Original Article

Induction of melanocyte precursors from neural crest cells surrounding the neural tube-like structures developed *in vitro* using mouse ES cell culture

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Neural crest cells differentiate into various cell types including melanocytes. In this study, we wanted to induce neural crest cells locating outside of neural tube by culturing mouse ES cells with retinoic acid (RA) and bone morphologic protein 4 (BMP4) *in vitro*, which mimicked the appearance of neural crest cells in the fetus. Histological examination of the cell aggregates composed of ES cells treated with RA and BMP4 disclosed emergence of neural tube-like structures surrounded by slug expressing neural crest cells. We detected melanocyte associated mRNAs such as pax3, sox10, Mitf and tyrosinase by RT-PCR of the aggregates. The neural crest cells expressed KIT and tyrosinase, indicating their differentiation to the melanocyte lineage. To accelerate the melanocyte induction, the cells were further treated by stem cell factor and endothelin 3 and then were transplanted to mouse femoral quadriceps muscles. They adhered to the recipient muscle tissue and retained the characteristics of melanocyte precursors, including expression of slug, KIT, endothelin receptor B and tyrosinase. Collectively, the neural crest cells derived from ES cells have a potency to differentiate into melanocyte precursors and they may be applicable for use in certain disease conditions such as vitiligo vulgaris in future.

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Introduction

Melanocytes are derived from neural crest cells which emigrate out of the dorsal area of the neural tube[1]. Neural crest cells (NCCs) are origins of a variety of cell types including neural cells of peripheral nervous system, craniofacial skeleton and cartilage, smooth muscle and melanocytes[2]. Expression of the zinc-finger transcriptional factors, slug and snail, is known as an early response to neural crest-inducing signal[3]. One of the *paired box* (pax) genes, pax3, is expressed in NCCs and functions in the development of neural crest derivatives[2,4,5].

In the early phase of melanocyte development from NCCs, two ligand receptor systems, stem cell factor (SCF) and its receptor KIT (encoded by c-kit), and endothelin 3 (EDN3) and its receptor endothelin receptor B (EDNRB), indispensably act for survival/differentiation and expansion/emigration of melanoblasts, respectively[6]. Pax3 and the high-mobility group box protein, Sox10, bind to the promoter region of microphthalmia-associated transcription factor (Mitf), a master transcription factor governing the differentiation of melanocytes, and regulate its expression[7-10]. Another transcription factor, lymphoid enhancer factor 1 (Lef1), interacts with β-catenin together with T cell factor and enhances the expression of Mitf. Lef1 binds to the Mitf promoter and transducing Wnt signaling[11,12]. Tyrosinase, tyrosinase related protein 1 (Tyrp1) and dopachrome tautomerase (Dct, also known as Tyrp2) are the three major enzymes of tyrosinase-related family specifically expressed in pigment cells[13]. Tyrosinase, Tyrp1 and Dct were involved in the biosynthetic pathway of melanins and all the three gene promoters contain an E-box motif, bound by Mitf, suggesting importance of Mitf in pigment cell specific gene regulation.

Embryonic stem (ES) cells are pluripotent cells capable of differentiating into cells of all the three germ layers[14-16]. They have potent self-renewing capability for providing unlimited numbers of cells. Thus, ES cells are a possible candidate as cell source for transplantation therapy. In our previous study, we induced neural tube-like structures from mouse ES cells *in vitro* and obtained NCCs expressing slug and snail that resided at periphery of the tube-like structures[17]. The NCCs efficiently expanded by the addition of bone morphological protein (BMP) 4.

In this study, we induced differentiation of melanocyte precursors from the NCCs residing around the neural tube-like structures. They expressed the transcription factors and proteins essential for the melanocyte development, and adhered to mouse tissue when transplanted. The melanocyte precursors obtained *in vitro* are an excellent tool for analysis of melanocyte differentiation and for treatment of hypomelanoses such as vitiligo vulgaris.

Materials And Methods

Induction of melanocyte precursors from mouse ES cells

Mouse ES cell line, E14.1; a kind gift of Dr Klaus Rajewsky, Cologne University (Passage number 11-15, normal karyotype) was used for this study. Undifferentiated ES cells were cultured on the mitomycin C treated mouse fetal fibroblasts in DMEM supplemented with 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1 x non-essential amino acids, 1 x pyruvate, 15% fetal calf serum and 1000 U/ml of recombinant mouse leukemia inhibitory factory (LIF, Life Technologies, Grand Island, NY).

