Wnt/β-Catenin and Kit Signaling Sequentially Regulate Melanocyte Stem Cell Differentiation in UVB-Induced Epidermal Pigmentation

Takaaki Yamada1,2, Seiji Hasegawa1,2, Yu Inoue1, Yasushi Date1, Naoki Yamamoto3, Hiroshi Mizutani1, Satoru Nakata1, Kayoko Matsunaga2 and Hirohiko Akamatsu4

UV radiation is a well-known inducer of epidermal pigmentation that is utilized in therapy for vitiligo, one of the skin depigmentation disorders. Although it has been reported that melanocyte stem cells (McSCs) play essential roles in hair pigmentation, the relationship between McSCs and epidermal pigmentation remains unclear. Repetitive UVB irradiation on the dorsal skin of F1 mice of HR-1 × HR/De caused apparent epidermal pigmentation, and it was characterized by increase in the number of melanocytes. Interestingly, differentiation of McSCs into melanoblasts in hair follicles was followed by induction of epidermal melanocyte differentiation. Administration of a neutralizing antibody for Kit receptor that depletes resident melanoblasts could not suppress increased number of melanocytes. UVB irradiation also induced robust expression of Wnt7a as well as Kitl in epidermis, and β-catenin translocation into nucleus in McSCs. Intradermal injection of IWR-1 (inhibitor of Wnt response 1), a chemical inhibitor of β-catenin activation, and small interfering RNA (siRNA) against Wnt7a suppressed increase in the number of epidermal melanocytes. Taken altogether, it was demonstrated that Wnt7a triggered McSCs differentiation through β-catenin activation, and Kitl might induce following migration of melanoblasts to epidermis. These findings will help in developing therapeutic technologies for vitiligo and other pigmentary disorders.

INTRODUCTION

UV radiation, a well-known inducer of epidermal pigmentation, stimulates keratinocytes to release cytokines and growth factors that promote melanogenesis in melanocytes residing in interfollicular epidermis (simply referred to as the epidermis). There have been believed to be melanocyte reservoirs in hair follicles because therapeutic UV irradiation for vitiligo, a pigmentary disorder characterized by the development of white macules related to the selective loss of melanocytes, causes perifollicular repigmentation (Falabella and Barona, 2009; Falabella, 2009). It has also been reported that hair follicles play important roles in repigmentation of vitiligo by traditional Chinese medicine without UVB irradiation (Cui et al., 1991). This phenomenon raised the possibility that there are stem cells of melanocyte lineage in hair follicles that can differentiate into melanoblasts/melanocyte and migrate from hair follicles to the epidermis in response to UV radiation.

Nishimura et al. (2002) first demonstrated the existence of melanocyte stem cells (McSCs) in the bulge area of the hair follicle. When the hair cycle progresses from the resting phase (telogen) to growth phase (anagen), McSCs divide and differentiate into melanoblasts, progenitors of melanocytes, which are still immature and unpigmented (Nishimura et al., 2005; Steingrímsson et al., 2005). After migrating into hair bulbs, they further differentiate into mature melanocytes and supply melanins to surrounding keratinocytes. It was reported that Wnt/β-catenin signaling regulated McSC differentiation in hair cycle progression. Wnt molecules are a large family of secreted proteins and their signaling is mediated by binding to a receptor, Frizzled (Fzd). Wnt and Fzd proteins have 19 and 10 families, respectively (Nusse, 2008). In the best-characterized Wnt signaling pathway, termed the canonical pathway, Wnt binds to Fzd receptors and coreceptor Lrp (low-density lipoprotein receptor–related protein) 5/6, and blocks the degradation of cytoplasmic β-catenin protein by inhibiting its phosphorylation. Stabilized β-catenin is transferred to the nucleus and binds to DNA as a transcription factor to induce various cellular events.
such as proliferation, differentiation, migration, and adhesion. Lang et al. (2005) demonstrated that β-catenin displaced Groucho corepressor and activated the expression of dopachrome tautomerase (Dct) in McSCs at anagen in the hair cycle. Rabbani et al. (2011) also demonstrated that activation of canonical Wnt signaling induced endothelin-1 expression in hair follicle stem cells (HFSCs) and then endothelin-1 promoted McSC differentiation. The signaling pathway also directly induced the differentiation of McSCs into melanocytes. We previously reported that McSCs expressed canonical Wnt signaling receptors, Fzd4, Fzd7, Lrp5, and Lrp6 (Yamada et al., 2010), and this finding supports the fact that McSCs can respond to stimulation by Wnts; thus, it is thought that Wnt/β-catenin signaling is essential for McSC regulation.

