#### CONFLICT OF INTEREST

The authors have declared no conflicting interests.

#### AUTHOR CONTRIBUTION

AD, SK and BD performed the experimental work. AR provided the  $IL-10^{FL/FL}$  mice and discussed the data. TJ initiated and designed the study, and SFM and TJ supervised the project and wrote the manuscript. All authors contributed to revising the manuscript and approved the final version.

#### Keywords

allergic contact dermatitis, contact hypersensitivity, immune regulation, skin, T cell

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#### SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

FIGURE S1 Vert-X mice display a normal CHS response to DNFB FIGURE S2 DNFB induces IL-10-eGFP expression in skin draining lymph node cells

**FIGURE S3** IL-10-eGFP<sup>+</sup>CD8<sup>+</sup> T cells of the skin show an activated, effector memory phenotype

**FIGURE S4** CHS response to TNCB or oxazolone induces IL-10-eGFP expression in  $CD8^+$  T cells in the skin

**FIGURE S5** IL-10-eGFP expression of CD8<sup>+</sup> T cells is independent of CD4<sup>+</sup> T cell help

FIGURE S6 IL-10 deficiency in IL-10  $^{\rm FL/FL}$  E81-Cre $^+$  mice is selective for CD8 $^+$  T cells

DATA S1 Supplementary methods

# AP736 induces miR-125b expression for the efficient whitening and anti-ageing action in human epidermal cells

# 1 | BACKGROUND

AP736, a recently developed whitening compound, is a benzylbenzamide derivative that strongly inhibits melanin synthesis by inhibiting tyrosinase expression and activity, and suppressing the expression of tyrosinase-related protein (Tyrp)-1 and Tyrp-2, and microphthalmiaassociated transcription factor (MiTF).<sup>[1,2]</sup> MicroRNAs (miRNAs) are known to regulate skin biology, including the pigmentation process in melanocytes.<sup>[3]</sup> For example, miR-340 regulates UVB-induced dendrite formation and melanosome transfer in melanocytes.<sup>[4]</sup> miRNA signatures in vitiligo also regulate melanocyte biology.<sup>[5]</sup> Recently, Kim et al.<sup>[6]</sup> in our research group found that miR-125b is a potent inhibitor of steady-state melanogenesis. In addition, other research groups have reported that miR-125b

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downregulates the expression of matrix metalloproteinase (MMP)-7 and MMP-13 in cutaneous carcinoma cells, and MMP-2 and MMP-9 in HepG2 cells.<sup>[7,8]</sup>

These studies have suggested that AP736-induced miR-125b expression can be an effective strategy for exerting whitening effects through inhibition of melanogenesis as well as promoting anti-ageing effects through inhibition of MMPs in skin cells. However, intracellular cAMP reduces the expression of miR-125b in melanocytes,<sup>[6]</sup> and ROS also inhibit miR-125b expression by increasing promoter methylation of the miR-125b gene.<sup>[9]</sup> Therefore, decreasing cAMP levels or suppressing ROS production by AP736 can be helpful for increasing miR-125b expression, resulting in whitening and anti-ageing effects, especially in UVB-stimulated epidermal cells.

## 2 | QUESTIONS ADDRESSED

In this study, we examined whether AP736 inhibits melanogenesis in human melanocytes by increasing miR-125b expression, a depigmentation inducer. We also tested whether AP736 suppression of MMP expression and ROS production, which contribute to the anti-ageing efficacy of AP736, involves increased miR-125b expression in human keratinocytes.

## 3 | EXPERIMENTAL DESIGN

We used human melanocytes, keratinocytes and the artificial 3D human skin model, Melanoderm (MatTek Corp, Ashland, MA, USA), to investigate the antimelanogenic and anti-ageing effects of AP736 and associated changed in miR-125b expression. Detailed methods can be found in the supplement.

