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ORIGINAL PAPER

CXCL12 regulates differentiation of human immature melanocyte precursors as well as their migration

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Abstract

Melanocyte stem cells (McSCs) are localized in the bulge region of hair follicles and supply melanocytes, which determine hair color by synthesizing melanin. Ectopic differentiation of McSCs, which are usually undifferentiated in the bulge region, causes depletion of McSCs and results in hair graying. Therefore, to prevent hair graying, it is essential to maintain McSCs in the bulge region, but the mechanism of McSC maintenance remains unclear. To address this issue, we investigated the role of CXCL12, a chemokine which was previously suggested to induce migration of melanocyte lineage cells, as a niche component of McSCs. Immunohistological analysis revealed that CXCL12 was highly expressed in the bulge region of human hair follicles. CXCL12 mRNA expression level was significantly lower in white hairs plucked from human scalps than in black hairs. CXCL12 attracted the migration of early-passage normal human epidermal melanocytes (eNHEMs), an in vitro model of McSCs, which had characteristics of immature melanocyte precursors. We also found that CXCL12 suppressed their differentiation. These results suggest that CXCL12 regulates differentiation of McSCs as well as their proper localization, and maintaining McSCs by regulating CXCL12 expression level in the bulge region may be a key to preventing hair graying.

Keywords Melanocyte stem cell · CXCL12 · Stem cell niche · Differentiation · Bulge

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Introduction

Hair follicle is a skin appendage which goes through a cycle of three phases: the growth phase, called anagen, the regression phase called catagen, and the resting phase called telogen [12]. All epithelial cells in the hair follicle are generated by hair follicle stem cells (HFSCs) in the bulge region, which is the lowest part of the permanent portion of the hair follicle. During catagen, cells constituting the transient portion of hair follicle, which is lower than the permanent portion (i.e., lower than and adjacent to the bulge region), cause apoptosis, and the transient portion shrinks. After telogen, hair follicles enter anagen, and HFSCs regenerate a transient portion by supplying epithelial cells.

Hair turns white with age, and as a rule of thumb it was said that 50% of hair becomes white in 50% of the population at the age of 50 years (50/50/50 rule; [10]). Recent study revealed that the percentage of people showing at least 50% gray hair coverage at the age of 50 years was smaller than that stated by the 50/50/50 rule, according to ethnic or geographical origin and natural hair color [16]. Hair color is determined in the hair

bulb by the amount of melanin, which melanocytes transfer to adjacent hair matrix cells when producing the hair shaft. Thus, the loss of hair pigment could be caused by the following events: disappearance of melanocytes in the hair bulb, decreased melanogenic potential in melanocytes, or defects in melanosome transfer from melanocytes to hair matrix cells. Among these, the primary cause of hair graying is thought to be the loss of melanocytes in the hair bulb [5].

In 2002, it was reported that mature melanocytes in hair bulb were supplied by immature cells termed melanocyte stem cells (McSCs) located in bulge region [13]. McSCs differentiate to supply melanocytes to the hair bulb in a synchronized manner with the hair cycle, i.e. when hair cycle enters anagen from telogen. The understanding of hair graying mechanisms has advanced prominently in this decade after the identification of McSCs. In mice, genotoxic stress may induce McSC differentiation ectopically in the bulge region, which results in the depletion of McSCs and subsequent hair graying [9]. In general, stem cells are known to be regulated and maintained by their specific microenvironment, called niche [15]. McSCs are surrounded by HFSCs in the bulge region, and TGF-β1 secreted by HFSCs is essential to maintain the McSC undifferentiated state [14]. Therefore, HFSCs contribute to providing McSC niche. In *COL17A1* knockout mice, the number of HFSCs decreased, and McSCs were not maintained [19]. The current model of hair graying is that disruption of the McSC niche causes the depletion of McSCs, melanocytes are not supplied to the hair bulb, and hair pigment is lost. Based on this model, it is important to maintain the McSC niche to prevent hair graying, but the factors important for this process remain largely unknown.