There is accumulating evidence about the role and differentiation mechanism of McSCs in the hair pigmentation process; however, little is known about their role in epidermal pigmentation. Differentiation of McSCs into epidermal melanocytes was successfully observed only under the conditions of forced expression of kit ligand or endothelin-1 by epidermal keratinocytes (Nishimura et al., 2002; Rabbani et al., 2011). Although these findings support that McSCs might be a reservoir of epidermal melanocytes, there is no study report on the relationship between McSCs and epidermal pigmentation induced by UV irradiation; therefore, the detailed molecular mechanism of the repigmentation process in therapy against vitiligo remains unclear. To address this issue, we conducted a study on the relationship between McSCs and epidermal pigmentation in F1 mice of HR-1 × HR/De. These mice possess melanin and epidermal melanocytes in dorsal skin and are a suitable animal model to investigate the molecular mechanism underlying UV-induced epidermal pigmentation (Furuya et al., 2009). We examined the changes in the number and localization of melanocytes during 4-week UVB exposure and found that UVB irradiation induced McSC differentiation into melanoblasts in hair follicles. The melanoblasts migrated to the epidermis and started to undergo melanogenesis (differentiation into mature melanocytes). Furthermore, we also confirmed that UVB-induced robust Wnt7a expression in epithelial lineages triggered McSC differentiation by β-catenin activation.

RESULTS

UVB irradiation increased the number of melanocytes followed by epidermal pigmentation

We first analyzed the gross skin color changes caused by UVB irradiation in F1 mice of HR-1 × HR/De, and observed that their dorsal skin became apparently darker on days 14 and 28 than before UVB irradiation (Figure 1b). To confirm whether this skin pigmentation was caused by melanocytogenesis, not melanogenesis by resident melanocytes, the number of melanocytes in the epidermis was investigated by immunostaining against tyrosinase-related protein-1 (Tyrp1), one of the melanocyte lineage markers involved in melanin synthesis. As shown in Figure 1c and f, the number of Tyrp1+ cells in the epidermis began to increase on day 7 before skin pigmentation and markedly increased on day 14, when the epidermis was obviously pigmented. Analysis of the dopa reaction and Fontana–Masson staining also confirmed the result (Figure 1d and e). mRNA expression levels of melanin synthesis-related enzymes, Tyrp1, Dct, and tyrosinase (Tyr), also increased on day 7 and further elevated on day 14 in accordance with the observation of changes in the number of Tyrp1+ cells (Figure 1g). These results demonstrated that UVB-induced epidermal pigmentation was mainly caused by melanocytogenesis, not melanogenesis by resident melanocytes, followed by their melanogenesis.

UVB irradiation induced the emergence of melanoblasts from melanocyte stem cells in hair follicles

To investigate the source of emerging Tyrp1+ cells in the epidermis on day 7 after UVB irradiation, mice epidermis was collected on days 0, 1, 3, and 7 to analyze the number and localization of Tyrp1+ cells. Although Tyrp1+ cells did not increase in UVB-irradiated epidermis compared with the unirradiated control before day 7, Tyrp1+ cells transiently appeared in hair follicles (Figure 2a). The number of follicular Tyrp1+ cells peaked on day 3 and then decreased by day 7 (Figure 2b). Based on these results, we hypothesized that UVB induces the differentiation of McSCs into melanoblasts (Tyrp1+ cells) in hair follicles, and then melanoblasts migrate into the epidermis. To investigate the involvement of McSCs in UVB-induced emergence of Tyrp1+ cells in hair follicles, epidermis and hair follicles were obtained separately from fresh-frozen sections using laser microdissection. mRNA expression levels of Fzd4 and Fzd7 in hair follicles, the McSC markers, were analyzed. As a result, Fzd4 and Fzd7 expressions transiently decreased in hair follicles by UVB irradiation on days 1–3, and were hardly detectable in the epidermis, in accordance with our previous study (Figure 2c). Immunohistochemical analysis of Fzd4 and Dct, one of the most popular markers of melanocyte lineages including McSCs, revealed that Fzd4+/Dct+ cells (McSCs) transiently decreased and, conversely, Fzd4/Dct+ cells (melanoblasts) emerged on days 1–3 (Figure 2d and f). In other words, there was an inverse correlation between the number of McSCs and melanoblasts. Dual immunohistochemistry for Dct and Kit also confirmed that McSCs (Dct+/Kit- cells) were transiently decreased and melanoblasts (Dct+/Kit+ cells) emerged on days 1–3 (Figure 2e and f). These results indicated that mRNA reduction of Fzd4 and Fzd7 in hair follicles was attributed to the decrease in the number of McSCs. McSCs differentiated into melanoblasts that in turn migrated to the epidermis followed by pigmentation. In order to demonstrate our hypothesis, we analyzed mRNA expression level of Kit ligand (Kit ligand, also called as stem cell factor) after UVB radiation, and administered ACK2 (a neutralizing antibody for the Kit receptor) to the dorsal skin of mice before UVB irradiation. Kitl involved in the survival and migration of melanoblast/melanocyte and blockade of Kitl signaling pathway depletes melanocyte lineage except McSCs, which can survive without Kitl (Nishimura et al., 2002). As shown in Figure 2g, UVB irradiation elevated mRNA expression level of Kitl. After administration of ACK2, although resident Tyrp1+ cells in epidermis disappeared (Figure 2h), increased number of Tyrp1+ cells were observed in hair follicles on day 3 and in epidermis on days 7 and 14.
by UVB irradiation (Figure 2i). These results indicated that increased epidermal melanocytes were derived from McSCs and suggested that Kitl/Kit signaling pathway was involved in the migration of differentiated melanoblasts from McSCs into epidermis and further differentiation into mature melanocytes.

