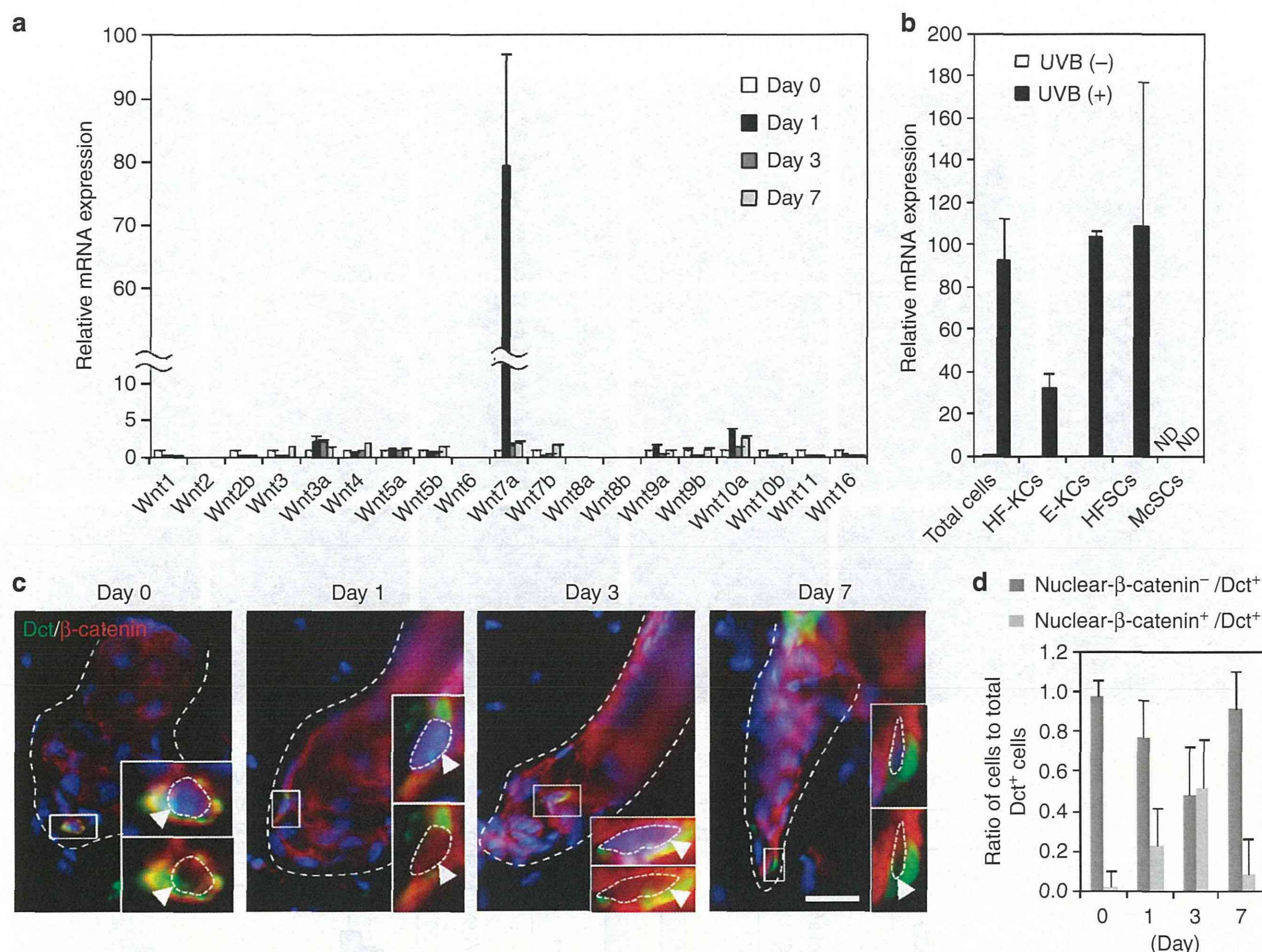
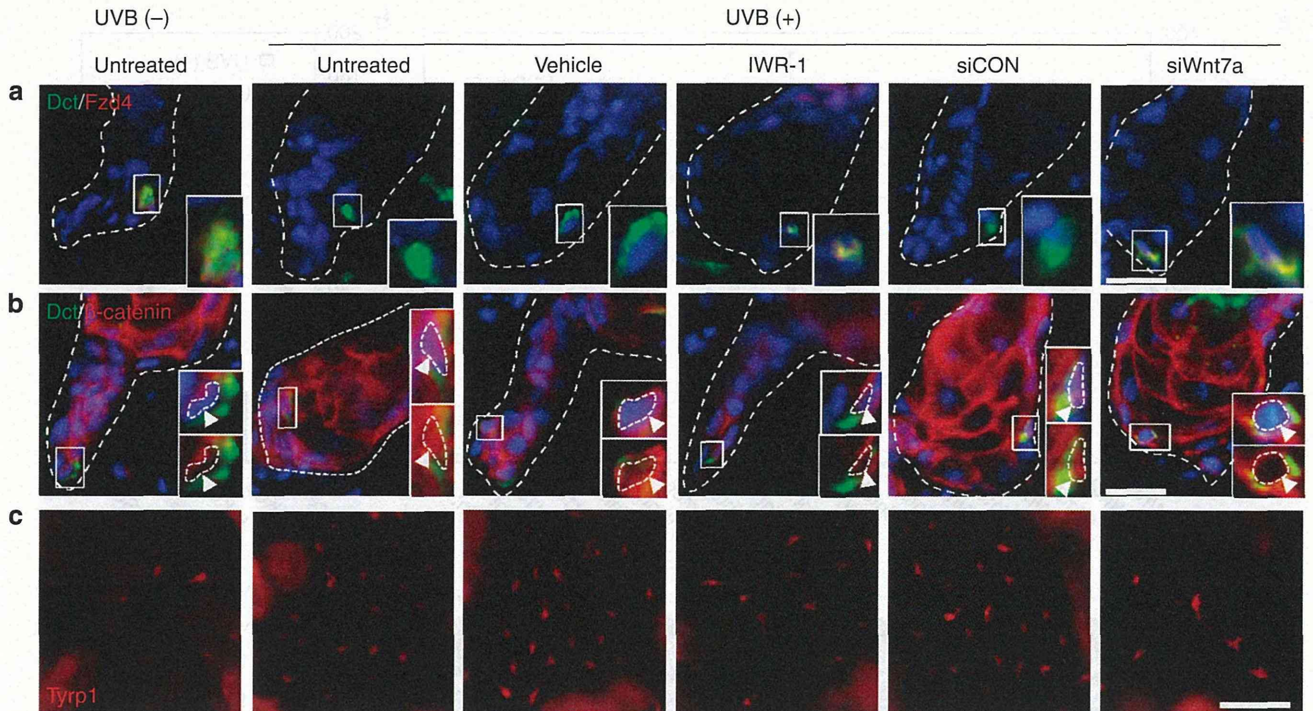


Figure 3. Wnt/β-catenin signaling was activated by UVB irradiation. (**a**) mRNA expression levels of Wnt genes in epidermis were analyzed by real-time PCR. (**b**) After separation of hair follicle stem cells (HFSCs), hair follicle keratinocytes (HF-KCs), epidermal keratinocytes (E-KCs), and melanocyte stem cells (McSCs) by FACS as described in Materials and Methods, gene expression analysis was performed (ND, not detected). (**c**, **d**) Intracellular localization of β-catenin in Dct^+ cells was analyzed by immunostaining in skin sections (scale bar = 20 μm). The ratio of each population to total Dct^+ cells was calculated (**d**). Dct, dopachrome tautomerase. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Right lower panels show enlargement of the boxed region. Arrowheads indicate nucleus.

Figure 4a-f shows that IWR-1 injection suppressed the decrease in the number of McSCs, nuclear translocation of β-catenin on day 1, the increase in the number of epidermal melanocytes, and the mRNA expression levels of melanogenic enzymes on day 14. Wnt7a siRNA injection reduced the mRNA level of Wnt7a induced by UVB irradiation to 66 ± 3.2% when compared with control siRNA injection, and inhibited nuclear translocation of β-catenin and the decrease in the number of McSCs on day 1 (Figure 4a, b, and g). Wnt7a knockdown also suppressed the increase in the number of epidermal melanocytes on day 14 and mRNA expression levels of melanogenic enzymes as well as IWR-1 injection (Figure 4c, h and i). These results showed that β-catenin activated by Wnt7a was involved in McSC differentiation in the process of UVB-induced epidermal pigmentation.

Finally, we analyzed the UVB-induced pigmentation mechanism in human cells. Previously, normal human epidermal keratinocytes (NHEKs) and normal human epidermal melanocytes (NHEMs) were used to investigate the suppressive effect of TGF-β on McSC differentiation as an *in vitro* model (Nishimura *et al.*, 2010). We used this *in vitro* model to study how Wnt/β-catenin signaling regulates McSC differentiation. UVB irradiation elevated WNT7A mRNA expression in NHEKs, but not in NHEMs (Figure 5a). 6BIO (Noda *et al.*, 2009; Yan *et al.*, 2009), a well-known chemical activator of the canonical Wnt signaling pathway, and recombinant WNT7A protein enhanced Dct mRNA expression in NHEMs (Figure 5b). In mouse McSCs, activated β-catenin in early anagen follicles promotes Dct expression, which is kept at a lower level in resting telogen stage hair follicles (Lang *et al.*, 2005). NHEM/NHEK co-culture experiment demonstrated that UVB irradiation on NHEKs elevated Dct expression in NHEMs, and IWR-1 inhibited its elevation (Figure 5c). These results indicated that keratinocyte-derived Wnt triggered McSC differentiation through Wnt/β-catenin signaling. Taken together, it was demonstrated that Wnt/β-catenin signaling regulates McSC differentiation into epidermal melanocytes induced by UVB irradiation.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a
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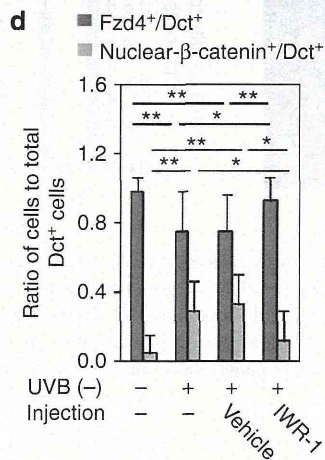
c
Tyrp1