#### 4 | RESULTS

In our previous report, we evaluated the whitening effect of AP736 (Figure S1) in melanocytes and an artificial skin model.<sup>[2]</sup> In the present study, we sought to define whether AP736 inhibits melanin synthesis by inducing miR-125b expression. Accordingly, we analysed the expression levels of miR-125b in normal human primary melanocytes following treatment with AP736. As shown in Figure 1A, AP736 significantly increased miR-125b expression approximately threefold. Furthermore, because cAMP decreases miR-125b expression [4] and AP736 inhibits cAMP production,<sup>[2]</sup> we tested whether AP736 restores cAMP-inhibited miR-125b expression in WM266-4 human melanocytes. As expected, the cAMP inducer, forskolin, inhibited miR-125b expression; notably, this inhibitory effect was reversed by AP736, which, like the cAMP inhibitor MDL-12330A (positive control), dramatically increased miR-125b expression in forskolin-treated melanocytes (Figure 1B). These data suggest that AP736 induces miR-125b, possibly through inhibition of cAMP, although other mechanisms are involved in AP736-induced miR-125b expression.



FIGURE 1 AP736 increased miR-125b expression and appeared depigmentation in human primary melanocytes and an artificial skin tissue model. (A) Expression level of miR-125b using real-time PCR. Normal human melanocytes (NHMs) were treated with AP736 (2uM) for 24 h. (B) Expression level of miR-125b using real-time PCR. WM-266-4 (human melanoma cells) were treated with MDL-12330A (5 µmol/L) or AP736 (3 µmol/L) in the presence of forskolin (15uM) for 24 h. (C) Colour of an artificial skin tissue model. Skin tissues (Melanoderm) were exposed to 20 mJ/cm<sup>2</sup> UVB and then incubated with AP736 (300  $\mu$ mol/L) for 18 d as described in methods. (D) The degree of depigmentation in skin tissue model. The  $\Delta L$  value between vehicle and AP736-treated groups. (E) The H&E and F&M staining of tissue sections. The slides were fixed in formaldehyde solution and embedded in paraffin wax for the staining. The red arrowheads indicate the keratinocytes possessing transferred melanin, and the blue arrowheads indicate the dendritic stretch of melanocytes. (F) The densitometry of melanin staining. (G) Expression level of miR-125b using real-time PCR in an artificial skin tissue. Total RNA of skin tissues was extracted from cell lysates using TRIzol reagent. Data are representative of at least three repetitions. Values are expressed as the mean±SE. Statistical difference from vehicle by Student's t-test or forskolin treatment group by one-way ANOVA test. (##P<.01, \*\*P<.01, \*\*\*P<.001)

Next, we confirmed that AP736 also induces miR-125b expression in an artificial skin model, where it showed whitening efficacy. As shown in Figure 1C,D, AP736 lightened the colour of the skin tissue and significantly changed the  $\Delta$ L value (the degree of pigmentation), as expected. Haematoxylin and eosin (H&E) and Fontana–Masson (F&M) staining of tissue sections revealed that the normal structure of skin tissue was maintained, melanin was rarely transferred from melanocytes to keratinocytes, the dendritic stretch of melanocytes was inhibited and melanin content were decreased in the presence of



FIGURE 2 AP736 increased miR-125b expression and inhibited MMP-1 and MMP-3 expressions in human primary keratinocytes. (A) Expression level of miR-125b using real-time PCR. Normal human epithelial keratinocytes (NHEKs) were treated with AP736 for 24 h after UVB exposure. (B) DPPH scavenging ability of AP736. Free radical scavenging activity of AP736 was tested using cell-free system, as described in supplementary method of Data S1. (C) ROS inhibitory ability of AP736. AP736 was treated to UVB-exposed HaCaT human keratinocytes, and ROS production was determined using Dcf-da assay, as described in supplementary method of Data S1. (D)-(E) The secreted protein level of matrix metalloproteinase. NHEK cells were treated with AP736 (2uM) in the presence of UVB exposure, and the collected medium was used for MMPs antibody array. Among MMPs, the secreted protein level of MMP-1 (D) and MMP-3 (E) was significantly changed by AP736 in UVB-exposed cells. Data are representative of at least three repetitions. Values are expressed as the mean±SE. Statistical difference by one-way ANOVA test. (##P<.01, \*P<.05, \*\*P<.01)

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AP736 (Figure 1E,F). Expression of the major melanogenic enzymes, tyrosinase, Tyrp-1 and Tyrp-2, and MiTF were significantly suppressed by AP736 treatment (Figure S2A–D). As expected based on results in melanocytes, AP736 dramatically increased miR-125b expression in the skin tissue model (Figure 1G). These data suggest the possibility that AP736 exerts it depigmentation effect by inducing miR-125b expression, and thereby inhibiting melanogenic enzyme expression and melanin synthesis.