**UVB irradiation activated Wnt/β-catenin signaling in hair follicles**

We previously reported that McSCs expressed Lrp5/6 as well as Fzd4/7, which are receptors for canonical Wnt signaling pathway. Although UVB irradiation is well known to induce the secretion of various growth factors and cytokines, the relationship between UVB irradiation and Wnt signaling is poorly understood. Figure 3a showed that UVB irradiation markedly elevated Wnt7a mRNA expression only in all Wnt genes on day 1, and the upregulated Wnt7a expression returned to the basal level on day 3. To investigate the source of Wnt7a, HFSCs, hair follicle keratinocytes in the outer root sheath (HF-KCs), epidermal keratinocytes (E-KCs), and McSCs were isolated by FACS and the mRNA expression level was analyzed. As a result, robust expression of Wnt7a was observed in HFSCs, HF-KCs, and E-KCs, but not in McSCs (Figure 3b). UVB irradiation also induced the nuclear localization of β-catenin, a sign of canonical Wnt-signaling activation, in Dct+ cells (Figure 3c and d). These results suggested that UVB induced strong expression of Wnt7a in epithelial cell lineages and consequently activated Wnt/β-catenin signaling, and thus the signaling pathway was presumed to trigger the differentiation of McSCs into melanoblasts.

**Wnt/β-catenin signaling regulates McSC differentiation induced by UVB irradiation**

To investigate whether the Wnt/β-catenin signaling pathway is involved in McSC differentiation, IWR-1 (inhibitor of Wnt response 1), a chemical inhibitor of the signaling pathway (Lu et al., 2009), or small interfering RNA (siRNA) against Wnt7a, were directly injected into dorsal skin of mice. IWR-1 inhibits β-catenin activation by stabilizing Axin, a component of β-catenin destruction, and indeed inhibited nuclear translocation of β-catenin induced by UVB irradiation (Figure 4b).
**Figure 2. UVB irradiation induced melanocytogenesis in hair follicles.** (a, b) Changes in the number of melanocytes in the epidermis (upper panels) and hair follicles (lower panels) were analyzed by immunostaining for tyrosinase-related protein-1 (Tyrp1) in epidermal sheets (n = 10 per group, mean ± SE). Scale bar = 100 μm. (c) Gene expression analysis combined with laser microdissection enabled mRNA to be separately obtained from the epidermis and hair follicles showed a transient decrease of melanocyte stem cell (McSC) markers in hair follicles. (d-f) Transient decrease of McSCs (Fzd4+/Dct+, Dct+/Kit−) and emergence of melanoblasts (Fzd4+/Dct+, Dct+/Kit+) were confirmed by immunostaining (d, e), and the ratio of each population to total Dct+ cells was calculated (f). Dct, dopachrome tautomerase; Fzd, Frizzled. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Right lower panels show enlarged images of the boxed region (scale bar = 10 μm). (g) Change in mRNA expression level of Kitl was analyzed by real-time PCR. (h, i) After ACK2 treatment, the number of Tyrp1+ cells was analyzed by immunostaining for Tyrp1 in the epidermal sheets on days 0, 3, 7, and 14. Arrowheads indicate Tyrp1+ cells. Scale bar = 100 μm.
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.

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.
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<table>
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<tr>
<th>UVB (–)</th>
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**a**

Dct/Fzd4

**b**

Dct/β-catenin

**c**

Tyr

**d**

Fzd4+/Dct+/Dct cells

**e**

Ratio of cells to total Dct+ cells

**f**

Relative mRNA expression

**g**

Fzd4+/Dct+/Dct cells

**h**

Ratio of cells to total Dct+ cells

**i**

Relative mRNA expression
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 4. 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 5. 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 if 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.

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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 ± 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 cm²) 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 cm²); 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 cm²). 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% H₂O₂ 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 mm² 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−/CD49f+homo), E-KCs (CD34+/CD49f+), 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, NHECs were seeded onto 24-well plates at a density of 4 x 10⁴ cells per well and cultured for 48 hours. Culture medium was changed to PBS (−) and UVB was irradiated at a dose of 10 μJ cm⁻². PBS (−) was replaced with fresh defined keratinocyte serum-free medium and 4 x 10⁴ 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 semi-quantitative 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

REFERENCES