BMP4-Induced Differentiation of Human Hair Follicle Neural Crest Stem Cells into Precursor Melanocytes from Hair Follicle Bulge

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Background: Vitiligo is a skin depigmentation disorder, for which, repigmentation treatment with combined follicular unit extraction (FUE) graft and narrowband ultraviolet B (NBUVB) is considered superior to micro-punch graft therapy. BMP4 can induce MITF expression in Neural crest stem cells (NCSCs), and α-MSH subsequently promotes the differentiation of MITF-expressing cells along the melanocyte lineage.

Objective: To investigate why FUE grafting is superior to epidermal mini grafting in promoting hair follicles (HF) melanocyte cell survival and longevity, we planned the in vitro experiments HF bulge NCSCs differentiate into melanocyte precursors under the co-treatment of BMP4 and α-MSH.

Methods: Cells that migrated from the HF bulge of scalp were cultured and assessed using immunofluorescence. Transcriptome analysis was performed on RNA sequencing results.

Results: Basic fibroblast growth factor promotes the proliferation and survival of NCSCs, with spontaneous differentiation into SOX10+/SOX2+ glial progenitors, but not into SOX10+/MITF+ precursor melanocytes. Both BMP4 and α-MSH promoted the differentiation into MITF-expressing cells. RNA sequencing revealed a downregulation in neuregulin-1 (NRG1) and semaphorin 3C (SEMA3C), and upregulation in WNT10A. Furthermore, FUE grafting had a source of reservoir melanocytes superior to mini-grafting in treatment for vitiligo. Conclusion: We obtained SOX10+/MITF+ precursor melanocytes through the co-treatment of BMP4 and α-MSH. According to the RNA sequencing results that NRG1 and SEMA3C were downregulated and WNT10A was upregulated, we postulated that HF NCSCs differentiated into melanocyte by co-treatment of BMP4 and α-MSH. Overall, FUE grafting is a more robust and substitutive treatment option for vitiligo. (Ann Dermatol 32(5) 409∼416, 2020)

Keywords: Hair follicle, Melanocytes, Neural crest, Neuregulin-1, Stem cells

INTRODUCTION

The main components linked to the pathogenesis of vitiligo are genetic background, oxidative stress, and immune regulation. In the immune-mediated destruction of melanocytes, type 1 response, Th17 cell responses, and cytotoxic T cells have been implicated under the regulatory T cell-deficient conditions. Narrowband ultraviolet B (NBUVB) phototherapy is currently the dominant repigmentation treatment for vitiligo because of its stimulatory properties in the proliferation and migration of melanocytes. However, a major pitfall of NBUVB phototherapy is some refractoriness as the repigmentation grade declines, especially during the 2nd year of NBUVB phototherapy relative to the 1st year. This response is proposed to be due to the ex-
haustion of functional melanocytes or from the depletion of reservoir melanocytes during long-term repeated sessions of NBUVB treatment

Various approaches are typically undertaken to replenish reservoir melanocytes, such as autologous cultured melanocyte graft, non-cultured epidermal cell suspension graft, mini grafting, and follicular unit extraction (FUE) grafting. In particular, FUE grafting is an attractive therapeutic option for vitiligo as this method has shown good repigmentation efficacy after NBUVB treatment and a long duration of sustained repigmentation. Even so, another potential source of precursor melanocytes is required for providing cells to treat vitiligo in clinical practice that can address both cosmetic acceptability and ease of application. Specific melanocyte stem cell (MSC) markers are not well known, as opposed to late-stage differentiated melanocytes markers, although MSCs are postulated to exist in the hair follicle (HF). Despite the good repigmentation efficacy and long duration of sustained repigmentation obtained from FUE grafting treatment, the detailed underlying mechanisms of action remain to be elucidated. Therefore, an in-depth study is needed to assess the undifferentiated stem cells, to address underlying questions for better and sustained repigmentation in FUE grafting therapy. Furthermore, it is important to understand the differentiation mechanism of the undifferentiated stem cells into the precursor melanocytes with the help of some fate-determining factors aside from UV radiation. Neural crest stem cells (NCSCs) would be most suitable as they have unique markers and are easily obtained from the HF bulge.

According to a previous study using pluripotent embryonic stem cells (ESCs), OCT4 and NANOG-expressing cells gave rise to multipotent NCSCs that expressed SOX10 and p75. After BMP4 induction, NCSCs gave rise to precursor melanocytes that expressed MITF, but not DCT, SILVER, or TYRP1. These MITF-expressing cells developed into fully differentiated melanocytes under the stimulation of BMP4, EDN3, and/or WNT3A and SCF.