We induced melanocyte differentiation from the ES cells by treating the cells with all-*trans* retinoic acid (RA, Sigma-Aldrich, Saint Louis, MO) and recombinant human BMP4 (R&D systems, Minneapolis, MN). Undifferentiated ES cells were cultured in DMEM supplemented with 15 % FCS without LIF (We defined the starting day of this LIF-depleted culture as Day0, Fig.1). Four days later, floating cell aggregates, embryoid bodies (EB), were transferred onto fresh dishes and 1 μM RA was added at Day 4 and Day 6 (Fig. 1, arrows). At Day 8, the cell aggregates were re-plated and further cultured in FCS-depleted medium consisting of DMEM/F12, N2 supplement and fibronectin for up to Day 16. 10ng/ml BMP4 were added to the cell culture at Day 10, Day 12 and Day 14 (Fig.1, downward arrowheads). Alternatively, 10ng/ml BMP4, 10ng/ml SCF (R&D systems) and 10nM EDN3 (Sigma) were added to the cell culture for the cell transplantation at Day 10 (Fig.1, the upward arrowhead).

RT-PCR

The cultured cell aggregates with RA and BMP4 were recovered at Day 10, 14 and 16. Alternatively, the cell aggregates cultured with RA, BMP4, SCF and EDN3 were recovered at Day 12 and 14. Total RNA was extracted by AGPC method and cDNA was synthesized[18]. PCR program started with preheating at 94℃ for 120 sec and then cycled 35 times of the following three steps: denaturing at 94℃ for 30 sec, annealing at 54℃ for 30 sec, and elongation at 72℃ for 60 sec. Primers used in this study were as follows; βactin (expected size: 450bp), sense gatgacgatatcgctgcgctg, antisense gtacgaccagaggcatacagg; slug (491bp), sense ggtcaagaaacatttcaacg, antisense catattcctgtcacagtac; snail (219bp), sense gtggaaaggccttctctaggc, antisense cagactcttggtgcttgtgg; pax3 (448bp) sense ccaggatgatgcggcccggcccggg, antisense aggatgcggctgatagaactcactg; c-kit (268bp) sense gacgtcatgaagacttgctg, antisense acagcagcaaagcctgttgg; EDNRB (701bp) sense cgagctgttgcttcttggagtag, antisense aacggaagttgtcatatccgtgat; Lef1 (302bp) sense cacctacagcgacgagcact, antisense cgtgttgaggcttcacgtgc; Sox10 (536bp) sense acctttgatgtgactgagctggacc, antisense cccctctaaggtcgggatatggggt; Mitf (202bp) sense agagcagggcagagagtgag, antisense ccctggttgctgtagaggtc; Tyrp1 (199bp) sense tttcactgatgcggtctttg, antisense ctgacctggccattgaactt; Dct (204bp) sense accctgtgtttgtggtcctc, antisense cggcgtaattgtagccaagt; tyrosinase (198bp) sense aggatgctgggctgagtaag, antisense catgaagcaccagggtttct. A part of the above molecules were also examined their expression in undifferentiated ES cells. NCC-

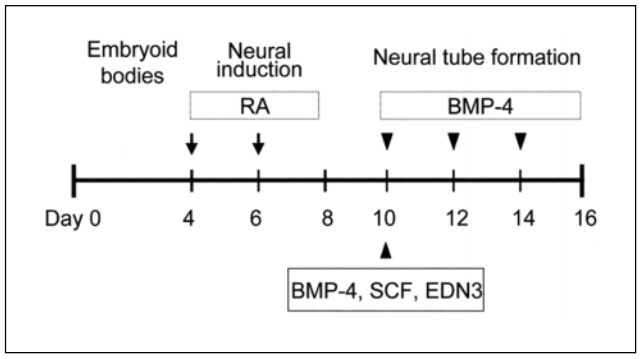

Fig.1 Cell culture protocol for differentiation of melanocyte precursors from ES-derived NCCs

Mouse ES cells were transferred to non-coated dishes on Day 0 and were cultured for four days to form EBs. RA was added twice on Day4 and Day6 to induce neural differentiation. On Day8, the EBs were transferred into FCS-depleted medium containing N2 supplement and fibronectin. BMP4 was added to the cell culture on Day10, 12 and 14. Alternatively, SCF and EDN3 together with BMP4 were added on Day10 for the cell transplantation experiment.