d
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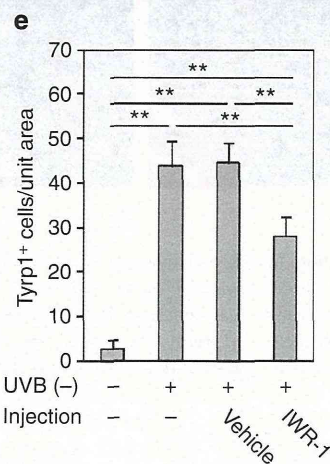
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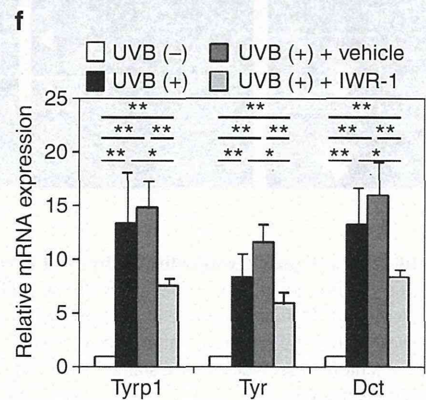
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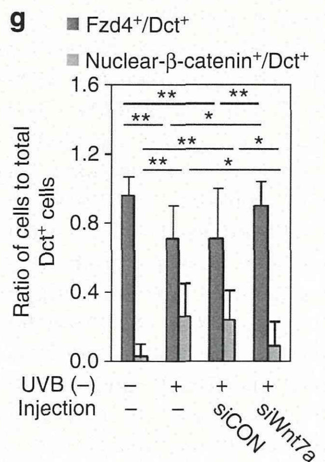
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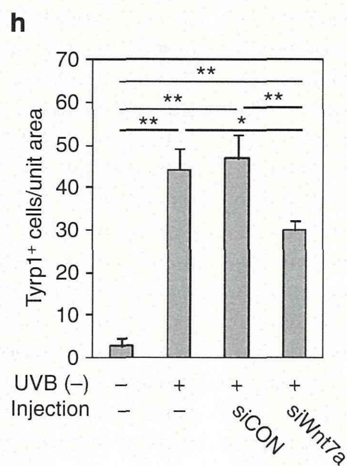
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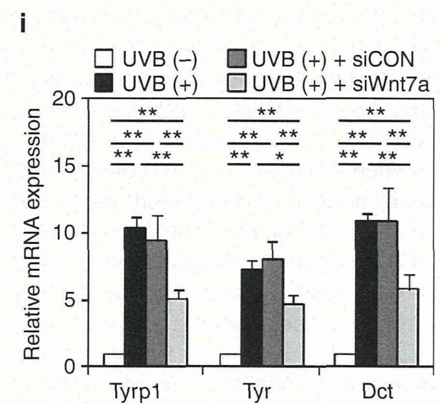
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UVB (+) + siWnt7a
Relative mRNA expression
Tyrp1
Tyr
Dct

DISCUSSION

UV radiation is a well-known inducer of epidermal pigmentation and has been used as the most important therapy for vitiligo treatment, a skin depigmentation disorder (Falabella, 2009; Falabella and Barona, 2009). Although UV radiation and other treatments for vitiligo such as topical corticosteroids, laser treatment, and melanocyte transplantation can induce repigmentation, these treatments require long-term and frequent ambulatory care or cause surgical stress and do not always achieve complete repigmentation. Therefore, elucidation of the relationship between McSC differentiation and UV radiation–induced skin pigmentation may be of great help to understand the differentiation mechanism of McSCs into epidermal melanocytes, and it could enable us to develop new treatment options for vitiligo. In this study, we addressed this issue and found that UVB-induced Wnt7A upregulation triggered McSC differentiation through β-catenin activation.

We first confirmed that epidermal Tyrp1$^+$ cells and melanogenic genes began to increase on day 7 before apparent epidermal pigmentation was observed on day 14 (Figure 1). Before the increase in the number of epidermal Tyrp1$^+$ cells, transient emergence of melanoblasts in hair follicles on day 3 and a decrease in the number of

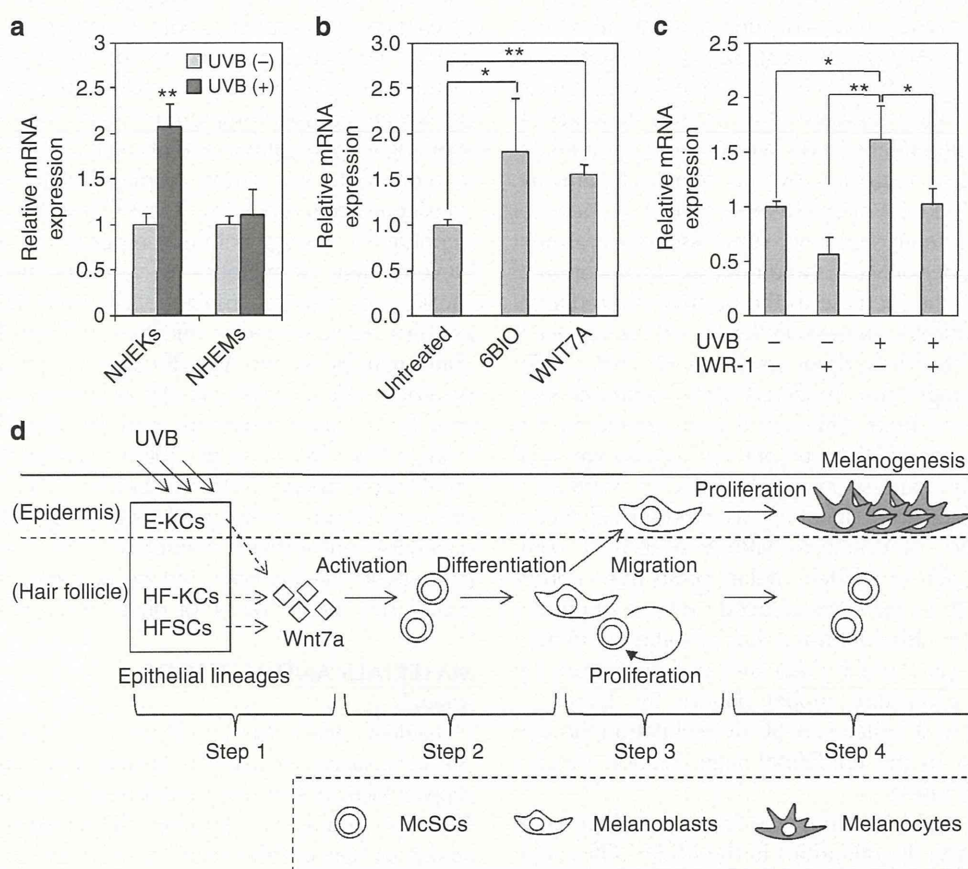

Figure 5. Wnt/β-catenin signaling activated the differentiation of human melanocytes *in vitro*. (**a**) After 24 hours of UVB irradiation (10 mJ cm^{-2}), changes in mRNA expression levels of WNT7A in normal human epidermal keratinocytes (NHEKs) and normal human epidermal melanocytes (NHEMs) were analyzed by real-time PCR ($n=4$ per group, mean ± SD). $^{**}P<0.01$ versus UVB (−) group as determined by *t*-test. (**b**) Effects of 6BIO and recombinant WNT7A on mRNA expression level of dopachrome tautomerase (Dct) in NHEMs was analyzed by real-time PCR ($n=4$ per group, mean ± SD). $^{*}P<0.05$, $^{**}P<0.01$ versus untreated control group as determined by *t*-test. (**c**) NHEMs were co-cultured with NHEKs just after UVB irradiation in the presence or absence of IWR-1 (inhibitor of Wnt response 1). After 24 hours, NHEMs were further cultured without NHEKs for 48 hours and then the mRNA level of Dct was analyzed ($n=4$ per group, mean ± SD). $^{*}P<0.05$, $^{**}P<0.01$ versus UVB (−)/IWR (−) group as determined by analysis of variance with Tukey–Kramer multiple comparison. (**d**) Schematic representation of UVB-induced epidermal pigmentation process. E-KC, epidermal keratinocyte; HF-KC, hair follicle keratinocyte; HFSC, hair follicle stem cell; McSCs, melanocyte stem cells.

Figure 4. Wnt/β-catenin signaling regulated melanocyte stem cell (McSC) differentiation. (**a**, **b**, **d**, **e**) Effects of intradermal injection of IWR-1 (inhibitor of Wnt response 1) or Wnt7a small interfering RNA (siRNA) on the number of McSCs (**a**) and intracellular localization of β-catenin in hair follicles (**b**) on day 1 were analyzed by immunostaining in skin sections. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Scale bar = 20 μm. Right lower panels show enlarged images of the boxed region. Arrowheads indicate nucleus. The ratio of each population to total Dct$^+$ cells was calculated (**d**, **g**). Dct, dopachrome tautomerase; Fzd, Frizzled; Tyr, tyrosinase; Tyrp1, tyrosinase-related protein-1. (**c**, **f–i**) Effects of intradermal injection of IWR-1 or Wnt7a siRNA on the number of melanocytes (**c**, **e**, **h**) and mRNA levels of melanogenic enzymes (**f i**) were analyzed by immunostaining in epidermis. Scale bar = 50 μm ($n=6$ per group, mean ± SD). $^{*}P<0.05$, $^{**}P<0.01$ versus UVB (−) group as determined by analysis of variance with Tukey–Kramer multiple comparison.