Here, we wanted to derive an alternative source of precursor melanocytes from HF multipotent NCSCs, for replenishing exhaustible resources in repigmentation treatment against vitiligo. We further aimed to investigate how BMP4 induces differentiation of HF NCSCs into precursor melanocytes using a migration cell culture model. This study focused on the advantages of FUE grafting as a tool of clinical evaluation of melanocyte turnover and melanogenesis in translational research, which connects experimental in vitro studies with expected clinical efficacies.

**MATERIALS AND METHODS**

**Assessment of vitiligo patients who underwent long-term NBUVB phototherapy**

We retrospectively reviewed the degree of repigmentation in 19 patients with vitiligo, who were treated with NBUVB therapy twice weekly for 2 years or longer (Fig. 1A, B). We evaluated the effectiveness of long-term NBUVB phototherapy for vitiligo using the Physicain’s Global Assessment. Repigmentation gradings were recorded as follows: No repigmentation (none) = grade 0, mild repigmentation (<25%) = grade 1, moderate repigmentation (25%–50%) = grade 2, good repigmentation (51%–75%) = grade 3, and excellent repigmentation (76%–100%) = grade 4. This study was approved by the Institutional Review Board of the Dong-A University Medical Center (IRB no. DAUHIRB-TEMP-20-143) and was conducted according to the 1983 Declaration of Helsinki. Written informed consent was obtained from patients before participation. We received the patient’s consent form about publishing all photographic materials.

**Isolation and culture of human HFs**

FUE grafting specimens were taken from occipital scalps of patients who were undergoing hair transplantation. A subcutaneous fat tissue containing lower HFs was dissected from the epidermis and dermis. The HFs were isolated under a binocular microscope with forceps and maintained in Williams E medium (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 95% O2 and 5% CO2. Dissected HFs were transferred into plastic dishes coated with bovine type I collagen and cultured in DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 20% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 95% O2 and 5% CO2. The HF explants were maintained for a week, and the medium was changed every 3 days. Explanted HFs were removed 1 week after identifying cultured cells that had migrated from the HF bulge regions. Once the cell outgrowth reached sub-confluency, the cultured bulge cells were harvested with 0.25% trypsin/10 mM EDTA in Hanks’ balanced salt solution and sub-cultured at a split ratio of 1:3 (from 1 well into 3 wells).

**Culture of migrated cells from the HF bulge**

The cultured HF bulge cells were plated in an eight-chamber slide and placed in serum-free DMEM for 24 hours. Basic fibroblast growth factor (bFGF) (10 ng/ml) was added to the culture medium, and anti-SOX10 antibody was used to identify NCSC proliferation. BMP4 (10 ng/ml) and α-MSH (0.1 μM) were added into the culture medium,
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Fig. 1. (A) Significant decline of narrowband ultraviolet B (NBUVB) phototherapy repigmentation grade during the 2nd period of NBUVB phototherapy relative to the 1st period. Values are presented as mean ± 95% confidence interval. (B) NBUVB therapy performed on vitiligo lesions for 1 year, with limited repigmentation. Epidermal melanocytes had reduced survival during post-epidermal graft adaptation, and NBUVB-induced repigmentation did not continue in the mini grafted sites (dotted circles). *p<0.001.

and anti-SOX-2 and anti-MITF antibodies were used to identify and calculate any proportional changes in cell type. Changes in cell composition were assessed 2 weeks after this treatment, by direct immunofluorescence. Subsequently, RNA was extracted from the cultured migrated cells after treatment with various concentrations of BMP4 and processed for RNA sequencing.

Immunofluorescence studies for NCSC, bipotent precursors, and precursor melanocytes