In addition to its effects in melanocytes, we also found that AP736 increased miR-125b expression in UVB-stimulated keratinocvtes (Figure 2A). To define the possible mechanism for miR-125b expression by AP736, we investigated whether AP736 suppresses ROS production because ROS inhibition can cause miR-125b expression.<sup>[7]</sup> As shown in Figure 2B,C, AP736 dramatically scavenged 2,2-diphenyl-1-picrylhydrazyl (DPPH) in a cell-free system and inhibited ROS production in UVB-stimulated HaCaT keratinocytes. These data suggest that AP736 suppresses ROS production, resulting in increased miR-125b expression in the presence of UVB irradiation. Previous reports have also shown that miR-125b inhibits expression of several MMPs.<sup>[5,6]</sup> Therefore, we investigated the effect of AP736 on the expression of MMPs in UVB-irradiated keratinocytes using MMP arrays. These analyses showed that UVB dramatically increased secretion of MMP-1 and MMP-3 by keratinocytes, whereas AP736 clearly inhibited both secretion (Figure 2D,E) and mRNA expression (Figure S3A,B) of MMP-1 and MMP-3 induced by UVB irradiation. Inhibition of MMP-1 by AP736 was also confirmed in an artificial skin model (Figure S3C). These results suggest that AP736 exerts antioxidative (ROS inhibition) and anti-ageing effects (MMPs inhibition) in UVB-irradiated keratinocytes. These actions of AP736 could involve an increase in miR-125b expression, although further studies, such as miR-125b knock down, will be required to confirm the detailed mechanism.

# 5 | CONCLUSION

In conclusion, our current data suggest the possibility that AP736 acts through a novel mechanism involving induction of miR-125b expression to cause the inhibition of melanin synthesis in melanocytes. AP736 also simultaneously suppresses MMP-1 and MMP-3 expression as well as ROS production, at least in part, through a signalling pathway involving miR-125b induction in keratinocytes. Taken together with previous reports, this study provides the first evidence to support a role for increased miR-125b expression in the antimelanogenic and anti-ageing actions of AP736.

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This work was approved by the Ethical Committee of the AMOREPACIFIC corporation. HS Baek, JA Hwang and IH Bae performed the research. KH Kim and YH Joo contributed essential tools. YJ Kim, HJ Shin, JH Lee and YH Park analysed the data. JW Kim and CS Lee designed the research study and wrote the manuscript.

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#### CONFLICT OF INTERESTS

The authors have declared no conflicting interests.

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#### SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 The structure of AP736 compound Figure S2 Expression levels of tyrosinase, Tyrp-1, Tyrp-2 and MiTF Figure S3 Expression levels of matrix metalloproteinase Data S1 Materials and Methods

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# High diversity of the T-cell receptor repertoire of tumor-infiltrating lymphocytes in basal cell carcinoma

#### Abstract

Whether specific T-cell clones are present in tumor infiltrating lymphocytes (TILs) in BCC is unknown. We employed deep sequencing of mRNA coding for the T-cell receptor (TCR) chains  $\alpha$ - and  $\beta$  to characterize the repertoire of TILs in BCC. V and J gene-usage and CDR3 length were computed to determine the clonality of TCR and degree of overlap in TCR repertoires between skin resident T-cells and TILs. We found high diversity of the TCR repertoire in BCC and control skin with random V-J gene usage and similar CDR3-length distribution. Lack of TCR repertoire restriction indicates absence of tumor-specific TIL clones in BCC.

#### 1 | BACKGROUND

Basal cell carcinoma (BCC) of the skin is the commonest cancer worldwide with a locally invasive growth pattern.<sup>[1]</sup> The tumor microenvironment is characterized by abundant tumor-infiltrating lymphocytes (TILs) thought to be involved in cancer immunoediting,<sup>[2]</sup> the process in which early interaction between tumor cells and lymphocytes favours tumor inhibition followed by disequilibrium promoting tumor growth in later stages.<sup>[3]</sup> This disequilibrium may be further perturbed by immunosuppression, as documented by significantly increased risk of BCC in solid organ transplant recipients and allogeneic hematopoietic stem cell transplant recipients.<sup>[4,5]</sup> We have recently studied TILs in BCC and identified recruitment of T-regs to BCC causing local immunosuppression.<sup>[6]</sup>