McSCs (Figure 2) were observed. Furthermore, after depletion of melanoblasts and melanocytes that Kit-dependently survive by administration of the neutralizing antibody for the Kit receptor, UVB radiation caused a transient emergence of melanoblasts in hair follicles on day 3 and an increase in the number of Tyrp1$^+$ cells on days 7 and 14 (Figure 2). Based on these results, it was suggested that UVB induced melanocytogenesis from McSCs before skin pigmentation. The transient decrease of McSCs can be explained by the immediate differentiation of McSCs into melanocytes without proliferation in order to respond to harmful stimulation such as UVB radiation and to protect skin. Interestingly, UVB radiation markedly elevated Wnt7a expression only in all Wnts in epithelial lineages (HFSCs, HF-KCs, and E-KCs) on day 1 and also induced the nuclear translocation of β-catenin in hair follicles (Figure 3). As Wnt/β-catenin signaling is important for McSC differentiation into hair bulb melanocytes as described above (Lang *et al.*, 2005; Rabbani *et al.*, 2011), it raised the possibility that differentiation of McSCs into epidermal melanocytes was also regulated by the signaling pathway. Indeed, administration of Wnt7a siRNA or IWR-1, a chemical inhibitor of Wnt/β-catenin signaling, suppressed the transient decrease in the number of McSCs and nuclear translocation of β-catenin as well as the increase in the number of epidermal melanocytes and mRNA expression levels of melanogenic enzymes (Figure 4); therefore, these results revealed that UVB-induced Wnt7A upregulation triggered McSC differentiation through β-catenin activation. Although it was considered that incomplete suppression of the number of melanocytes and melanogenic enzyme expression was because of insufficient inhibition of Wnt/β-catenin signaling, as represented in the knockdown efficiency of Wnt7a by siRNA ($66 \pm 3.2\%$ compared with control siRNA), resident melanoblasts in epidermis may partly contribute to the UVB-induced increase of epidermal melanocytes. We also confirmed that inhibition of Wnt/β-catenin signaling suppressed human melanocyte maturation using an *in vitro* co-culture model (Figure 5). Thus, we concluded that Wnt7a stimulates McSC differentiation through β-catenin activation in the epidermal pigmentation process induced by UVB radiation.

Based on the results of this study, we found that there were several similarities and dissimilarities in the McSC differentiation mechanisms between epidermal and hair pigmentation processes. Interestingly, both processes require Wnt/β-catenin signaling activation for McSC differentiation, which is triggered by epithelial lineage–derived Wnt molecules. UVB stimulates HFSCs, HF-KCs, and E-KCs to specifically express Wnt7a in the former process, whereas hair cycle transition from telogen to anagen causes Wnt secretion only by HFSCs in the latter process, in which the specific Wnt isoform remains to be elucidated. It is reasonable for an organism to exploit a common signaling pathway in distinct situations such as epidermal and hair pigmentation with respect to an efficient response to various stimuli. The epidermal pigmentation process is an extensive event that occurs in all areas of UVB-exposed skin, whereas hair cycle progression is regulated in small areas, bulge, and dermal papillae (Millar, 2002; Alonso and Fuchs, 2003; Sharov *et al.*, 2005; Enshell-Seijffers *et al.*, 2010). Therefore, it is thought that HF-KCs and E-KCs as well as HFSCs respond to UVB radiation and express a higher level of Wnt7a.

This study demonstrated that McSC differentiation was involved in UVB radiation–induced epidermal pigmentation. This process consists of four steps, as shown in Figure 5d: (1) Wnt7a upregulation by UVB radiation, (2) β-catenin nuclear translocation and rapid differentiation of McSCs into melanoblasts in hair follicles, (3) melanoblast migration into epidermis and McSC proliferation in bulge area, and (4) melanoblast proliferation and initiation of melanogenesis. We believe that our findings may be of great help in developing new therapeutic options for vitiligo. For example, various chemical agents in the signaling pathway may be exploited for treatment (Lim *et al.*, 2008; Zhong *et al.*, 2009; Nyati *et al.*, 2010), enabling patients to be relieved of consecutive therapeutic UV radiation or other invasive therapies. It has been reported that immature melanoblasts existed in the long-standing vitiligo epidermis (Tobin *et al.*, 2000), and are thought to contribute to vitiligo repigmentation. Therefore, it is considered that not only McSCs but also melanoblasts are important for repigmentation, especially in the glabrous skin. Development of methods to regulate the differentiation of McSCs as well as melanoblasts will eventually lead to establishment of more effective treatment for vitiligo. As abnormalities in the Wnt/β-catenin signaling pathway can possibly induce aberrant differentiation of McSCs, which may be a cause of various pigment disorders such as solar lentigo (Cario-Andre *et al.*, 2004; Noblesse *et al.*, 2006; Helm and Findeis-Hosey, 2008), we believe that further investigation of the relationship between the signaling pathway and pigment disorders will provide important clues to understand the pathogenic mechanisms and be of great help in developing therapeutic technologies for pigment disorders.

MATERIALS AND METHODS

Animals

F1 hairless mice of HR-1 × HR/De were obtained from Japan SLC (Shizuoka, Japan). All animal experiments were approved by both the Nippon Menard Research Laboratories Subcommittee on Research Animal Care and the Education and Research Center for Animal Models of Human Diseases of Fujita Health University on Research Animal Care.

UVB irradiation and sample collection

Seven-week-old mice were exposed to UVB three times a week by Toshiba FL-20 SE fluorescent lamps (Toshiba Electric, Tokyo, Japan). The daily dose was $100\,mJ\,cm^{-2}$. Dorsal skin was obtained on days 0, 1, 3, 7, 14, and 28 and immediately embedded in optimal cutting temperature compound or fixed with 4% paraformaldehyde to prepare fresh-frozen sections or paraffin-embedded sections, respectively. Epidermal sheets were separated from collected skin by forceps after incubation in 2 M sodium bromide at 37 °C for 2 hours.

Intradermal injection of ACK2, Wnt/β-catenin signaling inhibitor, and siRNA into mouse dorsal skin

ACK2 (neutralizing antibody for the c-kit receptor; BioLegend, San Diego, CA) was injected into the dorsal skin of 6-week-old mice

(20 μg per cm^2) three times a week (7, 5, and 3 days before initiation of UVB irradiation). A 10 mM IWR-1 stock solution in DMSO was diluted to 0.1 mM in phosphate-buffered saline (PBS) and then injected into the dorsal skin of mice (4 μg per cm^2); 1% DMSO in PBS was treated as the control. As previously reported (Murase *et al.*, 2009), a mixture of specific Stealth siRNAs directed against Wnt7a (oligo ID: MSS238710, MSS238711, MSS278848; Life Technologies, Carlsbad, CA) or negative control siRNA (Stealth RNAi siRNA Negative Control High GC; Life Technologies) was injected with *in vivo*-jetPEI (Polyplus-transfection SA, Illkirch, France) into the dorsal skin of mice (5 μg per cm^2). IWR-1 and siRNAs were treated for three consecutive days on days −1, 0, and 1.

Immunohistochemistry

Epidermal sheets were fixed with 4% paraformaldehyde and processed for immunostaining using anti-Tyrp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa Fluor 594–labeled anti-goat IgG (Life Technologies). Fresh-frozen sections were prepared by cryostat (Carl Zeiss, Thornwood, NY). These sections were fixed with acetone for 10 minutes at −20 °C, and were then incubated in 3% H_2O_2 in PBS for 5 minutes to quench endogenous peroxidase activity. TSA kit (Life Technologies) was used to detect Dct, as previously reported (Botchkareva *et al.*, 2001). Briefly, sections were blocked for 1 hour in a blocking reagent and were then incubated overnight at 4 °C with anti-Dct goat antibody and other primary antibodies as listed in Supplementary Table S1 online. After washing with PBS, the sections were further incubated for 1 hour at room temperature with horseradish peroxidase–labeled anti-goat IgG and corresponding Alexa 594-labeled secondary antibodies, followed by a 5-minute application of Alexa 488 tyramide. DAPI (4′,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA) was used for nuclear staining.

Dopa reaction and Fontana–Masson staining

To visualize dopa-positive melanocytes, epidermal sheets were fixed with 10% formalin for 30 minutes and then incubated in 0.1% L-dopa in PBS for 3 hours at 37 °C. Fontana–Masson staining was also performed on paraffin-embedded sections to display the amount and distribution of melanin.

Laser microdissection

Fresh-frozen sections were prepared with a cryostat on film slides. The sections were fixed in 75% ethanol and stained with 0.05% toluidine blue. After drying the sections, hair follicles and epidermis were separately isolated with a laser microdissection system Leica LMD6000 (Leica Microsystems, Wetzler, Germany).

Cell sorting

Mouse dorsal skin (0.5 mm^2 pieces) was incubated on 0.25% trypsin (BD Biosciences, San Jose, CA) for 2 hours at 37 °C and the epidermis was separated from the dermis with forceps. After incubation in 0.02% EDTA for 10 minutes at 37 °C, obtained cell suspension was filtrated and centrifuged, and cells were then isolated by FACS (FACSAria, BD Biosciences). In brief, the cells were stained with FITC-conjugated anti-CD34, phycoerythrin-conjugated anti-CD49f (BioLegend), and biotin-conjugated anti-Fzd4 (R&D Systems, Minneapolis, MN) antibodies for 30 minutes at 4 °C followed by allophycocyanin-conjugated StreptAvidin for 15 minutes at 4 °C. After staining with antibodies, HFSCs (CD34$^+$/CD49f$^+$), HF-KCs (CD34$^-$/CD49f^{Low}), E-KCs (CD34$^-$/CD49f^{High}), and McSCs (Fzd4$^+$) were sorted with FACS.

Cell culture

NHEKs and normal NHEMs were purchased from TOYOBO (Osaka, Japan) and maintained between passages 1 and 3 in defined keratinocyte serum-free medium and Medium 254 containing human melanocyte growth supplement (Invitrogen, Carlsbad, CA), respectively. After replacement of culture medium with PBS (−), cells were irradiated with UVB at a dose of 10 mJ cm^{-2} and incubated in fresh culture medium for 24 hours. Total RNA was then extracted for measurement of WNT7A expression level. NHEMs were incubated with 10 μM 6BIO (Enzo Life Sciences, Farmingdale, NY) or 10 μg ml^{-1} recombinant human WNT7A protein for 72 hours and total RNA was extracted. NHEKs and NHEMs were co-cultured using membrane inserts for 24-well culture plates with a pore size of 1.0 μm (BD Biosciences). Briefly, NHEKs were seeded onto 24-well plates at a density of 4×10^4 cells per well and cultured for 48 hours. Culture medium was changed to PBS (−) and UVB was irradiated at a dose of 10 mJ cm^{-2}. PBS (−) was replaced with fresh defined keratinocyte serum-free medium and 4×10^4 cells of NHEMs were seeded in the upper chamber coated with 0.1% gelatin. At 24 hours after co-culture, NHEMs were further cultured for 48 hours.

Real-time reverse transcriptase–PCR (RT–PCR)

Total RNA were extracted from epidermal sheets or culture cells using TRIzol Reagent (Invitrogen) and the RNeasy Micro Kit (Qiagen, Hilden, Germany) from laser microdissection samples. Complementary DNA was synthesized by reverse transcription and real-time semiquantitative RT–PCR was performed with the SuperScript III Platinum Two-Step qRT–PCR kit (Invitrogen), using the StepOnePlus real-time RT–PCR system (Applied Biosystems, Tokyo, Japan). Primer sequences are indicated in Supplementary Table S2 online. Amplification was normalized to a housekeeping gene, *glyceraldehydes-3-phosphate dehydrogenase* (*Gapdh*) or *18S ribosomal RNA*, and differences between samples were quantified based on the ΔΔCt method.