Studies on McSCs have suggested that McSCs need to stay in the bulge region with an undifferentiated state to maintain hair color unless they need to respond some stimuli, e.g. for hair cycle transition. Therefore, there should be some factors that prevent McSCs from migrating out of the bulge region, and we analyzed chemokines regulating cell migration as candidates for novel McSC niche factors. In this study, we focused on one of such chemokines, CXCL12, which has been reported to attract cultured human melanocytes [11, 22] and to be indispensable to regulate hematopoietic stem cells in bone marrow [18]. To investigate whether CXCL12 is involved in the maintenance of McSCs, we examined its expression pattern in human hair follicles and tested its effect on eNHEMs, an in vitro model of McSCs [14], which had characteristics of immature melanocyte precursors.

Materials and methods

Human skin biopsies

Samples of human scalp skin were collected from surplus surgical tissues at Fujita Health University Hospital after ensuring that the patients fully understood the study objective and other related information. Written informed consent was obtained from each subject. This study was conducted with an ethical approval from the Research and Ethics Committee of Fujita Health University (approval No. 15–235).

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections were prepared from skin biopsy tissues, and deparaffinized and boiled in Target Retrieval Solution (Dako, Glostrup, Denmark). After washing with PBS, sections were blocked with 5% normal donkey serum for 1 h and then incubated with anti-KRT15 antibody (GeneTex, San Antonio, TX, USA) and rabbit anti-KRT19 antibody (Abcam, Cambridge, MA, USA) overnight at 4 °C. After washing with PBS, the sections were incubated with anti-mouse IgG antibody conjugated to Alexa488 (Life Technologies, Carlsbad, CA, USA) or anti-rabbit IgG conjugated to Alexa594 for 1 h. Dual-staining for KRT15/CXCL12, KRT15/MITF, and CXCR7/MITF was performed using anti-KRT15 antibody, anti-CXCL12 antibody (R&D systems, Minneapolis, MN, USA), anti-MITF antibody (Thermo Fisher Scientific, San Jose, CA, USA) and anti-CXCR7 (R&D systems). All of these antibodies were derived from mice. Anti-CXCL12 antibody and anti-MITF antibody were labeled with Alexa594 using the Zenon labeling kit (Life Technologies). DAPI, 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) was used for nuclear staining. Fluorescent images were obtained using a Leica DMI 6000B fluorescent microscope (Leica, Wetzlar, Germany), and Leica Application Suite X (LAS X) was used as a software platform.

Real-time RT-PCR

Total RNA was extracted from plucked hair or cultured cells using RNAiso Plus (Takara Bio, Shiga, Japan) and cDNA was synthesized by reverse transcription. Real-time semi-quantitative RT-PCR was performed with SYBR Select Master Mix (Applied Biosystems, Tokyo, Japan) using a StepOnePlus Real-time RT-PCR system (Applied Biosystems) in accordance with the manufacturer's protocol. The sequences of primers used were as follows: 18S ribosomal RNA forward, 5′-CCGAGCCGCCTGGATAC-3′ and reverse, 5′-CAGTTCCGAAAACCAACAAAATAGA-3′;

CXCL12 forward, 5′-CATGCCGATTCTTCGAAAGC-3′ and reverse, 5′-CGAGTGGGTCTAGCGGAAAG-3′; *KRT15* forward, 5′-GGATGGACAGGTGGTTTCTTC-3′ and reverse, 5′-CAGGGACTGGAGTTTGC-3′; *SOX9* forward, 5′-GGTGCTGCTGGGAAACATT-3′ and reverse, 5′-TGC AGTGAACAAGCAAAGG-3′. Amplification was normalized to the housekeeping gene 18S ribosomal RNA, and differences between samples were quantified based on the ΔΔCt method. All PCR products were checked by melting curve analysis to exclude the possibility of multiple products or an incorrect product size.

Cell culture

Normal human epidermal melanocytes (NHEMs) at passage 2 were purchased from TOYOBO (Osaka, Japan) and maintained between passages 3 and 4 in Medium254 containing human melanocyte growth supplement (Life Technologies). The cells were seeded onto 3.5-mm plates at a density of 5×10^4 cells/well and cultured overnight. The cells were then incubated with recombinant human CXCL12 protein (Peprotec, Rocky Hill, NJ, USA) for 6 days to analyze mRNA expression of melanocyte differentiation-related genes. Fontana–Masson staining was performed according to a conventional method. To analyze the location of β-catenin, immunostaining was performed on NHEMs after fixation with 4% paraformaldehyde. After washing with PBS, cells were blocked with 5% normal donkey serum for 1 h and incubated with anti-β-catenin antibody (Cell Signaling Technology, Beverly, MA, USA) and anti-MART1 antibody (Thermofisher) for 1 h. Afterwards, cells were incubated with anti-rabbit IgG antibody conjugated to Alexa488 and anti-mouse IgG antibody conjugated to Alexa594 (Life Technologies) for 1 h.

