



ORIGINAL ARTICLE

NB-UVB Induces Melanocytic Differentiation of Human Hair Follicle Neural Crest Stem Cells

Dake Dong^{1,2}, Shujun Chen³, Cheng Feng^{2,4}, Huizi Xiong¹, Xiaowei Xu²

¹Department of Dermatology, Affiliated Hospital of Jiangnan University, Wuxi, Jiangsu, China, ²Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA, ³Department of Dermatology, Huashan Hospital, Fudan University, Shanghai, ⁴Department of Dermatology, the Second Affiliated Hospital of Xi'anJiaoTong University, Xi'an, Shanxi, China

Background: Phototherapy is an important method to treat vitiligo. However, it is unclear how phototherapy affects melanocyte precursors and skin neural crest stem cells.

Objective: To investigate the underlying mechanisms of narrow-band ultraviolet B (NB-UVB) induced melanocyte lineage differentiated from human scalp-derived neural crest stem cells (HS-NCSCs). **Methods:** HS-NCSCs were expanded from scalp hair follicles. The c-Kit⁺/CD57⁻ HS-NCSCs were isolated by cell sorting. Different doses of NB-UVB were used to irradiate these HS-NCSCs. Cell ultrastructure was examined by transmission electron microscope. Melanocyte marker expression was analyzed by Quantitative RT-PCR and Western blot. Cell proliferation and migration were also evaluated. **Results:** The c-Kit⁺/CD57⁻ HS-NCSCs expressed embryonic NCSC biomarkers. NB-UVB at a dose of 100 mJ of NB-UVB had little effect on the cell proliferation of differentiated melanocytes from c-Kit⁺/CD57⁻ HS-NCSCs, while 700 mJ inhibited cell proliferation significantly. The dendritic processes of differentiated melanocytes increased after radiation. The tyrosinase and Melanocortin 1 receptor (*Mc1R*) expression of differentiated melanocytes increased after

NB-UVB exposure. The effect of NB-UVB on tyrosinase expression was modulated by signaling inhibitors H89 and PD98059 as well as *Mc1R* level in the cells. The migration ability of differentiated melanocytes was enhanced under 100 mJ exposure. **Conclusion:** These data demonstrate that NB-UVB facilitates melanocytic differentiation of the HS-NCSCs and enhances migration of these cells. *Mc1R* and cAMP pathway play a critical role in NB-UVB induced melanocytic differentiation. (*Ann Dermatol* 32(4) 289 ~ 297, 2020)

-Keywords-

Human, Melanocortin 1 receptor, Melanocytes, Neural crest, Stem cells, Ultraviolet rays

INTRODUCTION

Phototherapy has been used as one of the treatment modalities for vitiligo in children and adults and several clinical studies showed high repigmentation rates^{1,2}. The phototherapy-activated repigmentation involves functional melanocyte precursors in the skin to divide, migrate, and differentiate into mature melanocytes through a complex process involving melanocytes and other cell lineages in the skin. However, how phototherapy induces melanocyte precursor differentiation and migration is largely unknown. Hair follicles are known to harbor a well-characterized niche for epithelial stem cells, melanocyte progenitor cells and other multipotent stem cells derived from the neural crest³⁻⁵. Another distinct population of stem cells with neural-crest-like stem cell features has also been discovered in both human and mouse hair follicles, which express neural crest stem cells (NCSC) markers and are multipotent⁶⁻⁸. The neural crest is a transient, multipotent, migratory cell

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Corresponding author: Xiaowei Xu, Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, 3400 Spruce Street, Philadelphia, PA 19104, USA. Tel: 215-662-6503, Fax: 215-349-5910, E-mail: xug@pennmedicine.upenn.edu
ORCID: <https://orcid.org/0000-0003-4098-7690>

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population that is unique to vertebrate embryos and gives rise to many derivatives, ranging from the neuronal and glial components of the peripheral nervous system to the ectomesenchymal derivatives of the craniofacial area and pigment cells in the skin.

In this study, we cultured and isolated skin neural crest stem cells from human scalp tissue, and these cells expressed NCSC markers. We used Narrow-band ultraviolet B (NB-UVB) to irradiate human scalp-derived neural crest stem cells (HS-NCSCs), and assessed the effects of NB-UVB on proliferation, melanogenesis and migration of c-Kit⁺/CD57⁻ HS-NCSCs and investigated the mechanism. We discovered that NB-UVB facilitates melanocytic differentiation of the HS-NCSCs and enhances migration of these cells. Melanocortin 1 receptor (*MclR*) and cAMP pathway play a critical role in NB-UVB induced melanocytic differentiation.