Statistical analysis

All experiments were carried out in duplicate or triplicate and were replicated three times. Data are presented as the mean ± SD. $P<0.05$ was considered significant. Statistical analysis was performed using analysis of variance with Tukey–Kramer multiple comparison.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Biol. Pharm. Bull. **36**(11) 1722–1730 (2013) Vol. 36, No. 11

Analysis of the Effects of Hydroquinone and Arbutin on the Differentiation of Melanocytes

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Received March 12, 2013; accepted August 12, 2013

Hydroquinone (HQ) is a chemical compound that inhibits the functions of melanocytes and has long been known for its skin-whitening effect. According to previous studies, the Tyrosinase (Tyr) activity inhibitory effect and melanocyte-specific cell toxicity are known depigmenting mechanisms; however, details of the underlying mechanisms are unknown. Arbutin (Arb) is also known for its Tyr activity inhibitory effect and is commonly used as a skin-whitening agent. However, the detailed depigmenting mechanism of Arb is also not yet fully understood. Few studies have attempted to elucidate the effects of HQ and Arb on undifferentiated melanocytes. In this study, we examined the effects of HQ and Arb throughout each stage of differentiation of melanocytes using a mouse embryonic stem cell (ESC) culture system to induce melanocytes. The results showed that HQ in particular downregulated the early stage of differentiation, in which neural crest cells were generated, and the late stage of differentiation, in which melanogenesis became active. On the other hand, Arb had no effect on the differentiation of melanocytes, and only suppressed melanogenesis by specifically suppressing elevations in Tyr expression in the late stage of differentiation.

Key words melanocyte; hydroquinone; arbutin; embryonic stem cell; differentiation

Melanocytes in the epidermis of the skin produce melanin, which is responsible for determining the color of human skin and hair. The generation and differentiation of melanocytes have been studied for many years and are now well understood; for example, melanocytes originate from undifferentiated neural crest cells derived from the neural tube during the embryonic stage,[1] these cells then migrate to the dermis and epidermis until they mature and ultimately colonize hair follicles. Melanocyte precursors in hair follicles partially exist as undifferentiated melanocyte stem cells around the bulge area. These melanocyte stem cells have been shown to proliferate and differentiate into mature melanocytes as necessary and colonize the epidermis and hair follicles.[2–4] In addition, mature melanocytes transfer melanosomes to peripheral keratinocytes and hair matrix cells, which may affect the color of skin and hair.[5] Given these findings, melanocytes undergo diverse stages of differentiation.

If an abnormality occurs in the differentiation and proliferation of melanocytes and the mechanism of melanin synthesis, it may cause various diseases; for example, when a mutation occurs in microphthalmia-associated transcription factor (MITF-M), which is a master gene of melanocytes seen in neural crest cells immediately after their migration from the neural tube, it may cause the abnormal differentiation and proliferation of neural crest cells, resulting in Waardenburg syndrome type 2, which is characterized by white spots on the skin, iris heterochromia, and deafness.[6] Paired box gene 3 (PAX3) and SRY-box containing g gene 10 (SOX10) are expressed in the neural tube before the first neural crest cells delaminate, and regulate MITF-M expression. When mutations occur in PAX3 and SOX10, it may cause Waardenburg syndrome types 1, 3, and 4.[6]

Melanin arises from the amino acid, tyrosine. Tyrosinase (Tyr) catalyzes three different reactions in the biosynthetic pathway of melanin: 1) the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA); 2) the oxidation of DOPA to DOPA quinone; and 3) the oxidation of 5,6-dihydroxyindole (DHI) to indole-quinone.[7] When these proteins develop abnormalities and lose their activity, melanin production does not occur, which eventually causes oculocutaneous albinism type I (OCA1).[6]

The most important role of melanin is to protect the skin against UV (ultraviolet) radiation. Melanosomes are transferred from melanocytes to neighboring keratinocytes in order to form perinuclear melanin caps that protect DNA from UV damage. Melanocytes are important for the human body; however, when melanogenesis becomes hyperactive in melanocytes, the skin consequently develops epidermal hyperpigmentation including melasma, freckles, and senile lentigines. UV is one of the major external factors that affects the proliferation and differentiation of melanocytes. In mice, factors such as Endothelin 1 (Edn1), Kit ligand (Kitl), and granulocyte/macrophage colony-stimulating factor (GM-CSF) are secreted from epidermal keratinocytes and fibroblasts in response to a stimulus from ultraviolet A (UVA) and ultraviolet B (UVB), and promote the differentiation and proliferation of melanocytes.[8] Senile lentigines appear as a result of an increase in melanocytes that have been matured due to UV exposure and constantly repeated melanin synthesis.

When an abnormality occurs in the generation, differentiation, and melanin synthesis of melanocytes, it can cause various types of pigmentation disorders or pigmented spots on the skin. The high visibility of these skin diseases negatively impacts on the quality of life of patients. Therefore, it is necessary to understand the mechanism controlling the differentiation and proliferation of melanocytes and to search for

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materials capable of controlling abnormal melanocytes.

Hydroquinone (HQ) is a chemical compound that inhibits the functions of melanocytes and has long been known for its skin-whitening effect. According to previous studies, the Tyr activity inhibitory effect and melanocyte-specific cell toxicity are known depigmenting mechanisms[9,10]; however, details of the underlying mechanisms are unknown. Arbutin (Arb), which is a hydroquinone glycoside, is also known for its Tyr activity inhibitory effect[11–13] and is commonly used as a skin-whitening agent. However, the detailed depigmenting mechanism of Arb is also not yet fully understood.

Conventional studies have used mature melanocytes in experiments to analyze the effects of HQ and Arb on melanocytes. However, to our knowledge, there was hardly any study that used undifferentiated melanocytes. Therefore, the effects of HQ and Arb on differentiation processes remain completely unknown. Not only mature melanocytes, but also melanocytes undergo various stages of differentiation (including neural crest cells, melanocyte stem cells, and melanoblasts) in the human body. We consider it of importance to analyze the effects of HQ and Arb on the differentiation process of immature melanocytes.

ES cells are pluripotent cells derived from the inner cell mass of a blastocyte, having indefinite proliferative potential and multipotency.[14–17] Yamane *et al.* established a melanocyte inducement system using mouse ES cells in 1999.[18] Their inducement system has made it possible to replicate the development and differentiation processes of melanocytes and analyze the effects of materials on each development stage. We previously searched for materials that could control the differentiation of melanocytes using this melanocyte inducement system.[19]

In this study, we examined the effects of HQ and Arb on the development to maturation processes of melanocytes using this melanocyte differentiation inducement system. The results showed that HQ in particular downregulated the early stage of differentiation, in which neural crest cells were generated, and the late stage of differentiation, in when melanogenesis became active. On the other hand, Arb had no effect on the differentiation of melanocytes, and only suppressed melanogenesis by specifically suppressing elevations in Tyr expression in the late stage of differentiation.

MATERIALS AND METHODS

Cell Culture BRUCE-4 ESCs (MILLIPORE, Billerica, MA, U.S.A.), derived from mouse ESCs of the cell line C57BL/6J, were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 15% fetal bovine serum (FBS) (Sigma, St. Louis, MO), ES Cell Qualified L-Glutamine Solution (CHEMICON International, Inc., Temecula, CA, U.S.A.), ES Cell Qualified 2-Mercaptoethanol (CHEMICON), ES Cell Qualified Non-Essential Amino Acids (CHEMICON), ES Cell Qualified Nucleosides (CHEMICON), and ESGRO (CHEMICON), according to the manufacturer's protocols. Mouse embryonic fibroblasts (MEF) (MILLIPORE) treated with 10 μg/mL of Mitomycin C (Sigma) were used as feeder layer cells.

ST2 cells (Riken Cell Bank, Ibaraki, Japan) were maintained in Minimum Essential Medium Alpha Modification (Invitrogen) supplemented with 10% FBS.

To induce the differentiation into melanocytes, ST2 cells were used as feeder layer cells. ESCs were seeded on ST2 feeder layer cells in 24 well plates (500 cells/well), and were cultured in Minimum Essential Medium Alpha Modification supplemented with 10% FBS, 100 nM dexamethasone (Sigma), 20 pM basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, U.S.A.), 10 pM cholera toxin (Bio Academia, Osaka, Japan), and 100 ng/mL Endothelin 3 (Edn3) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Microscopic observations revealed the formation of mouse ESC colonies on ST2 feeder layer cells on day 6 of the culture and pigmented melanocytes around these colonies on approximately day 18. Melanin synthesis was accelerated from day 18 through to day 24. We previously conducted detailed gene expression analyses using the ESC culture system and identified that the expression of each melanocyte marker was progressively increased (Supplementary Fig. 1).[20]

HQ (Wako) was dissolved in ethanol at a concentration of 1 M as a stock solution, and was then added to cell cultures at a final concentration of 3–100 μM. Arb (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in sterile water at a concentration of 100 mM as a stock solution, and was then added to cell cultures at a final concentration of 30–1000 μM.

Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis During the differentiation of ESC into melanocytes, total RNA was extracted from cells at various stages using TRIZOL® Reagent (Invitrogen), and cDNA was synthesized by reverse transcription. Real-time PCR was performed with the SuperScript™ III Platinum® Two-Step qRT-PCR kit (Invitrogen), using the 7300 Real Time PCR System (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocols. The primer sequences used were as follows:

Gapdh (Glyceraldehyde-3-phosphate dehydrogenase):
sense primer 5′-TGC ACC ACC AAC TGC TTA GC-3′
anti-sense primer 5′-TCT TCT GGG TGG CAG TGA TG-3′

Pax3 (paired box 3):
sense primer 5′-TCG GCC TTG CGT CAT TTC-3′
anti-sense primer 5′-CAG GAT CTT AGA GAC GCA ACC A-3′

Sox10 (SRY-box containing gene 10):
sense primer 5′-TGG AGG TTG CTG AAC GAA AGT-3′
anti-sense primer 5′-GAG CCT CTC AGC CTC CTC AA-3′

Mitf-m (microphthalmia-associated transcription factor):
sense primer 5′-TGC CTT GTT TAT GGT GCC TTC T-3′
anti-sense primer 5′-TCC CTC TAC TTT CTG TAA TTC CAA TTC-3′

Tyr (tyrosinase):
sense primer 5′-ACG ACC TCT TTG TAT GGA TGC A-3′
anti-sense primer 5′-TTT CAG AGC CCC CAA GCA-3′

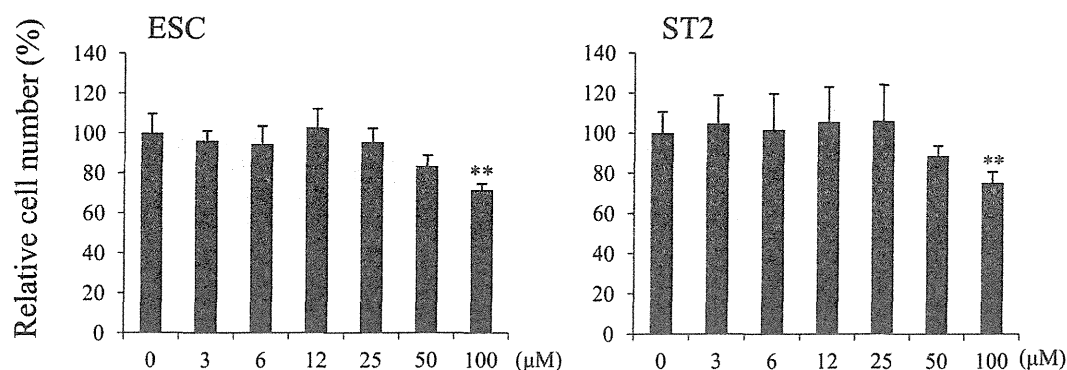
Dct (dopachrome tautomerase):
sense primer 5′-CCG GCC CCG ACT GTA ATC-3′
anti-sense primer 5′-GGG CAG TCA GGG AAT GGA TAT-3′

Tyrp1 (tyrosinase-related protein 1):
sense primer 5′-CAA CGC TAT GCT GAG GAC TAT GA-3′
anti-sense primer 5′-GCG GCT ATC AGA CCA TGG A-3′

The contents of the selected genes were normalized to Gapdh. All PCR products were checked by melting curve analysis to exclude the possibility of multiple products or an incorrect product size. PCR analyses were conducted in triplicate for each sample.

Cell Viability Assay ESCs and ST2 cells were seeded

a HQ



b Arb

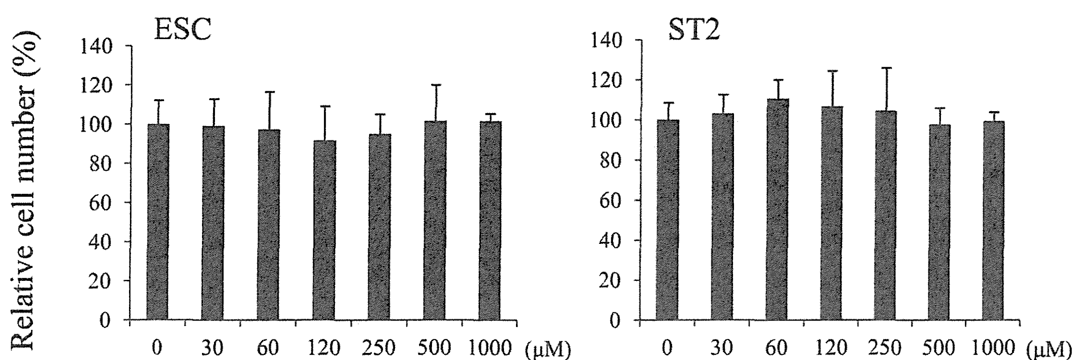


Fig. 1. Effects of HQ and Arb on the Cell Proliferation of ESCs and ST2 Cells

(a, b) Relative cell numbers (%). ESCs and ST2 cells were cultured with various concentrations of HQ (a) and Arb (b). The relative cell numbers were measured in each well after 24 h. Data are expressed as the mean±S.D. of three experiments, $^{**}p<0.01$, significantly different from the control.

in 96 well plates (1×10^4 cells/well), and the culture medium was replaced the next day by each culture medium containing HQ and Arb at various concentrations. After 24 h, relative cell numbers were measured using Cell Counting Kit-8 (DOJINDO Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. In brief, after removing the medium, 100 μL of CCK-8 solution was added to cells, and they were then incubated for another hour. Optical density values were tested at an absorbance of 450 nm using a microplate reader (Molecular Devices, Inc., Menlo Park, CA, U.S.A.).

Melanin Content Measurement ESC and ST2 cells were co-cultured in 24 well plates and induced to differentiate into melanocytes. The melanin content per relative cell number under each culture condition was determined after 24 d of induction. In brief, after removing the medium, 300 μL of CCK-8 solution was added to the cells, and they were then incubated for another hour. A total of 100 μL of each lysate was put in another 96 well plate, and optical density values were tested at an absorbance of 450 nm by the microplate reader. After calculating the relative cell number, the melanin content of cultured cells was measured. Cells were washed twice with phosphate buffered saline (PBS), lysed in 300 μL of 2 N NaOH, and boiled for 2 h at 60°C to solubilize the melanin. A total of 200 μL of each lysate was put in another 96 well plate, and absorbance at 475 nm was measured using a microplate reader (Molecular Devices).

Statistical Analysis The Student's *t*-test was used for statistical analysis, and multiple groups were evaluated by a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison.

RESULTS

1. Effects of HQ and Arb on the Viabilities of ESCs and ST2 Cells First, we examined the effects of HQ and Arb on the viabilities of each cell (ESC and ST2 cells). The results showed that 100 μM of HQ significantly inhibited the proliferation of ES cells and ST2 cells (Fig. 1a). On the other hand, Arb did not inhibit the proliferation of ES cells or ST2 cells to the same level as it did at 1000 μM (Fig. 1b).

2. Effects of HQ and Arb on Melanin Synthesis To analyze the effects of HQ and Arb on the differentiation of melanocytes, we co-cultured ESCs and ST2 cells in 24 well plates and induced them to differentiate into melanocytes while continuously adding HQ and Arb (HQ: 6.25, 12.5, and 25 μM, Arb: 125, 250, and 500 μM). After 24 d of induction, the melanin content per relative cell number under each culture condition was determined.

HQ and Arb showed no inhibitory effect on cell growth; however, the melanin content per relative cell number decreased in a concentration-dependent manner (Figs. 2a, b). Subsequent studies were conducted at a concentration of 25 μM

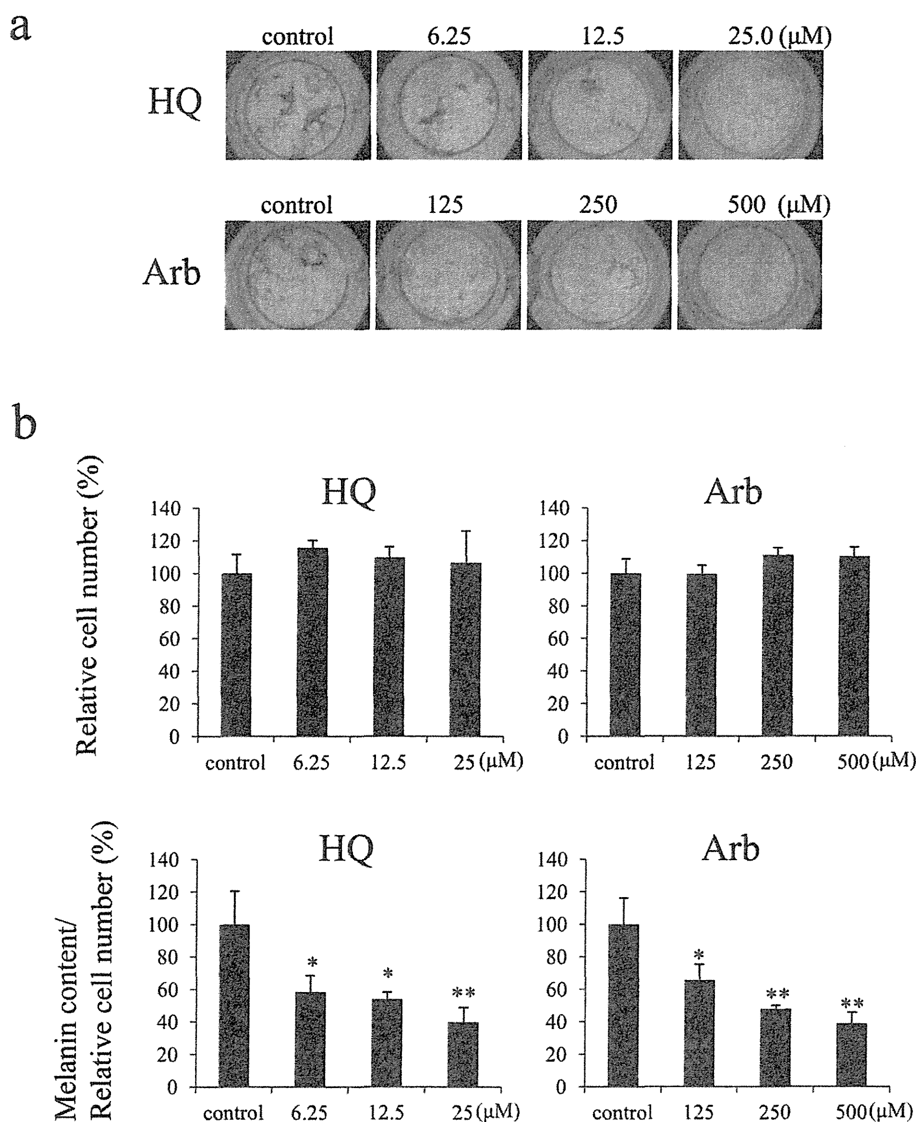


Fig. 2. Effects of HQ and Arb on Melanin Synthesis

(a) Representative dishes after induction. (b) Relative cell numbers (%) and melanin content/relative cell numbers (%). ESCs were differentiated with HQ (6.25, 12.5, and 25.0 μM) and Arb (125, 250, and 500 μM). Relative cell numbers and melanin content were measured in each dish after 24 d. The figure represents relative cell numbers and melanin content/relative cell numbers normalized to relative cell numbers. Data are expressed as the mean±S.D. of three experiments, * $p<0.05$, ** $p<0.01$, significantly different from the control.

of HQ and 500 μM of Arb due to their high efficacies.