Knockdown experiment

NHEMs were seeded onto 24-well plates at a density of 2×10^4 cells/cm^2 and cultured for 24 h. The culture medium was replaced with OPTI-MEM (Life Technologies) containing 50 nM of AccuTarget small interfering RNA (siRNA) as a negative control, or siRNA mixture (Bioneer, Daejeon, Korea) targeting CXCR4 or CXCR7 (No. 1037826, 1171128, and 1171129 for *CXCR4*; No. 1033035, 1033038, and 1033042 for *CXCR7*) to avoid off-target effects. After a 4-h incubation, OPTI-MEM was replaced with fresh culture medium and the cells were cultured for another 24 h.

Cell migration assay

Cell migration assay was performed in transwell dishes with 8-μm pore filters (Corning Costar, Cambridge, MA, USA). NHEMs were seeded into the upper chamber with Medium254 without supplement Medium254 (−), and 700 μL Medium254 (+) with or without recombinant human CXCL12 protein or SCF protein (ATGen, Los Angeles, CA) in the lower chamber. After a 16-h incubation in 5% CO_2 at 37 °C, the cells remaining on the upper face of the filter were removed with a cotton wool swab. The chamber was then fixed for 30 min at room temperature with methanol and rinsed in water. Cells that had migrated through the pores and adhered to the lower surface of the membrane were stained with Giemsa stain and counted in six random high-power fields.

Statistical analysis

Data are presented as the mean ± SD. $P<0.05$ was considered significant. Statistical analysis was performed using a Student's *t* test or Peritz test.

Results

Expression of CXCL12 in the bulge region of human hair follicles

A hair follicle has a region called bulge near the site where the arrector pili muscle is attached, as shown in H&E staining of the human scalp (Fig. 1a). The bulge region is the lowest part of the permanent portion, which is maintained throughout the hair cycle, and hair follicle stem cells (HFSCs) and melanocyte stem cells (McSCs) are known to reside in this region. As shown in Fig. 1b, cells expressing KRT15, which is an HFSC marker, are localized in the bulge region, and co-expressed another HFSC marker KRT19 (Fig. 1c). MITF (microphthalmia-associated transcription factor)-positive McSCs are observed between KRT15-positive HFSCs at intervals (Fig. 1d). A chemokine CXCL12 has been reported to attract cultured human melanocytes [11, 22] and is known to function in the niche for hematopoietic stem cells [18], and we thought that it might also play a role as a niche factor in hair follicles. We examined the expression of CXCL12 in the bulge region, and found that CXCL12 signals were observed in the area where KRT15-positive HFSCs were present (Fig. 1e). In addition, we performed immunohistochemistry against MITF and a CXCL12 receptor CXCR7, and found that McSCs expressed CXCR7 (Fig. 1f). Furthermore, co-staining against CXCR7 and CXCL12 showed that CXCL12 were localized adjacent to McSCs (Fig. 1g). Next, we analyzed mRNA expression levels of CXCL12 in different portions of plucked hair samples. The hair bulb, the lowermost part of the hair follicle, was excluded from the plucked hair, and the remaining hair was separated into three portions—the bulge region, and the regions higher