MATERIALS AND METHODS

Primary HS-NCSC cultures and cell sorting

Primary HS-NCSCs were isolated from 12~15 weeks human fetal scalp tissues, which were purchased from Advanced Bioscience Resources (Alameda, CA, USA). Scalp tissues were cut into small pieces and digested into single cells by trypsin, which were cultured in DMEM/F12 (1:1 ratio) with 2% B27 supplement, 1% N2 supplement, 20 ng/ml EGF, and 40 ng/ml bFGF⁹. The cells were maintained in a humidified incubator with 5% CO₂ at 37°C, and further passaged at 1:3 every 1~2 week, with experiments being carried out from 3 to 6 passages. The c-Kit⁺/CD57⁻ cells from HS-NCSCs were negatively selected using the Magnetic Nanoparticles with Magnet (EasySepTM; Stemcell Technologies, Vancouver, BC, Canada), which firstly incubated with anti-CD57 antibody (Becton, 561906; Dickinson and Company, Franklin Lakes, NJ, USA). The study is approved by the Institutional Review Board (Protocol number 707906) at the University of Pennsylvania.

Immunofluorescent staining

HS-NCSCs were seeded at the concentration of 2×10⁵ cells/well (6-well plates) onto gelatin-coated chamber slides. Cells were fixed in PBS containing 4% paraformaldehyde and permeabilized by incubation with PBS containing 0.2% Triton X-100 and 10% FBS for 30 min at room temperature. The cells were then incubated overnight at 4°C with primary antibodies: *Nestin* (ab105389; Abcam, Cambridge, MA, USA), *p75* (#8238; Cell Signaling Technology, Danvers, MA, USA) and *Sox10* Ab (ab155279; Abcam). After several washes with PBS, second antibodies at a dilution of 1:100 were added respectively and in-

cubated for 1 h at 37°C. The nuclei were counter-stained with DAPI. Then the slides were mounted and observed under fluorescence microscope.

Analysis of stem cell markers by reverse transcription polymerase chain reaction (RT-PCR)

Total RNA of HS-NCSCs was isolated using the RNeasy Mini kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Potential DNA contamination in the RNA sample was removed by RNase-Free DNase treatment. 1 μg of DNase-treated total RNA was reverse transcribed using the first-strand cDNA synthesis kit for RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA). *β-tubulin*, *p75*, *Sox2*, *Slug*, *Oct4*, *NF-M*, *Snail*, *Musashi*, *Sox10* and *Nestin* were amplified using ExTaq DNA polymerase (Thermo Fisher Scientific). The PCR products were visualized on 2% agarose gels and ethidium bromide staining.

NB-UVB radiation

Experimental cells were seeded at the concentration of 2×10⁵ cells/well (6-well plates) onto gelatin-coated chamber slides for 2 days, then were treated with 311 nm NB-UVB radiation by handheld light (#UV-109B; Waldmann Co., Villingen-Schwenningen, Germany) for 4 times every three days. The incident dose rate was 13.00 mW/cm², and the test dose ladder is from 0.1 and 0.7 J/cm², which radiation time correspond to 8 s and 53 s. NB-UVB was irradiated directly on the c-Kit⁺/CD57⁻ HS-NCSCs in the culture dishes. After the first exposure, the culture medium was replaced to the melanogenic differentiation medium, which contains Medium 254 and 1% HMGS (Invitrogen, Carlsbad, CA, USA).

Transmission electron microscope (TEM)

For TEM, fresh small cell blocks were fixed in the modified Karnovsky's fixative. Subsequently, the specimens were postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 1 h. Then, the material was dehydrated through a graded series of ethanols and propylene oxide, embedded in Epon 812 and sectioned on a Reichert ultramicrotome (Reichert Ultracut S; Reichert-Jung, Buffalo, NY, USA) to obtain semithin sections. Next, the sections were stained with 1% methylene blue in 1% sodium borate and preliminarily examined under a light microscope to select Epon blocks. The blocks were then used to prepare ultrathin sections, which were placed on grids, contrasted with uranyl acetate and lead citrate, examined using an Opton EM 900 transmission electron microscope (Zeiss, Oberkochen, Germany), and photographed with TRS camera (CCD camera for TEM 2K inside).

Quantification of melanogenic factors by real-time RT-PCR

Cells were lysed and total RNA was purified using the RNeasy Mini kit. For real-time RT-PCR analyses, 1 μ g of DNase-treated total RNA was reverse transcribed. The amplification of the cDNA of tyrosinase, *Tyrp1* and *Dct* was accomplished using the QuantStudio 3 detection system (Applied Biosystems) in the presence of the commercially available Power SYBRTM Green PCR Master Mix (Thermo Fisher Scientific) in a 40-cycle PCR respectively. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The mRNA levels of each target gene were normalized to the levels of β -actin.