3. Analysis of the Effects of HQ and Arb, Added at Each Stage of Differentiation, on Melanin Synthesis after 24-d Differentiation Induction In order to identify the effects of HQ and Arb at each stage of differentiation, we added HQ and Arb to the melanocyte differentiation inducement system in a specific pattern (days 0–6, days 6–12, days 12–18, and days 18–24), and analyzed their effects on terminal melanin synthesis after a 24-d culture.

The results showed that the final amount of melanin produced was significantly decreased when HQ was added on days 0–6 and days 18–24 of induction (conditions No. 1, 4–7, and 9–15, Fig. 3a). On the other hand, no downregulation in melanogenesis was observed when HQ was added on days 6–18 (conditions No. 2, 3, and 8, Fig. 3a).

When Arb was added on days 18–24 (conditions No. 4, 7, 9, 10, and 12–15, Fig. 3b), the final amount of melanin produced was significantly decreased. On the other hand, no downregulation in melanogenesis was observed when Arb was added on days 0–18 (conditions No. 1–3, 5, 6, 8, and 11, Fig. 3b).

HQ and Arb showed no inhibitory effect on cell growth under each culture condition (Supplementary Fig. 2).

Based on the above findings, it was suggested that HQ specifically showed suppressive effects in the early stage of differentiation, in which neural crest cells were generated (days 0–6), and the late stage of differentiation, in which melanogenesis became active (days 18–24). It was also suggested that Arb showed no effects on days 0–18, but had suppressive effects exclusively on the stage of melanogenesis (days 18–24).

4. Analysis of the Effects of HQ and Arb, Added at Each Stage of Differentiation, on the Expression of Marker Genes In order to analyze the effects of HQ and Arb on melanocyte-specific genes, we performed detailed gene expression analyses under conditions No. 5, No. 10, and No. 15 in which the compounds were added at the early stage of induction (days 0–12), at the late stage of induction (days 12–24), and throughout the entire period (days 0–24), respectively.

The final amount of melanin produced in the HQ group was

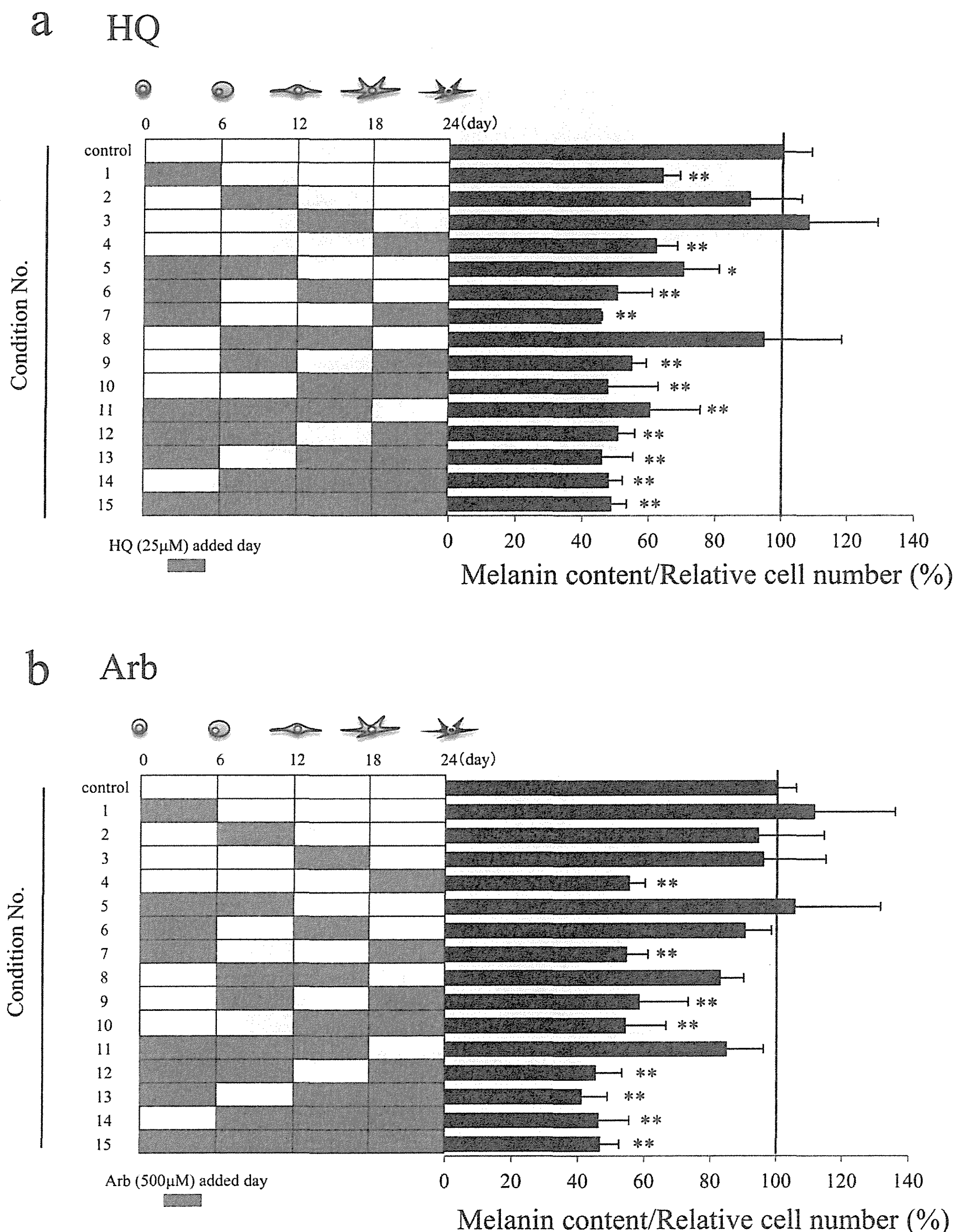


Fig. 3. Effect of the Time of HQ and Arb Addition on Melanin Synthesis on Day 24

(a) Melanin content/relative cell number (%) under conditions No. 1 to No. 15. HQ (25 μM) and Arb (500 μM) were added at various times during melanocyte induction and we measured the melanin content and relative cell numbers of each dish on day 24. The figure represents the melanin content normalized to relative cell numbers. Data are expressed as the mean±S.D. of three experiments. * $p<0.05$, ** $p<0.01$, significantly different from the control.

decreased under conditions No. 5, No. 10, and No. 15 (Figs. 3a, 4a). The results also showed that the increased expression of each marker was markedly downregulated under condition No. 5, while the expression of each marker was gradually increased in the control group (Fig. 4b). Downregulation in the expression of each marker was also observed under condition No. 15 (Fig. 4d). The final amount of melanin produced under condition No. 10 was significantly decreased (Fig. 3a), whereas significant changes in the expression of all marker genes were not observed (Fig. 4c).

The final amount of melanin produced in the Arb group was decreased under conditions No. 10 and No. 15 (Figs. 3b, 5a). The results also showed that the expression of all markers was gradually increased under condition No. 5, similar to the control (Fig. 5b). On the other hand, no significant change was observed in the expression of most marker genes, while a significant suppression was reported in the expression of Tyr under conditions No. 10 and No. 15 (Figs. 5c, d).

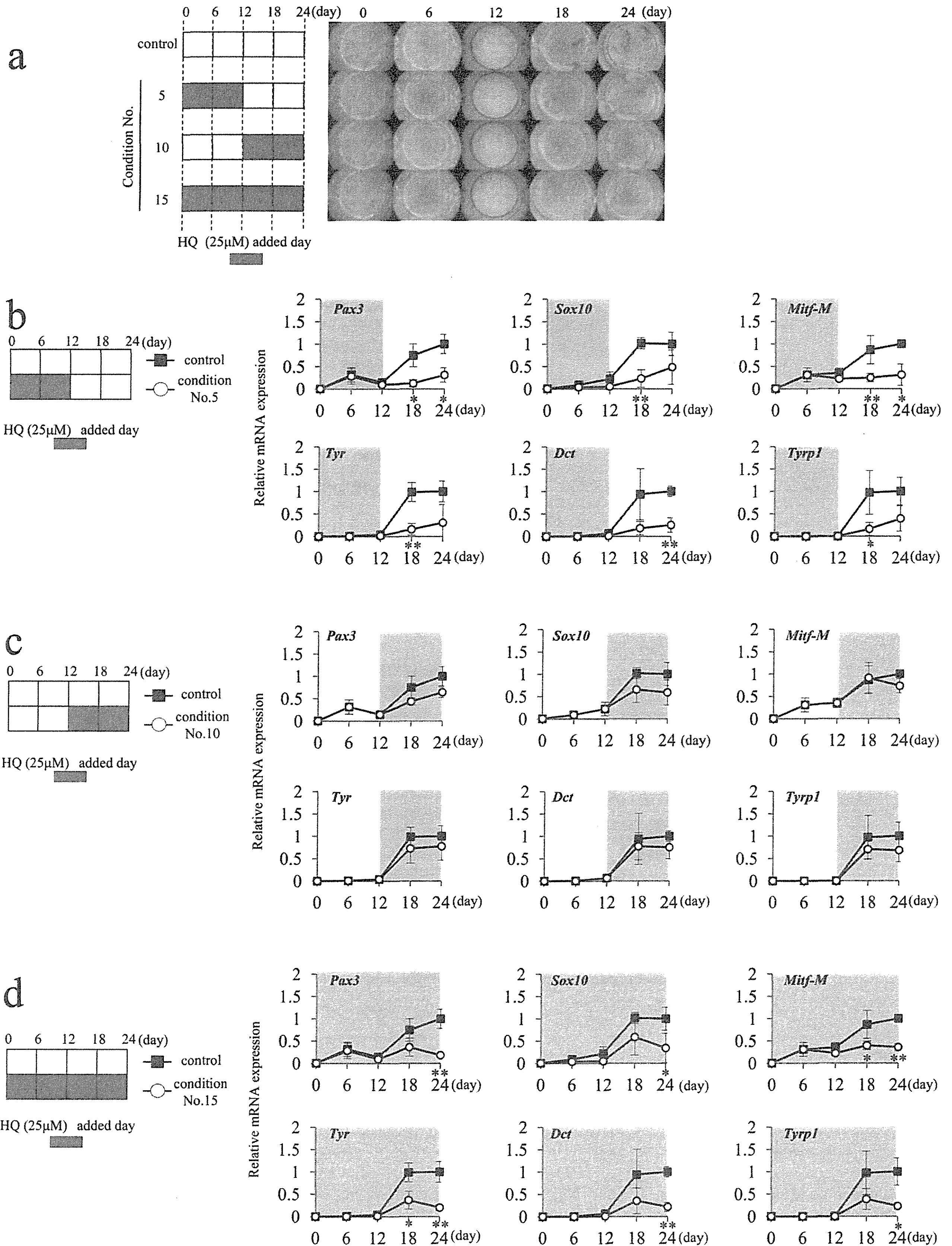


Fig. 4. Effect of the Time of HQ Addition on the Expression Profiles of Melanocyte Cell Lineage Markers

(a) Images of representative dishes under conditions No. 5, 10, 15, and the control. (b–d) Real-time PCR for melanocyte cell lineage markers observed under conditions No. 5, 10, 15, and the control. The expression level of each marker gene was normalized to that on day 24 (terminally differentiated melanocytes of the control). Data are expressed as the mean±S.D. of three experiments. $*p<0.05$, $**p<0.01$, significantly different from the control. Symbols: open circles, conditions No. 5, 10, and 15; closed squares, control.

