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Melanocyte stem cells express receptors for canonical Wnt-signaling pathway on their surface

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ABSTRACT

It has been reported that melanocytes play important roles in skin and hair pigmentation and are differentiated from melanocyte stem cells (MSCs) residing in the bulge area of hair follicles. Recently, interest has been growing in MSCs because regulation of the upstream of differentiated melanocytes is essential for the determination of skin and hair pigmentation; however, their precise characteristics remain to be elucidated. The aim of this study is to explore cell-surface markers expressed on MSCs in order to understand their characteristics.

To explore genes specifically expressed in the bulge region, we classified a hair follicle into four areas, hair bulb, hair bulb to bulge (lower bulge), bulge, and epidermis to bulge (upper bulge), and collected these areas from back skin sections of C57BL/6 mice by laser microdissection. Real-time RT-PCR performed on these areas revealed that *Frizzled* (*Fzd*)*-4*, *Fzd7*, *low density lipoprotein receptor-related protein 5* (*Lrp5*), and *Lrp6*, receptors for Wnt molecules, were expressed higher in the bulge area than other areas. Furthermore, FACS analysis showed that populations of $Fzd4^+$ cells and $Fzd7^+$ cells were different from those of Kit^+ cells (precursor of melanocytes: melanoblasts). $Fzd4^+$ and $Fzd7^+$ cells isolated by FACS required a longer culture period to differentiate into mature melanocytes than Kit^+ cells. Up-regulation of mRNA expressions of melanocyte markers (*dopa chrometautomerase*: *Dct*, *tyrosinase*: *Tyr*, *tyrosinase-related protein 1*: *Tyrp1*) was observed in $Fzd4^+$ and $Fzd7^+$ cells following Kit^+ cells during differentiation. These results suggested that $Fzd4^+$ and $Fzd7^+$ cells were more immature than melanoblasts, therefore raising the possibility that $Fzd4^+$ and $Fzd7^+$ cells are MSCs.

1. Introduction

Since adult stem cells in various organs maintain tissue homeostasis and play important roles in the repair of damaged tissues, their dysfunction and depletion are thought to be closely related to aging. It has been reported that several kinds of adult stem cells reside in skin, such as epidermal stem cells [1–3], hair follicle epithelial stem cells [4–6], and skin-derived precursor cells (SKP cells) that reside in the dermis [7]. These adult stem cells may be profoundly involved in skin aging as well as skin construction and restoration.

Melanocytes are pigment cells playing pivotal roles in skin and hair pigmentation by producing melanin [8,9]. They are differentiated from melanoblasts, progenitors of melanocytes, which are derived from neural crest cells, and migrate into the epidermis during embryogenesis [10–12]. Recently, it has been reported that stem cells committed to melanocyte lineage (melanocyte stem cells; MSCs) resided in the bulge area of hair follicles [13]. They are thought to supply melanoblasts and melanocytes in adults and are therefore most likely to be related to hyperpigmentation and hair graying with aging. For example, Nishimura et al. reported that one of the mechanisms of hair graying was the ectopic differentiation of MSCs [14,15], which ectopically differentiated into mature melanocytes with aging or by DNA damage in their niche environment, and concurrently disappeared.

Previous studies revealed some phenotypes of MSCs. MSCs in the bulge area survived independently of Kit, a receptor for stem cell factor, unlike other melanocyte lineage cells, melanoblasts and melanocytes [13,16,17]. Osawa et al. performed immunohistochemical analysis and demonstrated that MSCs only expressed dopachrome tautomerase (Dct) and Pax3, not other melanocyte markers, tyrosinase (Tyr), tyrosinase-related protein 1(Tyrp1),

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microphthalmia-associated transcription factor (Mitf) and Sox10, which are expressed on melanocytes in the hair matrix [18]. Therefore, MSCs and mature melanocytes are distinctly different, although they share some similar features. Although analysis of MSC characteristics and their changes during differentiation into melanocytes is extremely helpful to elucidate the mechanism of hair graying and develop therapies for pigment disorders, their precise characteristics remain unclear because of difficulties in their isolation and culture [19]. Thus, by finding cell-surface markers specifically expressed on MSCs, simple isolation of MSCs and better in vitro study will be achieved, and will contribute to further research into the detailed characterization of MSC.

Wnts are a large family of secreted proteins and their signaling is mediated by binding to a receptor, Frizzled (Fzd). Wnts and Fzd proteins have 19 and 10 families, respectively. In the best characterized Wnt-signaling pathway, termed the canonical pathway, Wnt binds to Fzd receptors and to low density lipoprotein receptor-related protein (Lrp) 5/6, and induces various cellular events, such as proliferation, differentiation, migration and adhesion [20,21]. As Wnt-signaling has been implicated in the control of MSCs in the bulge area [22,23], it was speculated that Wnt-signaling is important for MSC maintenance in their niche environment. Assuming that the regulation of Wnt-signaling in MSCs is the switch for differentiation into mature melanocytes, receptors of Wnt-signaling are likely to be candidates for markers specifically expressed on the surface of MSCs. In this study, we explored the cell-surface markers specifically expressed on MSCs in the bulge area of mouse back skin, focusing on receptors for Wnt-signaling, and consequently obtaining important findings on MSC markers.