Western blotting

Cells were lysed in 6 \times SDS-PAGE sample buffer. The lysates of cells were loaded on a 12% polyacrylamide gel and separated under reducing conditions, using Rainbow-

colored protein molecular weight markers as a reference. At the end of the electrophoresis, the gel was soaked in Western transfer solution before electrophoretic transfer of the protein onto polyvinylidene difluoride membrane at 120 v for 120 min. The membrane was blocked for 1 h in TBST containing 5% dried skimmed milk powder. After three washes in TBST, the membranes were incubated with primary antibody at a 1:1,000 dilution: Tyrosinase (ab170905; Abcam), *Mcl1R* (ab125031; Abcam) and β -actin (12620; Cell Signaling Technology), which for 1 h at room temperature followed overnight at 4^oC. After three washes with TBST, the membranes were then incubated with a 1:1,000 dilution of HRP-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature and developed with the ECL Western blotting system according to the supplier's recommendations. The membranes were then exposed to X-ray film which was subsequently developed.

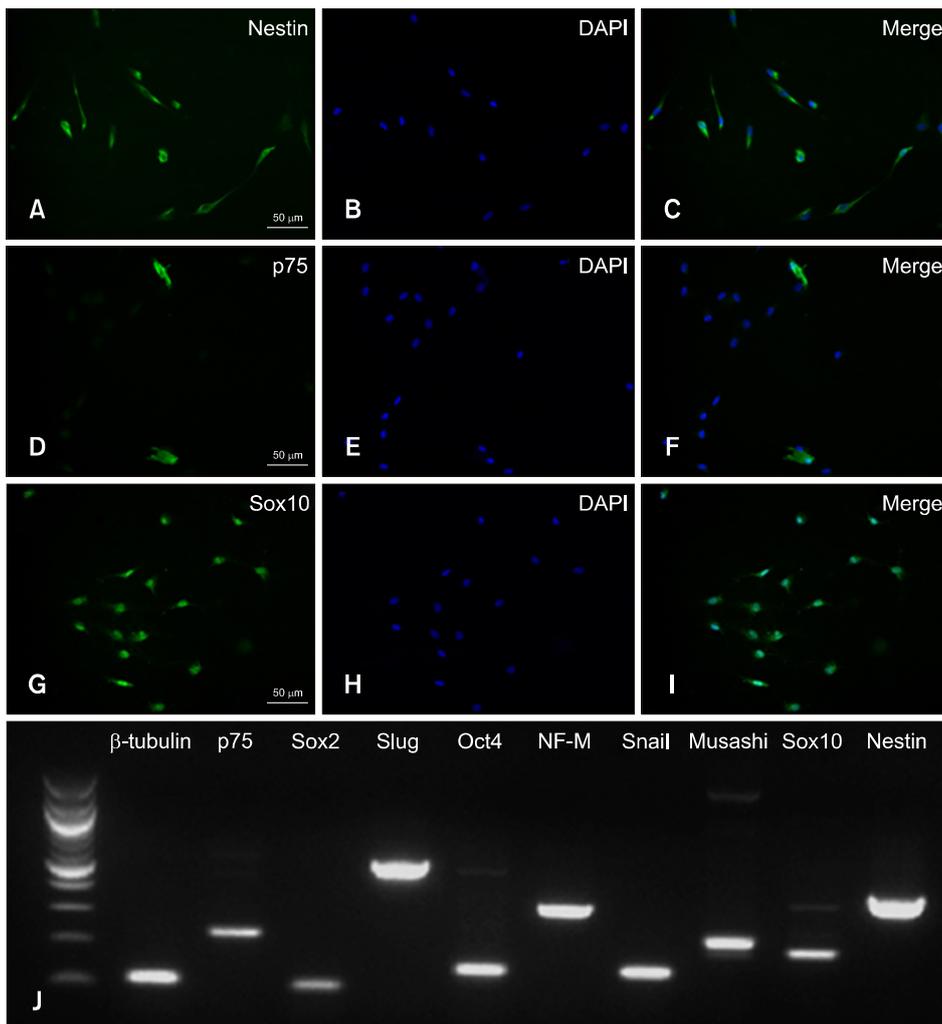


Fig. 1. Neural crest marker expression by human scalp-derived neural crest stem cells (HS-NCSCs). (A~C) *Nestin* expression in HS-NCSCs. Immunocytochemical stain was performed using indicated antibody. DAPI was used to visualize the nuclei. (D~F) *P75* expression in HS-NCSCs. (G~I) *Sox10* expression in HS-NCSCs. (J) Neural crest stem cell marker expression by HS-NCSCs. RT-PCR was performed to study the expression of stem cell markers.

Mc1R knockdown in the HS-NCSCs

c-Kit⁻/CD57⁻ HS-NCSCs were treated with shRNA against for *Mc1R* and control, which include shRNA luciferase and shRNA nontarget group, then using puromycin for 48 h after infecting. Then the remaining cells were used to receive the NB-UVB radiation.