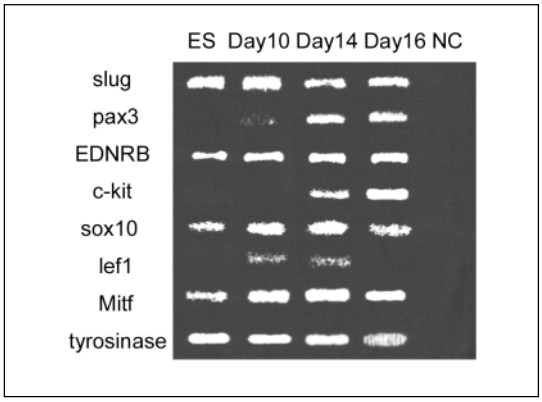

Fig.2 mRNA expressions of the melanocyte precursors induced by RA and BMP4

ES cells were first treated by RA on Day 4 and Day 6, and then treated by BMP4 on Day 10, 12 and 14 (see Fig.1). The cells were further cultured up to Day 16. Expression of mRNAs associated with the neural crest and melanocyte differentiation was studied by RT-PCR. The expression was also examined using un-differentiated ES-derived cDNAs (ES). De-ionized water was used as PCR template for negative control study (NC).

melan5 cells were kindly provided by Prof. Masako Mizoguchi in Department of Dermatology, St. Marianna University School of Medicine and cDNA of the cells was used for the positive control.

Immunohistochemistry

The cell aggregates with RA and BMP4 were recovered at Day 16, were quickly frozen and were sliced as 5 μm thickness of the tissue. After fixing with 4% paraformaldehide, the tissue sections were stained with the primary antibodies specific for slug (Santa Cruz Biotechnology, Santa Cruz, CA; dilution for staining, 1:100), Kit (Becton Dickinson, San Jose, CA; 1:400) and tyrosinase. The antibody specific for tyrosinase was kindly provided by Prof. Vincent Hearing in Laboratory of Cell Biology, National Institutes of Health (dilution for staining, 1:500). Reactivity was visualized with 3-amino-9-ethylcarbazole solution and the sections were counterstained with hematoxylin.

Transplantation of melanocyte precursors to mouse femoral quadriceps muscles

6-8 week old C57BL/6 mice were randomly divided into two groups. The cell aggregates cultured in the presence of RA, BMP4, SCF and EDN3 were harvested at day 14 and treated with trypsin. 1 x 10^6 of the cells was suspended in 10 μl PBS and injected into the femoral quadriceps muscles of mice in one group. The same volume of PBS was similarly injected in the other group. The muscles were recovered five days after the injection. Totally 8 mice were transplanted the cells.

Immunofluorescence staining

Tissue samples of the femoral quadriceps muscles were stained with two color immunofluorescence using antibodies for KIT, slug, tyrosinase and EDNRB (Biogenesis, Kingston, NH; dilution for staining, 1:50). Reactivity was then visualized using the following two systems: biotin-conjugated secondary antibodies (DakoCytomation, Glostrup, Denmark; 1:400)/Streptavidin-conjugated Cy3 (Jackson ImmunoResearch, West Grove, PA; 1:6000) and FITC-conjugated secondary antibodies (NBA Kit, Zymed, South San Francisco, CA; ready to use)/antiFITC-conjugated Alexa488 (Invitrogen/Molecular Probe, Carlsbad, CA; 1:400). Fluorescence was recorded by a confocal microscope (LSM510 META, Carl Zeiss, Jena, Germany).