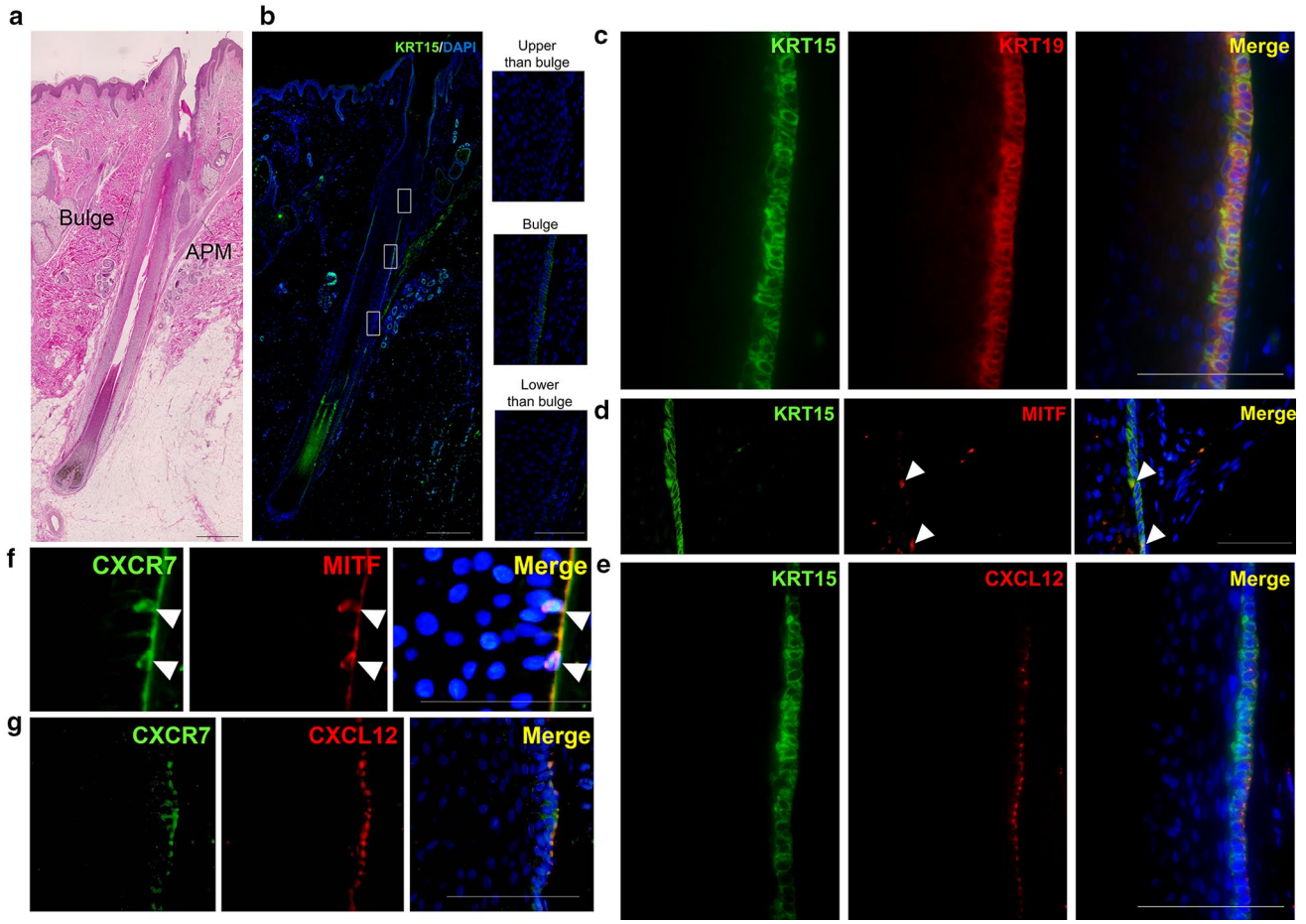

Fig. 1 Localization of CXCL12 protein in the bulge region of a human hair follicle. **a, b** H&E (**a**) and immunohistochemical (**b**) staining images of the human scalp sections (male, 32 years old). APM in (**a**), arrector pili muscle. **b** Merged image of double staining for a HFSC marker KRT15 (green) and nuclei (blue). Scale bar 500 μm. Enlarged image of the bulge region, where KRT15-positive cells exist (the middle boxed area) and of the areas without KRT15-positive cells (the upper and lower boxed areas) were shown as "Bulge", "Upper than bulge" and "Lower than bulge", respectively. **c–g** Immunohistochemical image in the bulge region of a human hair follicle. **c** Left panel, KRT15 (green); middle, KRT19 (red); right, merged image. Nuclei were stained with DAPI. Scale bar 100 μm. **d** Right panel, KRT15 (green); middle, a McSC marker MITF (red); left, merged image. Nuclei were stained with DAPI. Arrowheads indicate MITF-positive cells. Scale bar 100 μm. **e** Left, CXCL12 (red); middle, KRT15 (green); right, merged image. Nuclei were stained with DAPI, and arrowheads indicate CXCL12-positive cells. Scale bar 100 μm. **f** Left, CXCR7 (green); middle, MITF (red); right, merged image. Nuclei were stained with DAPI, and arrowheads indicate CXCR7/MITF-positive cells. Scale bar 100 μm. **g** Left, CXCR7 (green); middle, CXCL12 (red); right, merged image. Nuclei were stained with DAPI, and arrowheads indicate CXCL12-positive cells. Scale bar 100 μm