Cell signaling pathways investigation

After the first exposure of c-Kit⁻/CD57⁻ HS-NCSCs to NB-UVB, the culture medium was replaced to the melanogenic differentiation medium with 2.5 μ M H89, a selective protein kinase A (PKA) inhibitor; or 20 μ M PD98059, a MAPK kinase inhibitor, respectively (all are from Sigma).

Cell scratch assay

For cell migration assay, scratch wound was created using P-200 pipet tip, then culture medium was replaced with fresh melanogenic differentiation medium. Wound area was observed at 32 h and 56 h after wounding.

Statistical analysis

Results are expressed as the mean \pm standard deviation. A Student's t-test was used to determine significance among the groups. A value of $p < 0.05$ was considered to be significant. Analyses and graphical representation were performed using Graph-Pad Prism 5.01 software (GraphPad

Software, San Diego, CA, USA).

RESULTS

Isolation of HS-NCSCs

HS-NCSCs were isolated as we previous described^{5,10}. Immunocytochemical stain results showed that the attached cells were positive for *nestin* (Fig. 1A~C), *p75* (Fig. 1D~F) and *Sox10* (Fig. 1G~I). RT-PCR results showed that the attached cells expressed *nestin*, *p75*, *Sox10*, *Snail*, *Musashi*, *β -tubulin*, *Sox2*, *Slug*, *Oct4* and *NF-M* (Fig. 1J). These results demonstrated that these attached cells express NCSC markers.

Melanocytic differentiation from c-Kit⁻/CD57⁻ HS-NCSCs with increased dose of NB-UVB

c-Kit⁻/CD57⁻ cells were isolated from HS-NCSCs by negative selection using anti-CD57 and anti-c-Kit antibodies labelled with magnetic nanoparticles. The attached c-Kit⁻/CD57⁻ HS-NCSCs were irradiated with different doses of NB-UVB and then cultured in the melanogenic differentiation medium. We irradiated the cells four times with three-day interval. After every irradiation, we replaced the melanogenic differentiation medium immediately. We counted the number of attached differentiated cells at day 12. Morphology of the attached cells was similar to typical dendritic morphology of melanocytes. At 100 mJ of NB-

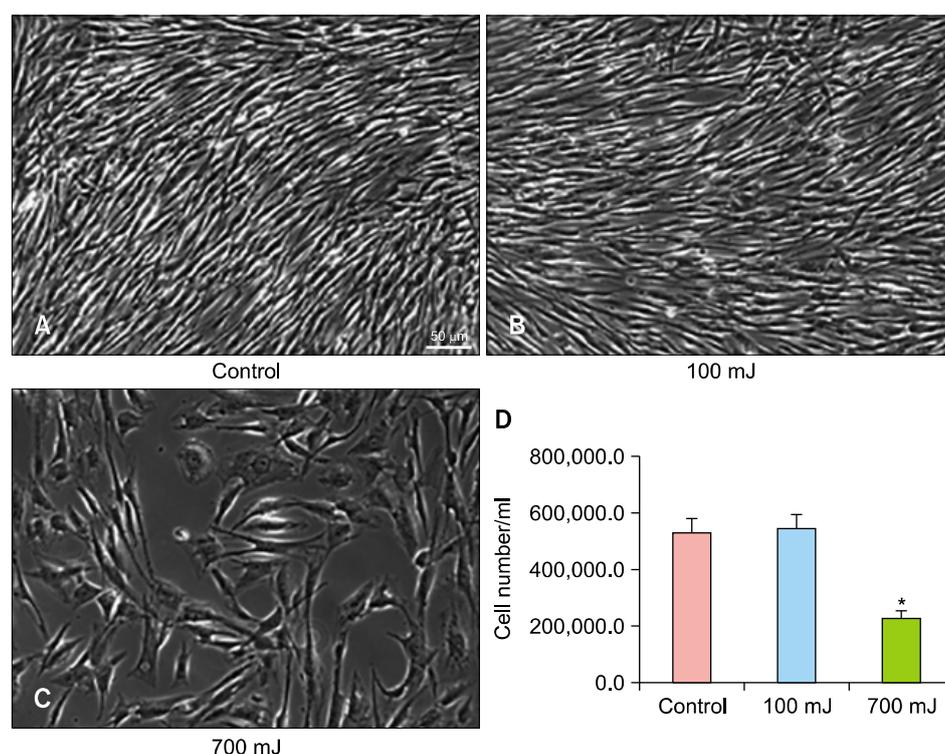


Fig. 2. Cell proliferation after narrow-band ultraviolet B (NB-UVB) exposure. (A) Differentiated melanocytes from c-Kit⁻/CD57⁻ HS-NCSCs in melanocyte media without exposure. (B) Differentiated melanocytes from c-Kit⁻/CD57⁻ HS-NCSCs under 100 mJ NB-UVB. (C) Differentiated melanocytes from c-Kit⁻/CD57⁻ HS-NCSCs under 700 mJ NB-UVB. (D) Cell number of c-Kit⁻/CD57⁻ HS-NCSCs after exposure to different doses of NB-UVB at day 12. HS-NCSCs: human scalp-derived neural crest stem cells. *Indicate $p < 0.05$.