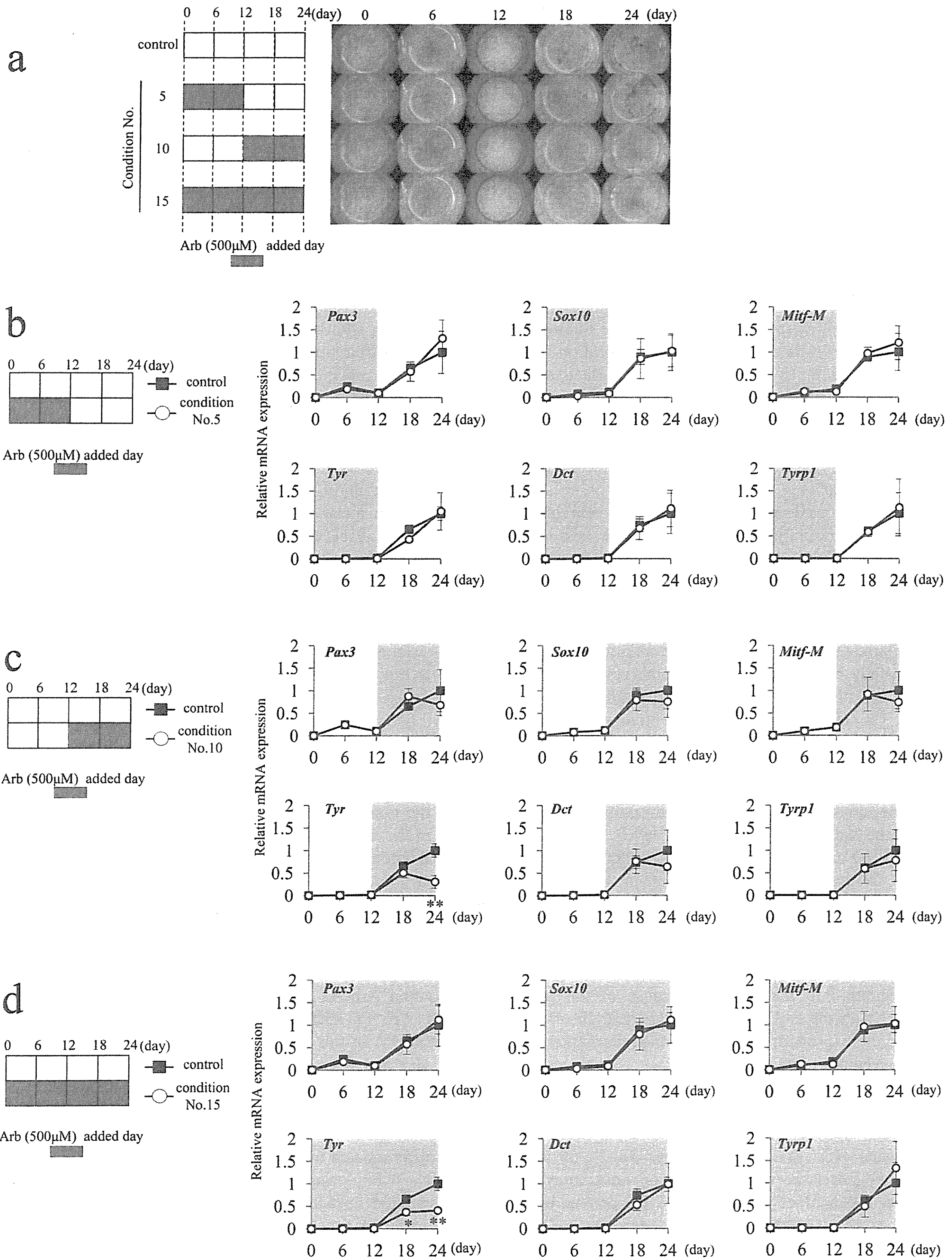


Fig. 5. Effect of the Time of Arb Addition on the Expression Profiles of Melanocyte Cell Lineage Markers

(a) Images of representative dishes under conditions No. 5, 10, 15, and the control. (b–d) Real-time PCR for melanocyte cell lineage markers observed under conditions No. 5, 10, 15, and the control. The expression level of each marker gene was normalized to that on day 24 (terminally differentiated melanocytes of the control). Data are expressed as the mean±S.D. of three experiments. $*p<0.05$, $**p<0.01$, significantly different from the control. Symbols: open circles, conditions No. 5, 10, 15; closed squares, control.

Based on the above results, the expression of all melanocyte differentiation markers was significantly downregulated when HQ was added in the early stage of differentiation. On the other hand, these results demonstrate that while Arb had no effect on the differentiation of melanocytes, it specifically suppressed the increased expression of Tyr in the differentiation process.

DISCUSSION

In this study, we examined the effects of HQ and Arb on the development to maturation processes of melanocytes using a melanocyte differentiation inducement system. When HQ and Arb were added continuously to the melanocyte differentiation inducement system using ES cells, the final amount of melanin produced was decreased in a concentration-dependent manner (Figs. 2a, b). In order to identify the effects of HQ and Arb on each stage of the differentiation of melanocytes, we added HQ and Arb to the melanocyte differentiation inducement system at each stage of differentiation and induced differentiation for 24 d. We then analyzed its effects on terminal melanin synthesis.

The results showed that HQ in particular downregulated the early stage of differentiation, in which neural crest cells were generated (days 0–6), and the late stage of differentiation, in which melanogenesis became active (days 18–24) (Fig. 3a). We believe that a downregulation in the final amount of melanin produced occurred when HQ was added on days 0–6 because HQ may have inhibited the early differentiation of ES cells. A previous study reported that HQ has embryotoxicity.[21] Therefore, HQ can also be considered to affect the early differentiation of ES cells and promote differentiation into other cells besides melanocyte lineage cells.[22] However, very few studies have attempted to elucidate the effects of HQ on the early differentiation of ES cells.

Benzene metabolites including hydroquinone are known to affect the various gene expression levels of cells.[23] Such effects of HQ may have inhibited the expression of genes necessary for melanocyte differentiation in the present study. More detailed analyses should be conducted in the future to identify why HQ controlled ES cells in the early stage of differentiation into melanocytes.

Nevertheless, we consider that ES cells lost their ability to differentiate into melanocytes because of the inhibitory effect of HQ on the early stage of differentiation of ES cells. Therefore, the expression of each melanocyte marker was not increased even after HQ was removed.

Although the final amount of melanin produced was not decreased when HQ was added on days 6–12 and days 12–18 of induction, the final amount of melanin produced was decreased when HQ was added on days 18–24 of induction when melanogenesis became active. A previous study demonstrated that HQ competitively inhibited the activation of Tyr, by acting as an alternative substrate of tyrosine.[9] Therefore, we concluded that HQ became highly effective after day 18 when Tyr activity was activated. The final amount of melanin produced under condition No. 10 was significantly decreased (Fig. 3a); however, significant changes were not observed in the expression of melanocyte markers (Fig. 4c). A previous study showed that HQ did not affect gene expression related to melanin synthesis such as Tyr.[24]

Arb had no effects on days 0–18; however, the final amount of melanin produced was decreased on days 18–24 only when melanogenesis became active (Fig. 3b). Since no change was observed in the expression of markers, for example, Pax3, Sox10, Mitf-M, Dct, and Tyrp1 (Figs. 5b–d), Arb was considered to have had no effects on the differentiation of melanocytes. In contrast, Arb markedly suppressed the expression of Tyr (Figs. 5c, d). Previous studies have shown that Arb works as a competitive inhibitor of Tyr and has no effect on the expression of Tyr, Tyrp1, or Dct itself.[11–13] These differences may have occurred because other studies used terminally-differentiated melanocytes and melanoma cells to examine the effect of Arb, while we used melanocytes that were in the process of differentiation. Arb may downregulate the expression of Tyr, in addition to the competitive inhibitory effect of Tyr against differentiating melanocytes.

Furthermore, previous studies have shown that Arb has multiple effects on cells, besides Tyr activity inhibitory effect, such as antioxidant and anti-inflammatory effects.[25,26] The expression of Tyr in this study may also have been indirectly suppressed by these functions of Arb. SB203580, a p38 MAP kinase inhibitor, is known to suppress the gene expression of Tyr in melanoma cells.[27] Further studies should be conducted to elucidate the mechanism by which Arb inhibited the mRNA expression of Tyr in this study.

In the present study, no significant difference was observed in the final melanin content (Figs. 3a, b) between when the expression of all melanocyte differentiation markers was decreased after HQ was continuously added (Fig. 4d) and when only the expression of Tyr was decreased after Arb was continuously added (Fig. 5d). These results may be attributed to Tyr being a key regulator of melanogenesis.[7] Therefore, we consider that, even if the differentiation of melanocytes had progressed in the Arb group, melanogenesis did not occur properly because of the decreased expression of Tyr, resulting in the same final melanin content as that in the HQ group.

In this study, we analyzed the effects of HQ and Arb on the differentiation of melanocytes using the mRNA expression of each melanocyte differentiation marker as indicators. On the other hand, the protein-level effects of HQ and Arb on each gene are also of importance; therefore, we need to elucidate these details through further research in the future.

Many researchers have focused on analyzing the effects of HQ and Arb on melanogenesis. However, the effects of HQ and Arb on differentiation processes remained completely unknown. In this study, we consistently examined the effects of HQ and Arb on melanocytes from their development to maturation using a melanocyte inducement system from ES cells, and found that each agent exhibited different effects depending on the stage of differentiation. Mature and also undifferentiated melanocytes have been reported in the human body.[2–4] Exploring specific compounds that can control undifferentiated melanocytes as well as mature melanocytes may help achieve fundamental control over skin pigmentation.