The cultured HF bulge cells were washed with PBS and immersed in cold methanol for 10 minutes. After blocking with 10% normal goat serum for 1 hour, the cells on slides were incubated with rabbit polyclonal anti-SOX10 antibody (1:50 dilution) at 4°C overnight, washed 3 times with PBS, and incubated with goat anti-rabbit FITC-conjugated antibody at a 1:50 dilution for 1 hour. The cells on slides were then washed with PBS and counterstained with 4,6-diamidino-2-phenylindole for 10 minutes. Negative controls for the immunostaining were made using normal rabbit immunoglobulin G. The cultured HF bulge cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at 4°C, then washed and permeabilized with 0.05% Tween 20 in PBS for 10 minutes. Blocking of nonspecific binding was performed using normal rabbit immunoglobulin G. The cultured HF bulge cells were fixed with 4% paraformaldehyde in PBS for 30 minutes at 4°C, then washed and permeabilized with 0.05% Tween 20 in PBS for 10 minutes. Blocking of nonspecific binding was performed using 10% serum of the animal in which the secondary antibody was raised. The following primary antibodies were used for indirect immunocytochemistry: anti-SOX2 (1:200) and anti-MITF (1:20). Incubation was performed overnight at 4°C. Corresponding secondary antibodies (conjugated to TRITC, FITC, or Cy5) were applied at 1:200 dilution for 2 hours at room temperature. BX51 microscope (Olympus, Tokyo, Japan), DP71 camera (Olympus), and Cell P software (Olympus) were used for image acquisition.

RNA sequencing

We performed RNA sequencing on cultured cell extract after BMP4 treatment at free, low (10 ng/ml), and high (50 ng/ml) concentrations. RNA quality was assessed by analysis of rRNA band integrity, using an Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). Ahead of cDNA library construction, 1 μg of total RNA and magnetic beads with Oligo (dT) were used to separate poly (A) mRNA. Subsequently, the purified mRNAs were disrupted into short fragments, and the double stranded cDNAs were immediately synthesized. The cDNAs were subjected to end-repair, poly (A) addition, and connected with sequencing adapters using the TruSeq RNA sample prep Kit (Illumina, San Diego, CA, USA). The suitable fragments automatically purified by BluePippin 2% agarose gel cassette (Sage Science, Beverly, MA, USA) were selected as templates for PCR amplification. The final library sizes and qualities were evaluated electrophoretically with an Agilent High Sensitivity DNA Kit (Agilent Technologies),
and the fragment was found to be between 350～450 bp. Subsequently, the library was sequenced using an Illumina HiSeq2500 sequencer (Illumina).

**Transcriptome data analysis**

Filtering: Low quality reads were filtered according to the following criteria: reads containing more than 10% of skipped bases (marked as ‘N’s), reads containing more than 40% of bases whose quality scores were less than 20, and reads whose average quality scores were less than 20. The whole filtering process was performed using in-house scripts.

Sequence alignment: Filtered reads were mapped to the human reference genome\(^{12}\) using the aligner TopHat\(^{13}\).

Gene expression estimation: Gene expression level was measured with Cufflinks v2.1.1\(^{13}\) using the gene annotation database of Ensembl release 72. Non-coding gene region was removed with mask option. To improve the measurement accuracy, multi-read-correction and fragbias correct options were applied. All other options were set to default values. Differentially expressed genes (DEGs) were identified using the Cuffdiff tool, with the default parameter setting with a significance of \(q\)-value < 0.05.

Gene Ontology analysis: The Gene Ontology (GO) database classifies genes according to the three categories of Biological Process, Cellular Component, and Molecular Function, and predicts the function of selected genes. To characterize the identified genes from DEG analysis, the GO-based trend test was performed using the Fisher’s exact test. \(p\)-values < 0.001 were considered statistically significant.

**FUE grafting vs. mini grafting as the optimal measures for replenishing melanocytes**

Four vitiligo patients (2 patients with vitiligo on their faces and 2 patients with vitiligo on their hands) were treated with FUE grafts of 2 mm diameter, with a total of 46 grafts. A total of 12 grafts of 2 mm diameter were engrafted on 2 patients with mini grafting. In both groups, after 1 week’s halt post-grafting, NBUVB phototherapy was administered twice a week for 6 months. The degrees of repigmentation were measured by the diameter of repigmentation using a dermscope.

**RESULTS**

**Isolation of outer root sheath cells, including NCSCs, migrated from the human HF bulge**

At 1 week post-explantation, cells with spindle or stellate morphology migrated from the dissected HFs, increasing in number over time. The area of outgrowth corresponded to the bulge region (asterisk), which is the HF niche. Many of these migrated cells from the human hair bulge showed SOX10 positivity, which is a marker for NCSC (Fig. 2).