UVB, the number of attached differentiated cells was similar to that in control (Fig. 2A, B), whereas there was a significant decrease of the number with increased NB-UVB doses at 700 mJ (Fig. 2C). At 700 mJ of NB-UVB, less than 40% of the attached cells showed melanocytic differentiation. High dose of NB-UVB has an inhibitory effect on cell proliferation (Fig. 2D).

Ultrastructural characteristics of melanocytes from c-Kit⁻/CD57⁻ HS-NCSCs under NB-UVB

TEM showed that the cell membrane was smooth in the melanocytes derived from c-Kit⁻/CD57⁻ HS-NCSCs with-

out NB-UVB (Fig. 3A). Interestingly, TEM showed that there were many dendritic processes emerged on the surface of cell membrane under NB-UVB at 100 mJ dose, many melanosomes were present in the cytoplasm (Fig. 3B).

Alteration of signaling pathways during melanocytic differentiated from c-Kit⁻/CD57⁻ HS-NCSCs after NB-UVB radiation

Tyrosinase, tyrosinase-related protein-1 (*tyrp1*) and tyrosinase-related protein-2 (*tyrp2*, *Dct*) are well-known key enzymes in melanin synthesis. Real-time RT-PCR showed

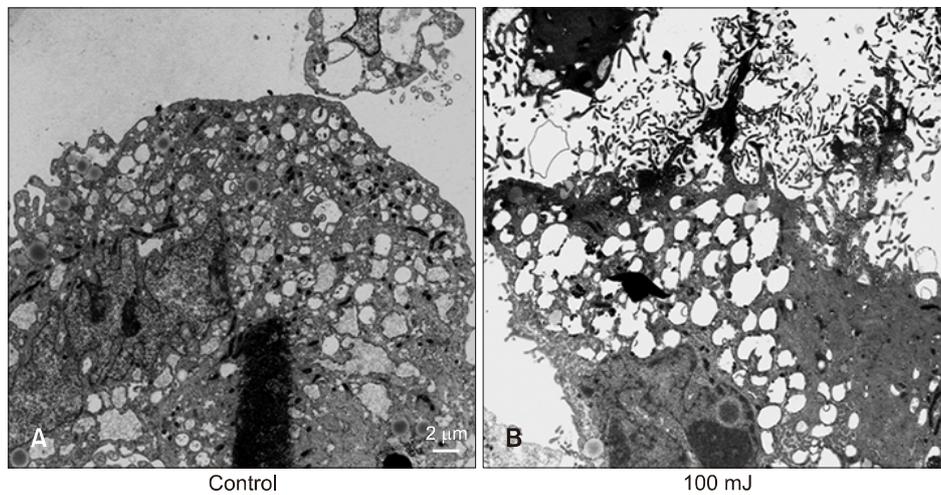


Fig. 3. Ultrastructure of differentiated melanocytes after narrow-band ultraviolet B (NB-UVB) exposure. Transmission electron microscopy was performed after differentiated melanocytes exposed to NB-UVB. The sections were stained with Uranyl acetate and lead citrate. (A) Differentiated melanocytes from c-Kit⁻/CD57⁻ HS-NCSCs without NB-UVB exposure. (B) Differentiated melanocytes from c-Kit⁻/CD57⁻ HS-NCSCs after exposure to 100 mJ NB-UVB. Significantly more dendritic processes are present after exposure to NB-UVB. HS-NCSCs: human scalp-derived neural crest stem cells.