Results

The aim of our study is to induce melanocyte precursors from NCCs that have been differentiated from mouse ES cells. We previously developed neural tube-like structures surrounded by slug positive NCCs using ES cell culture. ES cells were induced to differentiate into neural lineage by the stimulation with RA

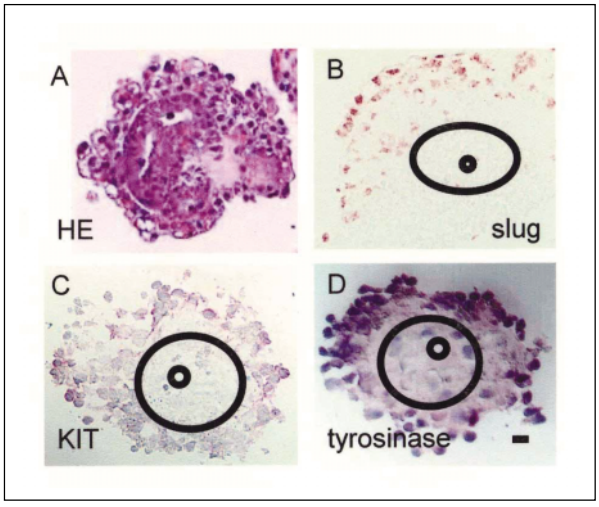

Fig.3 Expression of melanocyte related proteins in the melanocyte precursors derived from NCCs that had appeared around the neural tube-like structures *in vitro*

(A) HE staining of a cell aggregate at Day 14 treated by RA and BMP4 (three times). The neural tube-like structure was surrounded by the cells.
(B) A representative result of a cell aggregate at Day 16 containing a neural tube-like structure (double circles). Cells surrounding the tube-like structure expressed slug in their nuclei.
(C) A serial section of Fig3B. Most of the slug positive cells expressed KIT, suggesting their differentiation to the melanocyte lineage.
(D) A serial section of Fig3BC. A considerable number of the cells surrounding the tube-like structure expressed tyrosinase. The scale bar indicated 20 μm in AB and 10 μm in CD.

and then the cells were further cultured in the N2-supplemented media containing fibronectin and BMP4[17]. The NCCs were efficiently induced by the addition of BMP4, which was evidenced by the emergence of slug and snail expressing cells around the tube-like structures. Here, we cultured the slug positive NCCs for the induction of melanocyte precursors (Fig.1). ES cells were sequentially treated with RA and transferred to the medium including N2-supplement and fibronectin on Day8. BMP4 was added on Day10, 12 and 14. Alternatively, SCF and EDN3 were introduced to the cell culture together with BMP4 on Day10.

mRNA expression of the cell aggregates was analyzed by RT-PCR (Fig.2). As we reported previously, cell aggregates treated by RA and BMP4 expressed slug, a marker of NCCs (Fig.2). From Day 10 they started to express pax3, a marker of neural crest stem cells, and Sox10 and Lef1, essential transcription factors for Mitf expression. Mitf is a product of a master gene to direct the melanocyte differentiation. Expression of c-kit, a receptor for SCF, and EDNRB, that for EDN3, was found during Day 10 and Day14. The cell aggregates expressed Dct, Tryp1 and tyrosinase, all of which were specifically expressed in melanocytes (Fig.2 and Fig.4).

Next, we examined protein expression of slug, KIT and tyrosinase, which are representative markers of melanocyte differentiation. Fig.3A showed a representative section of a cell aggregate treated by RA and BMP4, containing a neural tube-like structure surrounded by NCCs. The NCCs surrounding the tube-like structure expressed slug in their nuclei (Fig.3B). Analyzing serial sections, most of the slug positive cells expressed KIT on their cell membrane (Fig.3C) and a considerable number of the KIT positive cells further expressed tyrosinase in their cytoplasmic region (Fig.3D). Thus, NCCs induced by treating with RA and BMP-4 differentiated to melanocyte precursors (slug and KIT expressing cells), probably receiving signals from SCF produced by the cultured cells, and a considerable population of the melanocyte precursors further differentiated to premature melanocytes (KIT and tyrosinase expressing cells).