**Effect of bFGF followed by BMP4 and \(\alpha\)-MSH treatment on the differentiation fate of NCSCs migrated from the human HF bulge**

After bFGF (10 ng/ml) was added to the migrated cell culture medium, the proportion of SOX10-positive cells increased to 68.4% due to stimulated proliferation, compared to 51.3% in bFGF-free condition (Fig. 3A). Untreated SOX10+ NCSCs spontaneously differentiated into SOX10+/SOX2+ glial progenitors (mean ratio, 0.906; SOX2+ cells/total cells) (Fig. 3B), but not into SOX10+/MITF+ precursor melanocytes. After BMP4 and \(\alpha\)-MSH (10 ng/ml each) were added, differentiation into MITF+ precursor melanocytes was promoted (mean ratio, 0.45; MITF+ cells/total cells) (Fig. 3C).

**Heatmap of RNA sequencing results**

Among the various neural crest genes, neuregulin-1 (NRG1) and semaphorin 3C (SEMA3C), were downregulated after BMP4 treatment (Table 1). WNT10A was upregulated upon BMP4 treatment (Table 1). Among various other genes, green color marked molecules, such as SNAI2 and EDN1, were downregulated after BMP4 treatment; levels of nestin, a marker for pluripotent ESC, remained unchanged.

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**Fig. 2.** At 1 week post-explantation from the hair follicle bulge, there is an increase in SOX10-positive cells; SOX10 is a neural crest stem cell-specific marker.
Fig. 3. (A) The effect of bFGF (10 ng/ml) treatment on the migrated cell culture. There is an increased proportion of SOX10-positive cells (68.4%) due to stimulated proliferation, compared to 51.3% grown under bFGF-free conditions. Values are presented as mean±95% confidence interval. (B) Spontaneous differentiation of majority of SOX10+ neural crest cells into SOX10/SOX2+ glial progenitors, after a 2-week culture period. (C) MITF expression in some cells during BMP4- and α-MSH-added culture conditions, mainly distributed in the nucleus. Mean ratio=0.45 (MITF+ cells/total cells). bFGF: basic fibroblast growth factor, NBUVB: narrowband ultraviolet B, FUE: follicular unit extraction. **p=0.002.

Table 1. RNA sequencing results

| Gene name | Description | Control | BMP4 10 ng/ml | BMP4 50 ng/ml |
|-----------|-------------|---------|---------------|---------------|
| NRG1      | Neuregulin-1| 76.23   | 18.86         | 17.93         |
| SEMA3C    | Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphoring) 3C | 124.2 | 48.57 | 44.7 |
| WNT10A    | Wingless-type MMTV integration site family, member 10A | 1.98 | 11.75 | 11.44 |
| ALDH1A2   | Aldehyde dehydrogenase 1 family, member A2 | 0.80 | 1.41 | 0.58 |
| Gbx2      | Gastrulation brain homeobox 2 | 0.57 | 0.91 | 0.2 |
| MEF2C     | Myocyte enhancer factor 2C | 0.57 | 0 |  |

NRG1 (neural crest development) and SEMA3C (neural crest cell migration) were downregulated more than 2-fold in a BMP4 concentration-dependent manner. WNT10A (neural crest cell proliferation) was upregulated more than 2-fold in a BMP4 concentration-dependent manner.

Fig. 4. Heatmap of RNA sequencing results. SNAI2 and EDN1 were expressed in BMP4-treated conditions. Nestin, SOX9, EDN3, KIT/KITL, and DCT were not expressed.
SOX9, SLF, EDN3, KIT/KITL, and DCT were not expressed, indicating no expression of differentiated melanocytes (Table 1, Fig. 4). These results show that the cell isolation experiments using migrated cell cultures from the hair bulge were successful; most of the migrated cells were NCSCs, and BMP4 treatment differentiated those into MSC progeny.

A representative case of treatment of a patient with vitiligo using FUE grafting vs. mini grafting with subsequent NBUVB therapy

A 68-year-old Korean male patient was affected with a left forehead vitiligo lesion that developed after a burn about 30 years ago. First, we treated him with NBUVB for a year; however, it did not achieve satisfactory results. Next, we tried epidermal mini grafting with NBUVB therapy, for 24 months, but the repigmentation response was poor with a few marginal repigmentation sites. Finally, FUE grafting was applied, followed by NBUVB treatment. Owing to a much better repigmentation than seen previously, the second session of FUE grafting was performed 6 months after the first session. Satisfactory repigmentation was attained during the subsequent NBUVB treatment. Therefore, FUE grafting had a more effective source of reservoir melanocytes, as seen from the 6.0-fold better repigmentation efficiency, compared to 4.55-fold efficiency in the mini grafting (Fig. 5A, B).