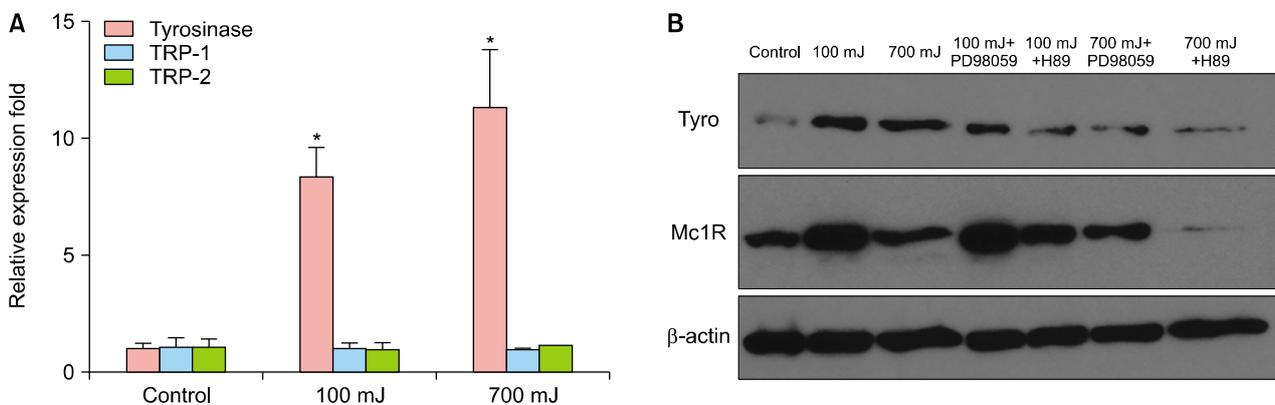


Fig. 4. Signaling pathway changes after narrow-band ultraviolet B (NB-UVB) exposure. (A) Effect of mRNA expression in differentiated melanocytes from HS-NCSCs after NB-UVB radiation. (B) Western blot analysis of Tyrosinase and *Mc1R* protein levels after NB-UVB radiation. H89 and PD98059 were added in the melanocyte culture medium, respectively. Molecular weight of Tyrosinase is 60 kD, *Mc1R* is 35kD and β -actin is 45kD. HS-NCSCs: human scalp-derived neural crest stem cells, *Mc1R*: Melanocortin 1 receptor, Tyro: tyrosinase. * $p < 0.05$, compared with control.

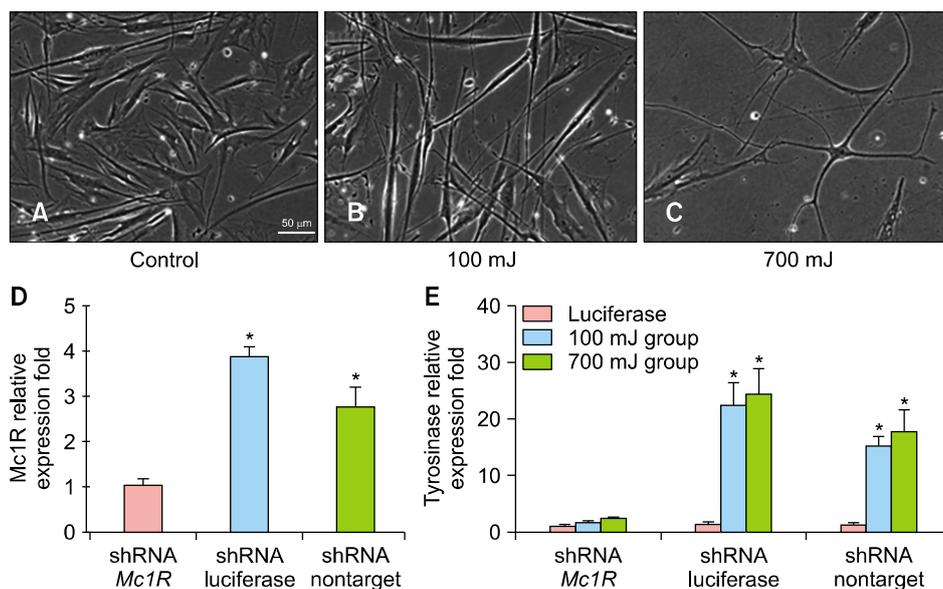


Fig. 5. Effect of *Mc1R* knockdown on response of differentiated melanocytes to narrow-band ultraviolet B (NB-UVB). HS-NCSCs were transfected with shRNA to *Mc1R*, luciferase or random nontarget and transfected cells were selected. (A) Differentiated melanocytes from *c-Kit*⁻/*CD57*⁻ HS-NCSCs with *Mc1R* knockdown in melanocyte medium without NB-UVB exposure. (B) Differentiated melanocytes from *c-Kit*⁻/*CD57*⁻ HS-NCSCs with *Mc1R* knockdown in melanocyte medium with 100 mJ NB-UVB exposure. (C) Differentiated melanocytes from *c-Kit*⁻/*CD57*⁻ HS-NCSCs with *Mc1R* knockdown in melanocyte medium with 700 mJ NB-UVB exposure. (D) *Mc1R* expression after knockdown. (E) Effect of tyrosinase mRNA expression after *Mc1R* knockdown in response to NB-UVB. HS-NCSCs: human scalp-derived neural crest stem cells, *Mc1R*: Melanocortin 1 receptor, Tyro: tyrosinase. **p* < 0.05, compared with control.