It is interesting to study whether the melanocyte precursors derived from ES cells accommodate to and survive in the *in vivo* condition. We thus transplanted the melanocyte precursors into the femoral quadriceps muscle of C57BL/6 mice. ES cells were further treated with SCF and EDN3 in addition to RA and BMP4 to accelerate melanocyte differentiation. Characteristics of the differentiated cells were evaluated by RT-PCR at Day 12 and 14 because the cells were considered to retain abilities of expansion and differentiation in the period. As results, the cells at Day 14 further treated with SCF and EDN3 expressed all the examined molecules which committed to the melanocyte development, thus, they were used for the subsequent transplantation (Fig.4). Five days after the transplantation, the graft was recovered for analysis of the adaptation of the melanocyte precursors *in vivo*. HE staining of the graft disclosed that the cells with high nucleus/cytoplasm ratio made a cluster surrounded by the muscle tissue (Fig.5AB). They were easily distinguished as grafted cells from the host muscle because of their unique histological feature. Some of the cells showing spindle and polygonal shapes seemed to start extending dendrites (Fig.5B). Most of the cells clustering

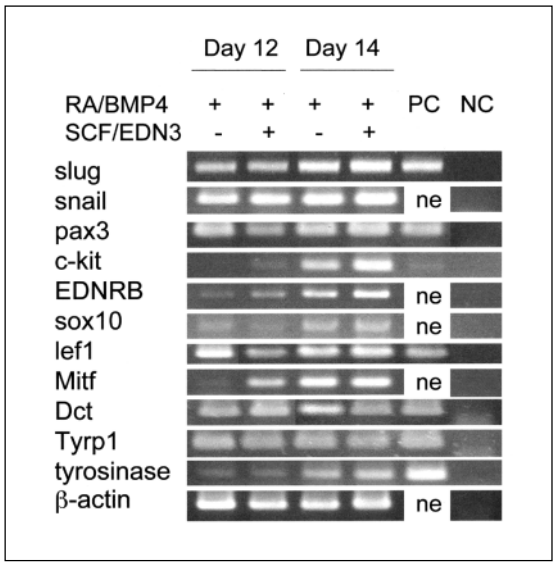

Fig.4 mRNA expressions of the melanocyte precursors induced by RA, BMP4, SCF and EDN3

ES cells were first treated by RA on Day 4 and Day 6, and then treated by BMP-4, SCF and EDN3 on day 10 (see Fig.1). The cells were further cultured up to Day 14. Expression of mRNAs associated with the neural crest and melanocyte differentiation was studied by RT-PCR. PCR templates for positive control study (PC) and negative control study (NC) were NCC-melan5 cDNAs and de-ionized water, respectively. ne, not examined.

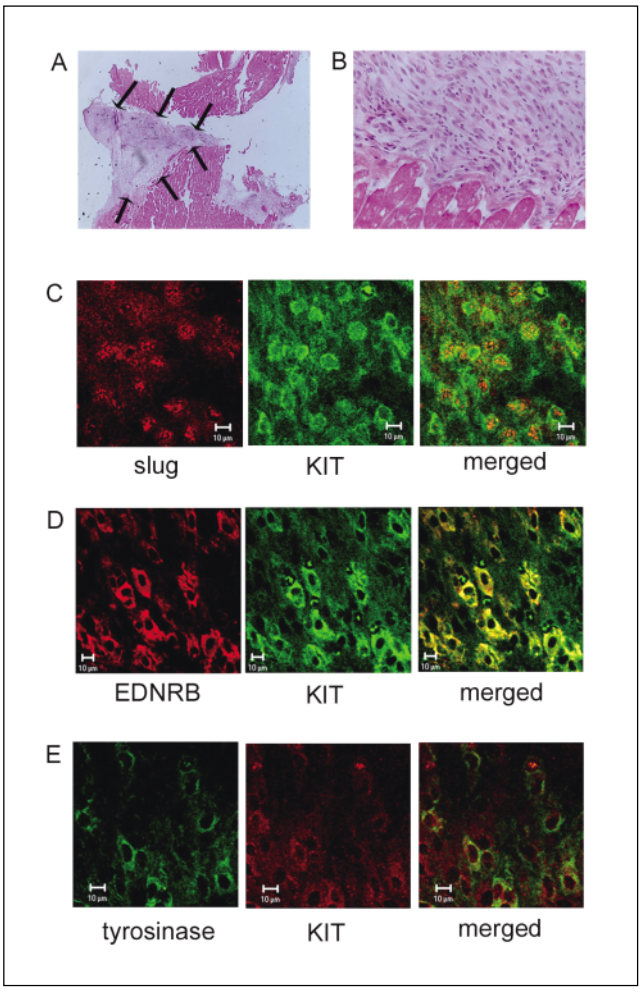

Fig.5 Transplantation of the melanocyte precursors to the femoral quadriceps muscles of recipient mice