DISCUSSION

Among the various pitfalls of NBUVB phototherapy as the main option for vitiligo treatment, one is the refractoriness and less responsiveness of repigmentation over time. Such poor repigmentation ability is postulated to result from the exhaustion of functional melanocytes or from depletion of reservoir melanocytes during the long-term repeated sessions of NBUVB treatment. Reservoir melanocytes are located in both the epidermis and the HF bulge region. The FUE grafting method is reportedly more effective and sustainable than the epidermal mini grafting for repigmentation after NBUVB phototherapy, since the reservoir melanocytes of the HF bulge have higher stem cell activity and a longer life span.

We herein propose the new possibilities of providing a pool of reservoir melanocytes in an undifferentiated stem cell state from the HF bulge region, where the repigmentation process can be started by precursor melanocytes rather than by epidermal melanocytes. If we can take advantage of the precursor melanocytes directly from the HF bulge stem cells, or if we can induce differentiation of HF bulge NCSCs into multiple staged melanocyte lineage cells, we can create a new regimen of re-
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We assessed whether HF bulge NCSCs differentiated into precursor melanocytes after BMP4 and \( \alpha \)-MSH induction in developmental steps. We then investigated the detailed expression of the fate-determining or regulatory factors and affirmed how such HF bulge NCSCs can be further differentiated into precursor melanocytes. Next, we undertook an in vivo trial to assure longer survival using the FUE grafting than the epidermal mini grafting under NBUVB phototherapy.

The bFGF-untreated SOX10+ NCSCs were spontaneously differentiated into SOX10+ SOX2+ glial progenitors, but not into MITF+ precursor melanocytes. However, following BMP4 and \( \alpha \)-MSH treatment, SOX10+ MITF+ precursor melanocytes were seen to increase. Overall, cells treated with BMP4 and \( \alpha \)-MSH showed SOX10+ MITF+ phenotype precursor melanocytes, as opposed to SOX10+ SOX2+ glial progenitors. Subsequently, we conducted RNA sequencing to find the fate-determining or regulatory factors to compare the effect of different experimental conditions to that of various concentrations of BMP4. Importantly, RNA sequencing results showed that NRG1 and SEMA3C were downregulated, hence indirectly denoting that the fate toward neuroglial lineage may be blocked. The former is essential for normal development of the nervous system and heart, and the latter functions as an attractant for growing axons. WNT10A showed upregulated expression upon BMP4 treatment. WNT10A, in the outer bulge, maintains stem cell potency throughout hair cycle quiescence and growth. WNT10A expression occurs at the onset of anagen in mice, and continuous WNT signaling is required for the maintenance of anagen. Wnt10A may also play a role in the induction of melanocyte development. Moreover, undifferentiated stem cells isolated from the HF bulge did not contain functional or later differentiation markers, such as SOX9, SLF, EDN3, KIT/KITL, DCT, and nestin, after BMP4 and \( \alpha \)-MSH treatment. Those molecules would be related to the acquisition of precursor melanocytes via downregulated NRG1 and SEMA3C and upregulated WNT10A. This speculation could be verified in the subsequent experiments of migrated cell culture from the HF bulge through direct immunofluorescence for MITF after BMP4 treatment.

In response to UV exposure, keratinocytes produce several factors with paracrine action on melanocytes, and these may have stimulatory or inhibitory effects on melanin production. Keratinocytes contribute to transient UV-induced melanogenesis by releasing numerous paracrine growth factors for pigmentation, such as \( \alpha \)-MSH, ET-1, stem cell factor, and others. In addition to \( \alpha \)-MSH, a functional \( \beta \)-MSH signal is present in the human epidermis that contributes to the control of melanogenesis through the cAMP pathway. \( \beta \)-MSH has a low affinity for the melanocortin 1 receptor (MC1-R), and a higher affinity for the melanocortin 4 receptor (MC4-R); its affinity is higher than that of \( \alpha \)-MSH. It has been reported that MITF and tyrosinase were induced, in association with melanin production, in the presence of \( \beta \)-MSH/MC4-R stimulation. Therefore, future studies would be necessary to determine whether MITF and tyrosinase would be induced in association with melanogenesis in the presence of \( \beta \)-MSH/MC4-R and/or \( \alpha \)-MSH/MC1-R stimulation in the precursor melanocytes that were differentiated from the HF bulge NCSCs.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

Research data are not shared.

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