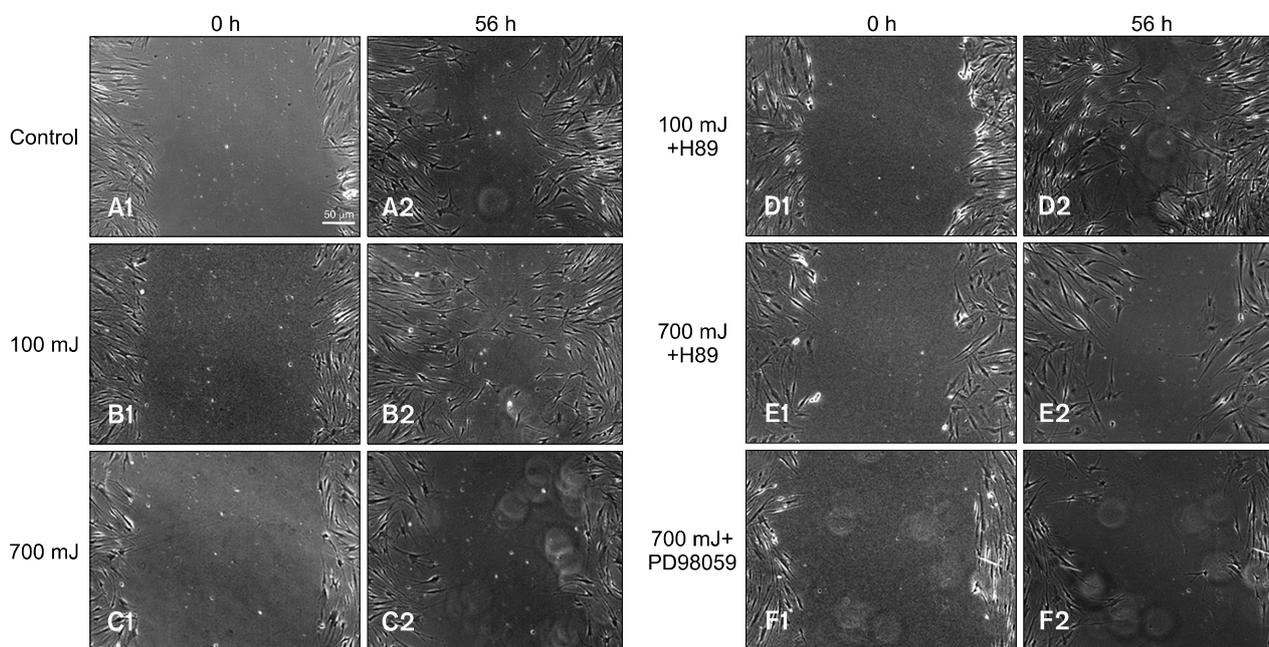


Fig. 6. Cell migration in response to narrow-band ultraviolet B (NB-UVB). Cell migration change of melanocytes differentiated from *c-Kit*⁻/*CD57*⁻ HS-NCSCs with different doses of NB-UVB at the time of 0 and 56 h. (A1, A2) Control group. (B1, B2) 100 mJ group. (C1, C2) 700 mJ group. (D1, D2) 100 mJ with H89 group. (E1, E2) 700 mJ with H89 group. (F1, F2) 700 mJ with PD98059 group. HS-NCSCs: human scalp-derived neural crest stem cells.

that the expression of *tyrosinase* was significantly increased after 100 or 700 mJ NB-UVB exposure. However, there was no difference in the gene expression of *Tyrp1* and *Dct* after NB-UVB radiation (Fig. 4A). Western blot analysis also showed the expression of *tyrosinase* was significantly increased in cells exposed to 100 or 700 mJ of NB-UVB exposure. The increase of *tyrosinase* expression was attenuated by addition of a MAPK inhibitor PD98059 or a selective PKA inhibitor H89, suggesting that these pathways are involved in the NB-UVB induced melanocytic differentiation. The expression of *Mc1R* were significantly increased at the dose of 100 mJ NB-UVB exposure (Fig. 4B).

Mc1R knockdown in c-Kit⁻/CD57⁻ HS-NCSCs

To study the role of *Mc1R* in the melanogenesis induced by NB-UVB, c-Kit⁻/CD57⁻ HS-NCSCs were transfected with *Mc1R* shRNA or controls including shRNA luciferase and shRNA random nontarget, and then treated with NB-UVB. The melanocytes differentiated from c-Kit⁻/CD57⁻ HS-NCSCs with *Mc1R* knockdown became enlarge than that of c-Kit⁻/CD57⁻ HS-NCSCs without *Mc1R* knockdown (Fig. 5A~C). The expression of *Mc1R* was significantly decreased in the group of c-Kit⁻/CD57⁻ HS-NCSCs with *Mc1R* knockdown, compared to the groups transfected with shRNA luciferase or shRNA nontarget, respectively (Fig. 5D). After NB-UVB irradiation, the expression of *tyrosinase* was significantly decreased in the group of c-Kit⁻/CD57⁻ HS-NCSCs with *Mc1R* knockdown, compared to the groups with shRNA luciferase or shRNA nontarget, respectively (Fig. 5E).