Acknowledgments We would like to sincerely thank Ms. M. Hori (Cellisis Co., Ltd., Aichi, Japan) and Ms. M. Hotta (Fujita Health University Institute, Aichi, Japan) for their support in analyzing our experimental data.

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Allergic contact dermatitis caused by phenylethyl resorcinol [4-(1-phenylethyl)-1,3-benzenediol], a skin-lightening agent in cosmetics

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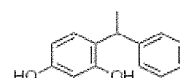
doi:10.1111/cod.12114

Key words: 4-(1-phenylethyl)-1,3-benzenediol; allergic contact dermatitis; cosmetics; phenylethyl resorcinol; skin-lightening agent.

A number of skin-lightening agents are currently available, including kojic acid, arbutin, and hydroquinone. However, all of these compounds have significant disadvantages. Kojic acid is believed to have a high sensitizing potential, leading to a relatively high incidence of contact sensitivity (1, 2). Arbutin (natural β-glycoside of hydroquinone) is suspected to be a hydroquinone precursor, and has been reported to cause allergic contact dermatitis (3). Hydroquinone is an aromatic compound that functions as an antioxidant, fragrance, reducing agent and polymerization inhibitor in cosmetics. However, to ensure safety, hydroquinone use is restricted to nail enamel (< 0.02%) and hair dye (< 1%) in the EU, and also to nail enamel (< 1%) and hair dye (< 1%) in the United States (4–6). Phenylethyl resorcinol [4-(1-phenylethyl)-1,3-benzenediol) (Fig. 1)], a potent inhibitor of tyrosinase, is a relatively new and highly efficient skin-lightening agent (7, 8). We describe the first case of allergic contact dermatitis caused by phenylethyl resorcinol.



Molecular formula: $C_{14}H_{14}O_2$
Molecular weight: 214.264
CAS no.: 85-27-8

Fig. 1. Chemical formula of phenylethyl resorcinol [4-(1-phenylethyl)-1,3-benzenediol].

Case Report

A 52-year-old Japanese female presented with a 3-year-history of itchy erythematous rash and hyperpigmented areas on both cheeks. She had no history of contact dermatitis. Before visiting our hospital, she had been

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Conflicts of interest: The authors have declared no conflicts.

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using several steroid ointments and antifungal creams prescribed by other dermatologists on both cheeks. These topical medicaments improved her symptoms, but the skin lesions relapsed. She reported that the facial erythema appeared after application of a skin-lightening essence for hyperpigmentation on the face.

We performed patch testing with her personal cosmetics and 17 cosmetic allergens, using Finn Chambers® (SmartPractice, Phoenix, AZ, USA) mounted on Scanpor® tape (Norgesplaster AS, Vennesla, Norway) on the upper back for 2 days. Reactions were read on D2, D3, and D7, in accordance with the International Contact Dermatitis Research Group recommendations. Positive reactions to the skin-lightening essence (+ on D2, D3, and D7) were observed. In the second patch test for cosmetic ingredients provided by the cosmetic supplier, the patient reacted to 1% and 0.1% phenylethyl resorcinol in pet. (+ on D2, D3, and D7). Other cosmetic ingredients and cosmetic allergens gave negative results. Patch tests with phenylethyl resorcinol in 1% pet. in 2 control subjects gave negative results.

Discussion

Cosmetics containing skin-lightening agents, such as kojic acid, arbutin, and hydroquinone, have been reported to cause allergic contact dermatitis (1–3). Other newer, skin-lightening agents, including 5,5′-dipropylbiphenyl-2,2′-diol, may also cause contact dermatitis (9). Phenylethyl resorcinol has been used as a skin-lightening agent in cosmetics in Japan since 2000, and this is the first report describing an adverse skin reaction to this agent. Phenylethyl resorcinol, also known as SymWhite® 377 (Symrise AG, Holzminden, Germany), is a synthetic compound, and is one of the natural lightening compounds found in *Pinus sylvestris*. Phenylethyl resorcinol is used as a lightening and brightening agent in skin care products, hair-lightening products, and cosmetics. It reduces pigmentation and is used to lighten pigmented skin. Phenylethyl resorcinol was at least 10 times more effective than kojic acid in a pigmented three-dimensional epidermal model (MelanoDerma™) *in vitro*, and lightens human skin at a concentration of 0.5% in *in vivo* tests on Asian subjects (8). Gold et al. reported the usefulness of a hydroquinone-free skin-brightening cream containing phenylethyl resorcinol in patients with melasma (10).

The concentration of phenylethyl resorcinol in cosmetics ranges from 1% to 3%, and the cosmetics used by the present patient contained phenylethyl resorcinol at concentrations of 1% and 2%. We expect that phenylethyl resorcinol will be used in many products for its skin-lightening effect. To minimize the risk of sensitization, the concentration of phenylethyl resorcinol in skin care products needs to be regulated. In addition, information regarding the presence of phenylethyl resorcinol and its concentration should be clearly stated in the product information brochures of cosmetic products.

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In conclusion, although it did not affect their conclusion that the microorganism population does not significantly change before and after soap washing, I believe that the discrepancy in their result on *Staphylococcus* reflects a very interesting and delicate aspect of studying skin microbiota.

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Reply: "Comment on *Staphylococcus* cell number discrepancy between culture-based and non-culture-based analyses: Quantitative effect of face washing on cutaneous resident microbiota in female subjects who wear make-up"

Dear Editor,
We greatly appreciate the helpful and insightful comments from Dr Dekio and would like to try to reply in every instance. First, we agree with his first hypothesis. It is known that up to a dozen *Staphylococcus* cells form clumps of microorganisms. Each clump is counted as one colony and culture-based quantification is much lower in *Staphylococcus* species counts than culture-independent quantification. Second, we would like to comment on his second hypothesis that the samples may be contaminated by cosmetics. In this study, according to the purpose, we obtained the samples from the women who wore make-up. Major preservatives in cosmetics including parabens are inactivated by polysorbate 80: a non-ionic emulsifier used in this study.[1] Moreover, we used serial dilutions, plating and counting live bacteria to determine the number of microorganisms. We confirmed a live germ in all culture media. From these facts, we think that the effects of antiseptics did not influence our results. In addition, the methodological difference between Dr Dekio's study and ours influenced the results. Our study was to estimate the change of the number of resident microorganisms before and after washing face, and this data clarified it.

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COD
Contact Dermatitis

• CONTRIBUTIONS TO THIS SECTION MAY NOT UNDERGO PEER REVIEW, BUT WILL BE REVIEWED BY THE EDITOR •

Allergic contact dermatitis caused by *N,N*-diethyl-*p*-phenylenediamine used in water quality analysis

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doi:10.1111/cod.12063

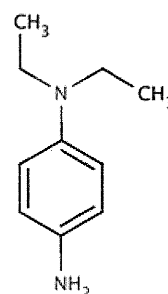
Key words: allergic contact dermatitis; *N,N*-diethyl-*p*-phenylenediamine; water quality analysis.

N,N-Diethyl-*p*-phenylenediamine (DPD; Fig. 1) is a frequently used chemical for measuring free chlorine (Cl_2, HOCl, ClO^-) and combined chlorine (NH_2Cl, $NHCl_2$) in drinking water. DPD can be used as an indicator for both acid–base and oxidation reactions (1). Herein, we report a case of allergic contact dermatitis caused by DPD used in water quality analysis.

Case Report

A 28-year-old woman with mild atopic dermatitis started work as an analyst of water quality 2 years previously, where she came into direct contact with chemicals, including DPD, without using gloves. Before starting this job, she had a 1-year history of hand dermatitis with erythema. After she started the new job, the hand dermatitis worsened; there was an itchy erythematous rash with small blisters, and erythema appeared on her arms, legs, and neck. She had never dyed her hair.

We performed patch testing with the Japanese baseline series and chemicals used at her workplace, using Finn Chambers® (SmartPractice, Phoenix, AZ, USA) mounted on Scanpor® tape (Norgesplaster AS, Vennesla, Norway). The tests were applied to the upper part of her back for 2 days, and read on D2, D3, and D7, according to International Contact Dermatitis Research Group criteria. The patient showed a positive reaction to DPD at the following concentrations: 1% aqua (D3, +; D7, +); 0.1% aqua (D3, +; D7, +); 0.01% aqua (D3, +; D7, +); and 0.001% aqua (D3, −; D7, +). She had no reactions to other ingredients and allergens, including *p*-phenylenediamine (PPD) and *p*-diethylaminobenzenediazonium (DDA). We also performed patch testing on 3 persons with DPD as normal controls, and they showed negative reactions. From these findings, the patient was diagnosed with allergic contact dermatitis caused by DPD.



CAS number: 93-05-0
Molecular formula: $C_{10}H_{16}N_2$
Molecular weight: 164.25

Fig. 1. Chemical structure of *N,N*-diethyl-*p*-phenylenediamine.

Discussion

DPD is a para-amino compound that has benzene ring with amine and diethylamine in para-substitution, and it is soluble in water, but not volatile. DPD is used in the form of DPD sulfate for measuring free chlorine and combined chlorine in drinking water (1, 2). This method is used in many countries. In this case, the patient came into contact with chemicals, including DPD, without the use of gloves, resulting in allergic contact dermatitis on her

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Conflicts of interest: The authors have declared no conflicts.

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hands caused by DPD. We also considered the cutaneous lesions other than her hand eczema to be autosensitization dermatitis. However, she came into contact with DPD as a powder, so there is a possibility of airborne contact dermatitis. Only 2 cases of allergic contact dermatitis caused by DPD have been reported. Kato et al. described a case of occupational allergic contact dermatitis caused by DPD in the Japanese literature in 1991 (3). The occupation of the patient in that case was water quality analyst, the same as in the present case. Their patient reacted positively to DPD and DDA. This reflects a cross-reaction between DPD and DDA, as their chemical structures are similar. However, our patient did not react to DDA and PPD. DPD is also used in the development of photographs, and Aguirre et al. described a case of contact dermatitis caused by the colour developers CD1, CD2, CD3, CD4 and DPD in 1992 (4). In the aforementioned 2 cases, the positive patch test concentrations of DPD were 5% aqua (3). and 1% pet. (4), but, in our case, the patient reacted positively to 0.001% aqua. This suggests that DPD has the potential to cause allergic contact dermatitis even at very low concentrations.

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