Melanocyte precursors derived from ES cells treated with RA, BMP4, SCF and EDN3 (see Fig.4) were injected to femoral quadriceps muscles of C57BL/6 mice. The muscles were recovered five days after the transplantation for the histological examination. Results of HE staining (AB) and two color immunofluorescence staining (CDE) were shown.
(A) HE staining showed relatively homogeneous cells accumulating at the site of transplantation in the muscle tissue. Arrows indicate the boundary of the transplanted site (x 20).
(B) A boundary between the grafted cells and the host muscle tissue. Cells with relatively high nucleus/cytoplasm ratio made a cell cluster in the host muscle tissue and most of them showed spindle or polygonal appearance (x 200).
(C) The transplanted site stained with two color immunofluorescence using antibodies for slug (red) and KIT (green). A number of slug and KIT expressing cells were detected in the grafted region.
(D) Most of KIT expressing cells (green) also expressed EDNRB (red).
(E) A considerable number of KIT expressing cells (red) contained tyrosinase (green), suggesting their differentiation to premature melanocytes.

within the muscle expressed slug, KIT, and EDNRB, indicating that they retained the characteristics of melanocyte precursors in the grafted site (Fig.5CD). A considerable number of the cells expressed tyrosinase, suggesting their differentiation to premature melanocytes (Fig.5E).

Discussion

We have induced melanocyte precursors from NCCs derived from mouse ES cells that had been treated with RA and BMP4. Because slug positive cells expressed KIT and a considerable number of KIT positive cells expressed tyrosinase, we considered that NCCs induced from ES cells further differentiated to melanocyte precursors (the slug and KIT expressing cells) including premature melanocytes (the KIT and tyrosinase expressing cells). After grafting to mouse muscle tissue, the melanocyte precursors developed *in vitro* from ES cells adapted to the *in vivo* condition.

NCCs are derived from the dorsolateral edge of the neural plate and differentiate into various types of cells including melanocytes[19]. Slug and snail, zinc finger transcription factors of the snail family, are known as the earliest response genes to neural crest inducing signals[3]. Overexpression of slug leads to an excess of neural crest derived melanocyte[20], indicating that slug expression is crucial for the neural crest-derived melanocyte induction. Slug expression was found in most of the cells surrounding the neural tube-like structures in the present study, suggesting that slug may promote melanocyte differentiation even in the case from ES cells (Fig.2, Fig.3B and Fig.4).

As a melanocyte-derivative marker, pax3 locates at higher position compared to sox10 in genetic hierarchies of neural crest development[21]. Mutational analyses revealed that pax3 is required to increase numbers of committed melanoblasts or restricted progenitor cells early in development, whereas Mitf affects the survival rate of the melanoblast within and immediately after emigration from the dorsal neural tube[22]. Our neural crest-derived melanocyte precursors expressed pax3, Sox10 and Mitf, indicating that the cells were systematically differentiated, being sequentially influenced by these transcription factors as in somatic developmental procedures. Wnt signaling may also contribute to induction of our melanocyte precursors because they also expressed Lef1.

KIT and its ligand SCF are required to sustain melanocyte development in different stages of embryogenesis during migration, colonization and population of skin[6]. EDNRB is expressed in mouse melanoblasts and its ligand EDN3 works in a paracrine fashion during melanocyte development. Mutation in genes encoding these molecules shows white coat spots due to deficiency of melanocyte differentiation[23-26]. Our melanocyte precursor cells expressed both EDNRB and c-kit, suggesting that they are capable to supply sufficient number of normally differentiating melanocyte precursors by receiving the signals from the ligands.

mRNA expression of a part of examined molecules was also detected in ES cell-derived cDNAs (Fig.2). It sometimes happens that a small portion of ES cells escapes from the undifferentiation-maintaining culture condition and expresses differentiation marker molecules. Such escapes happen very irregularly and not all the differentiation marker molecules associated with a particular directed-differentiation express in such cases as seen in the results of Fig.2. Indeed, once ES cells are transferred to the specific culture condition for inducing a particular directed-differentiation, the expression of differentiation marker molecules in ES cells once disappears and then reappears, and continues thereafter until maturation stages[27].