Cell migration in melanocytes differentiated from c-Kit⁻/CD57⁻ HS-NCSCs with NB-UVB

Using cell migration assay, we observed that the 100 mJ of NB-UVB (Fig. 6B1, B2) induced faster cell migration compared to the control (Fig. 6A1, A2), while 700 mJ dose (Fig. 6C1, C2) resulted slower cell migration. While, treatment with H89 or PD98059 did not induce significant changes compared to that in the control (Fig. 6D1, D2, E1, E2 and F1, F2).

DISCUSSION

Vitiligo is a common depigmentation condition that carries a great impact on patients' quality of life. Reversing vitiligo depigmentation requires activation of hair follicle and/or epidermal melanocyte precursors by phototherapy and/or drugs. Experimental observations in human vitiligo and genetically engineered mouse models have shown that, during UV exposure, melanocyte precursors pro-

liferate and migrate along the infundibulum outer root sheath to the interfollicular epidermis and repopulate the epidermal areas devoid of melanocytes¹¹⁻¹³. Our previous studies also found that NB-UVB directly induces melanocyte lineage differentiation of the mouse hair follicle-derived neural crest stem cells^{14,15}. Nevertheless, the currently available mouse models and mouse hair follicle-derived neural crest stem cells cannot accurately reproduce the human repigmentation process in patients undergoing phototherapy. In this study, we examined the effect of NB-UVB on human hair follicle-derived neural crest stem cells.

To better understand the effect of phototherapy on human repigmentation pathways, we focus on human scalp hair follicles, which contain neural crest stem cells, melanocyte precursors and differentiated melanocytes. Melanocytes in vertebrates are derived from the neural crest, which arises during gastrulation of embryogenesis at the dorsal edge of the neural plate¹⁶. Previous studies showed c-Kit is a mark of unpigmented melanocyte stem cells in hair follicles^{4,17,18}. In this study, we first demonstrated that the Kit⁻/CD57⁻ HS-NCSCs express various NCSC related markers. Cell proliferation assays showed that NB-UVB have little effect on differentiated cell proliferation at 100 mJ, but can significantly decrease the number of differentiated cells at 700 mJ. Interestingly, transmission electron microscopy showed that there are many dendritic processes emerged on the surface of cell membrane after NB-UVB radiation, which is likely due to melanocyte maturation after NB-UVB radiation. We noticed some dendritic processes are filled with melanosomes.

We discovered that *tyrosinase* expression in melanocytes differentiated from c-Kit⁻/CD57⁻ HS-NCSCs were upregulated using quantitative PCR and western blot analysis after irradiation, which suggests that NB-UVB stimulates the differentiation and maturation of melanocytes from c-Kit⁻/CD57⁻ HS-NCSCs. In addition, *Mc1R* protein expression was significantly increased after NB-UVB exposure. *Mc1R* is a G protein-coupled receptor that regulates the amount and type of melanin pigments in melanocytes. Stimulation of *Mc1R* with α -MSH leads to a G-protein coupled induction of cAMP driving the CRE-MITF pathway and promoting melanin synthesis and cell proliferation¹⁹⁻²². Our study show that the NB-UVB exposure induced expression of *tyrosinase* is repressed with H89, a selective protein kinase A inhibitor, supporting that CREB-MITF pathway play a critical role in NB-UVB induced melanogenesis. Using *Mc1R* knockdown c-Kit⁻/CD57⁻ HS-NCSCs, our data demonstrate that *Mc1R* is involved in the NB-UVB induced *tyrosinase* expression.

Migration of melanocytes from the hair follicle to neigh-

boring epidermis also plays an important role in repigmentation of vitiligo skin after NB-UVB radiation. We performed cell scratch assay to determine the effects of NB-UVB on cell migration, and found that the migration ability of differentiated cells from c-Kit⁺/CD57⁻ HS-NCSCs is enhanced after 100 mJ of NB-UVB exposure, but is inhibited at higher dose of NB-UVB. These data suggest that NB-UVB enhances the migration ability of differentiated melanocytes derived from HS-NCSCs.

In summary, this study supports that NB-UVB facilitates melanocytic differentiation of the HS-NCSCs and enhances migration of these cells. Both effects may contribute to the skin re-pigmentation in vitiligo patient after NB-UVB treatment.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

ORCID

Dake Dong, <https://orcid.org/0000-0002-3231-7606>
 Shujun Chen, <https://orcid.org/0000-0001-7940-8655>
 Cheng Feng, <https://orcid.org/0000-0002-8785-2681>
 Huizi Xiong, <https://orcid.org/0000-0003-1329-1612>
 Xiaowei Xu, <https://orcid.org/0000-0003-4098-7690>

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