVB-induced phosphorylation of p38, ERK, JNK and Akt in a dose-dependent manner (Fig. 1c). AP-1 and NF-κB are transcription factors downstream of the MAPK and Akt pathways and are involved in carcinogenesis, inflammation and transformation (7–9). Apocynin treatment significantly reduced UVB-induced activation of AP-1 and NF-κB in JB6 P+ cells (Fig. 1d, e). Further examination revealed that apocynin suppressed the promoter activity of UVB-induced COX-2, an enzyme known to play a critical role in inflammation and skin cancer development (Fig. 1f) (S2).

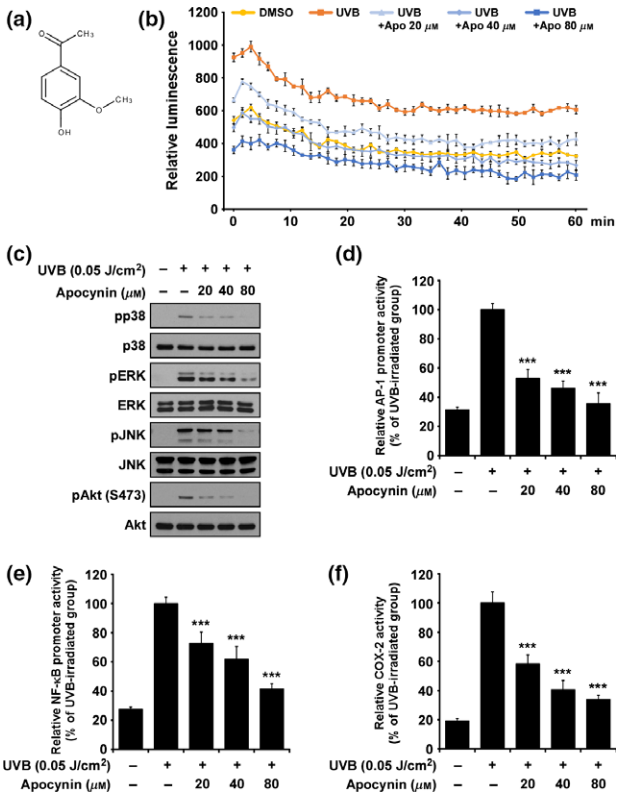


**Figure 1.** Inhibition of UVB-induced carcinogenic signalling by apocynin.(a) Structure of apocynin. (b) Apocynin suppresses UVB-induced NADPH oxidase activity. JB6 P+ cells were pretreated with apocynin for 30 min and irradiated with UVB (0.05 J/cm$^2$) for 30 min. NADPH oxidase activity was measured 30 min after UVB irradiation. NADPH oxidase activity was measured in triplicate. Data presented as mean ± SD. (c) Apocynin inhibits MAPK and Akt signalling. JB6 P+ cells were pretreated with apocynin for 30 min and irradiated with UVB (0.05 J/cm$^2$) for 30 min. (d-f) Apocynin inhibits UVB-induced AP-1 and NF-κB transactivation, as well as COX-2 promoter activity. Luciferase activity is displayed as a percentage of activity compared to cells treated with UVB only. Data presented as mean ± SD. ***, $P < 0.001$, significant difference between groups treated with both apocynin and UVB and the group treated with UVB alone.

The strong inhibitory effect of apocynin on UVB-induced carcinogenic signalling suggests a therapeutic potential for apocynin against skin inflammation and cancer. We next investigated whether apocynin can reduce UVB-induced acute inflammation *in vivo* and found that apocynin treatment significantly decreased UVB-induced ear oedema, a well-known inflammation marker (Fig. 2a, b). To further evaluate the chemopreventive effect of apocynin *in vivo*, we used a UVB-induced two-stage skin carcinogenesis model. Topical application of apocynin resulted in a marked preventive effect against UVB-induced tumor incidence (Fig. 2c, d). While exposure to UVB caused the development of skin tumors in all of the mice within 21 weeks, apocynin treatment significantly delayed the onset of UVB-induced skin tumors and only 62% of the mice in the 200 nmol-treated group developed tumors by the end of the study at 25 weeks (Fig. 2d). Importantly, treatment with apocynin at 40 or 200 nmol significantly reduced the number of UVB-induced tumors per mouse (Fig. 2e). Histological examination of skin tumors showed that apocynin treatment suppressed UVB-induced squamous cell carci-