SCF and EDN3 have been reported to efficiently induce more mature DOPA positive cells and melanocytes from cultures of NCCs[28-32]. Supplementation of SCF and EDN3 to the ES cell culture stimulated with RA and BMP4 promote efficient melanocyte precursors for the transplantation experiments. We reduced the amount of BMP4 in the case of SCF and EDN3 supplementation, considering that excess amount of BMP4 may interfere with the melanocyte differentiation. After transplantation, a large number of cells showed a shape of premature dendritic cells (Fig5B) and expressed slug, KIT and EDNRB (Fig5CD), indicating that they, at least partly shared the characteristics of both NCCs and differentiated melanocyte precursors. The cells expressing KIT and EDNRB were capable of receiving signals from SCF and EDN3, thus, may efficiently maturate to functional melanocytes. Presence of the tyrosinase expressing cells suggested their ability to synthesize melanins (Fig5E). Moreover, mRNA expression of Dct and Tyrp1 was found in the cell aggregates treated with RA and BMP4, and in that further treated with SCF and EDN3 (Fig.4), suggesting that those cell aggregates included highly differentiated melanocytes which have the ability to synthesize eumelanins. In the case of premature melanocytes (KIT and tyrosinase expressing cells) detected at the site of transplantation, they might have acquired the well-differentiated melanocyte characters *in vitro* and retained them even after the transplantation. Otherwise, they may be differentiated from melanocyte precursors after the transplantation. Further studies will be needed to assess this point and to consider which differentiation procedures of cells are more adequate for the cell transplantation.

Several methods for the melanocyte induction have been reported including that from ES cells[33]. An immortal line of melanoblasts was induced from cultures of neonatal mouse epidermis[34]. Mizoguchi et al. established immortal melanocyte precursor cell lines designated as NCCmelan5, NCCmelb4 and NCCmelb4M5 from mouse NCC lines[35-37]. Yamane et al. cocultured ES cells with bone marrow-derived stromal cells and succeeded in the induction of pigmented melanocytes[38]. The induced melanocytes contained abundant stage IV melanosomes, showing the ability of ES cells to terminally differentiate in the melanocytes. ES cell lines expressing the lacZ reporter gene under the control of the Dct promoter were established and they were used for the study of melanocyte differentiation[39].

There were few studies which induced melanocytes from NCCs derived from ES cells. In the present study, we induced melanocyte precursors from NCCs surrounding the neural tube-like structures and successfully transplanted the induced cells to mouse

tissue. We mainly focus KIT expression to detect melanocyte precursors. c-kit expression was detected in NCCs and maintained at high levels until presumptive melanocytes completely differentiate into pigment cells[40]. Luo et al. reported that c-kit and another receptor tyrosine kinase, TrkC, are expressed by distinct neural crest subpopulations and the c-kit expressing NCCs present fate-restricted melanocyte precursors[41]. From this point of view, KIT is a crucial molecule for melanocyte development from NCCs. Further, KIT signaling is indispensable for tyrosinase expression and influences gene expression during melanocyte development[42]. Our KIT expressing cells may be restricted their fate to melanocytes according to somatic development system. KIT expression may be also crucial even for differentiating melanocytes from ES-derived NCCs.

As far as we know, this is the first study to demonstrate successful transplantation of melanocyte precursors induced from ES-derived NCCs. Because differentiation of melanocytes from NCCs is according to somatic development system, our melanocyte precursors have a great advantage of cell transplantation therapy for hypomelanoses such as vitiligo vulgaris[1]. We transplanted the melanocyte precursors to total 8 mice and followed for 5 days after the transplantation. No teratoma fomation was seen in all the cases, however, longer period observation will be required to evaluate the safety of this transplantation. Further studies are needed to elucidate whether our melanocyte precursors differentiate to mature melanocytes to synthesis melanins and whether they retain the function even after transplantation to the site of skin lesion.

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