2. Materials and methods

2.1. Animals

C57BL/6 mice were obtained from Japan SLC (Shizuoka, Japan). The animals were cared for according to the International Guiding Principles for Biomedical Research Involving Animals (published by The Council for International Organization of Medical Science). The present experimental protocol was planned according to these guidelines and 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.

2.2. Histochemistry

Immunohistochemical analysis was performed according to a general method. Briefly, back skin collected from postnatal day (P) 9 mice were fixed with 4% paraformaldehyde, and then frozen sections were made by cryostat (Carl Zeiss Inc., Thornwood, NY, USA). These sections were processed for immunostaining using anti-Dct antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the Histofine® peroxidase-DAB system (Nichirei Bioscience, Tokyo, Japan). For immunofluorescence, anti-Fzd4 antibody (R&D Systems, Minneapolis, MN, USA), anti-Fzd7 antibody (R&D Systems), and anti-rat IgG antibody labeled with Alexa Fluor® 488 (Invitrogen Corp., Carlsbad, CA, USA) were used. DAPI (VECTASHIELD H-1200; Vector Laboratories, Burlingame, CA, USA) was used for nuclear staining.

2.3. Laser microdissection

Back skins collected from P9 C57BL/6 mice were immediately frozen in liquid nitrogen and stored at −80 °C. Frozen sections of these tissues were made by cryostat, and then placed on film slides. The sections were fixed in 75% ethanol for 30 s and rinsed in distilled water (DW) for 30 s. They were stained with 0.05% toluidine blue for 1 min and then rinsed in DW for 30 s, 75% ethanol for 30 s, 90% ethanol for 30 s, and 100% ethanol for 30 s. After drying the sections, laser microdissection (LMD) was performed using Leica LMD6000 (Leica Microsystems, Wetzler, Germany). Total RNA was extracted and purified with the RNeasy® Micro Kit (Qiagen, Hilden, Germany).

2.4. Flow cytometry

Basal cell suspensions containing epidermal and hair follicle cells were obtained from the skins of P1 mice according to conventional methods [24]. The suspension was filtrated and centrifuged, and then cells were analyzed and sorted by fluorescence-activated cell sorting (FACS) (FACSAria, BD Biosciences, San Jose, CA, USA). In brief, the cells were stained with anti-Fzd4 antibody, or anti-Fzd7 antibody followed by anti-rat IgG antibody labeled with Alexa Fluor® 488. Subsequently, anti-Kit antibody labeled with APC was reacted. After staining with antibodies, Kit^+, $Fzd4^+$, and $Fzd7^+$ cells were sorted.

2.5. Differentiation

After sorting, Kit^+, $Fzd4^+$, and $Fzd7^+$ cells were seeded on 96-well plates coated with type IV collagen (Nitta Gelatin, Osaka, Japan). For differentiation into melanocytes, the cells were cultured for 21 days with melanocyte differentiation medium: αMEM supplemented with 10% FBS, 50 ng/mL SCF (ATGen, Gyeonggi, South Korea), 100 ng/mL endothelin-3 (Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan), 0.5 mM dibutyryl-cAMP (Sigma, St. Louis, MO, USA). Total RNA was extracted every 7 days by the Cells Direct qRT-PCR Kit (Invitrogen, Carlsbad, CA, USA).

2.6. Real-time RT-PCR

After extraction of total RNA, cDNA was synthesized by reverse transcription. Real-time semiquantitative RT-PCR was performed with the SuperScript™ III Platinum® Two-Step qRT-PCR kit (Invitrogen), using the 7300 Real Time PCR System (Applied Biosystems, Tokyo, Japan). Primer sequences are indicated in Supplementary Table S1. Amplification was normalized to a housekeeping gene, *ribosomal protein L23* (*Rpl23*). All PCR products were checked by melting curve analysis to exclude the possibility of multiple products or incorrect product size. PCR analyses were conducted in triplicate for each sample.

3. Results

3.1. Analysis on gene expression profile in mouse hair follicle

Previous studies on *Dct-LacZ* transgenic mice, which carry the *LacZ* reporter under the control of *Dct* promoter, revealed that melanocyte stem cells (MSCs) existed in the bulge region of hair follicles [13,14]. Based on these reports, we tried to observe cells expressing dopachrome tautomerase (Dct) to confirm the existence of MSCs in the bulge region. As a result of immunohistochemical analysis of the back skin of P9 C57BL/6 mice, the localization of Dct-positive cells in the hair matrix and bulge region was observed (Fig. 1A).

To identify markers specifically expressed in MSCs, we explored genes whose expression was highest in the bulge area and lower in the bulb (where mature melanocytes reside) and the outer root sheath except for the bulge. Hair follicles of mouse back skin were classified into four sections (Fig. 1A): hair bulb, hair bulb to bulge