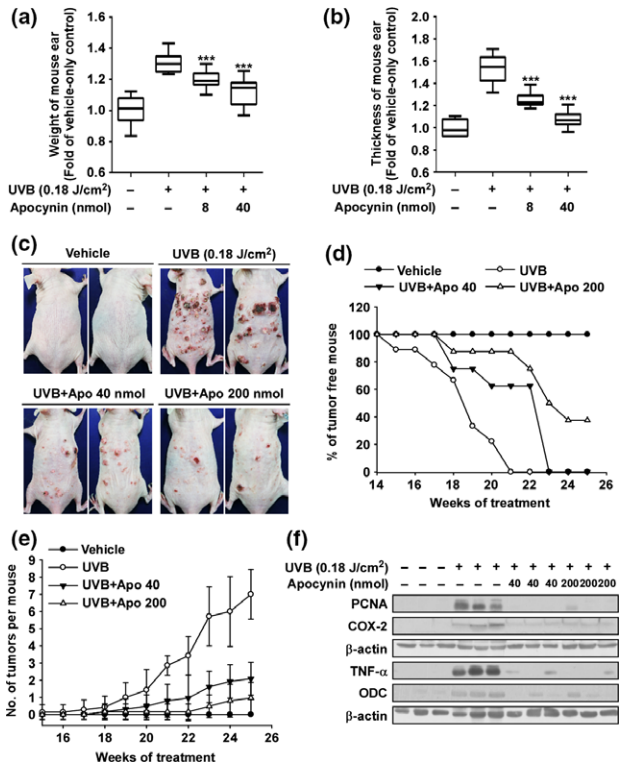


**Figure 2.** Apocynin suppresses UVB-induced skin inflammation and skin tumorigenesis. (a-b) Apocynin inhibits UVB-induced ear oedema development. Female SKH-1 hairless mice had both ears treated with apocynin at the indicated concentrations, or acetone alone after irradiation with UVB as described in the Methods. ***, $P< 0.001$, significant difference between groups treated with both apocynin and UVB and the group treated with UVB alone. (c-e) Apocynin reduces UVB-induced tumor formation *in vivo*. The treatment groups were as follows: not irradiated and acetone only; acetone and UVB irradiated; 40 nmol or 200 nmol of apocynin in 200 $\mu$l of acetone and UVB irradiated. The compound was applied topically to the dorsal skin of female SKH-1 hairless mice ($n$=8). (c) Representative image of experimental animals. (d-e) A tumor was defined as an outgrowth of > 1 mm in diameter that persisted for 2 weeks or longer. Tumor incidence and multiplicity were recorded each week until the end of the experiment. (f) Apocynin treatment suppresses expression of PCNA, COX-2, TNF-$\alpha$ and ODC in UVB-irradiated skin. Dorsal skin samples from three mice were randomly selected from each group and analysed by immunoblotting.

noma development (Table S1, S2 and Figure S1). We next examined whether apocynin affected molecular markers in UVB-irradiated skin. Apocynin treatment attenuated UVB-induced proliferating cell nuclear antigen (PCNA) expression, implying a reduction of UVB-induced proliferation (Fig. 2f). COX-2, TNF-$\alpha$ and ODC are well-known inflammatory markers that are involved in the development of skin cancer (S2–S4). Western blot analysis demonstrated that apocynin strongly downregulated chronic UVB-induced expression of COX-2, TNF-$\alpha$ and ODC in mouse skin (Fig. 2f).

## Conclusion

NMSC is the most common form of skin cancer in many regions of the world (1–4), and although rarely life-threatening, it inflicts a significant burden on healthcare systems (S12). Preventative measures include the avoidance of excessive sun exposure and the liberal use of sunscreen. However, sunscreen cannot ensure the prevention of UVB-induced skin damage and studies have exposed limitations to its efficacy in reducing skin cancer rates (S13–S17). The identification of novel chemoprotective agents that can suppress the formation of UVB-induced skin cancer may therefore provide new leads towards effective prevention strategies.

In the current study, we observed that the NADPH oxidase inhibitor apocynin can significantly suppress the formation of UVB-induced skin cancer. While UVB irradiation induced the expression of key carcinogenic factors in skin cells, treatment with apocynin significantly reduced these effects. It was found that the inhibition of NADPH oxidase activity by apocynin was at least partially responsible for the attenuation of signalling pathways involved in skin inflammation and carcinogenesis. It therefore stands to reason that UVB induces various inflammatory mediators and carcinogenesis through NADPH oxidase, and the pharmacological inhibition of such factors with compounds such as apocynin may represent a novel strategy for the prevention of NMSC.

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## Author contributions

S.B. conducted most of the experimental work. E.L. participated in Fig. 2. Y.J.J. participated in Fig. 1b. Y.K. performed histological analysis. S.B., E.L. and K.W.L. designed the experimental plans, analysed and interpreted the data. K.W.L. directed the project.

## Conflict of interest

The authors have declared no conflicting interests.

## Supporting Information

Additional supporting data may be found in the supplementary information of this article.

**Figure S1.** Representative images of skin tumors.
**Table S1.** Statistics for squamous cell carcinoma and papilloma.
**Table S2.** Histopathology of skin tumors.
**Data S1.** Materials and Methods.
**Data S2.** Supplementary References.

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