or lower than the bulge (Fig. 2a). The bulge region samples showed a prominently higher expression of *KRT15* and *SOX9*, which are HFSC markers, compared with other samples (Fig. 2b), which confirms that the method of dividing hair follicles was appropriate. We observed a significantly higher expression of CXCL12 in the bulge region than the non-bulge regions of the plucked hair samples (Fig. 2b), which is consistent with immunohistochemical analysis of the scalp, and supports the result that CXCL12 was localized in the bulge region. If CXCL12 has a role as a niche component, CXCL12 expression may be altered in white hair, where McSCs are not properly maintained. We compared CXCL12 expressions in the plucked white and black hair, and found significantly reduced CXCL12 expression in white hair (Fig. 2c). In addition, the expression of *CXCL12* mRNA was compared between plucked hair samples from young (27 ± 1.4 years) and old subjects (54 ± 5.4 years) to analyze the correlation between aging and CXCL12 expression, and it was found that CXCL12 expression was significantly lower in the old subjects than the young (Fig. 2d). These results raise the possibility that CXCL12 function as a niche component to maintain McSCs.

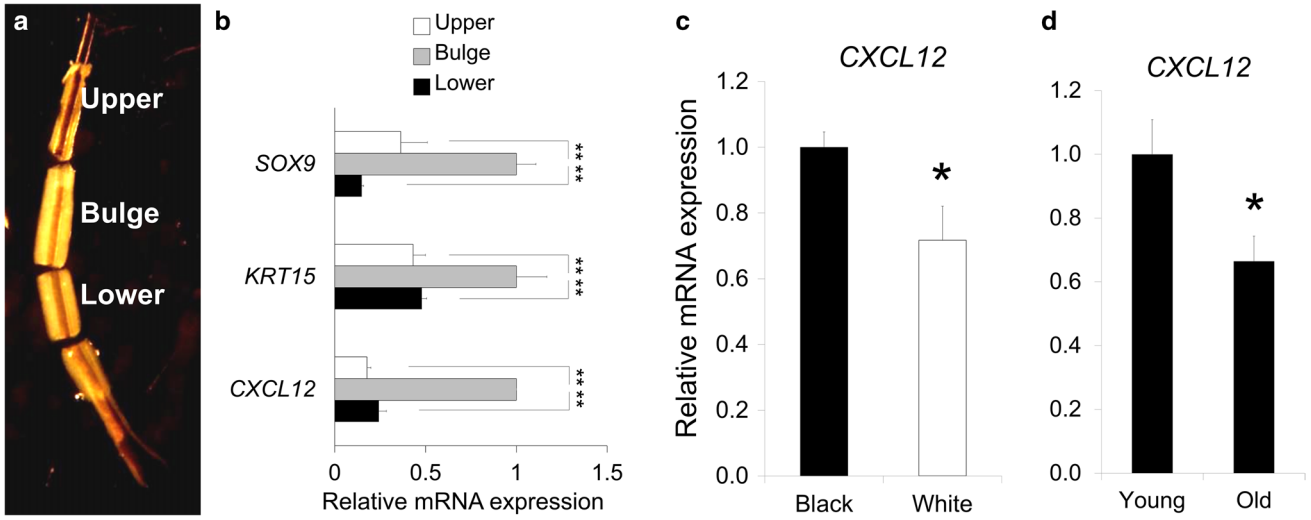

Fig. 2 Expression of *CXCL12* mRNA in a plucked human scalp hair. **a** Human scalp hairs plucked from the temporal region of the head were divided into four regions shown in A. Upper, the region above the bulge. Lower, the region below the bulge. **b** RNA was extracted from each of the three portions (i.e., the bulge, the region above, and the region below), and expression of *KRT15, SOX9* and *CXCL12* was examined by real-time RT-PCR. Data shown are the mean ± SE ($n = 5$). $^{**}P < 0.01$. **c** Black or white hairs were plucked from the temporal region and *CXCL12* gene expression was compared by real-time RT-PCR. Data shown are the mean ± SE ($n = 4$). $^{*}P < 0.05$. **d** *CXCL12* gene expression was compared between hairs from the young (27 ± 1.4 years) and old subjects (54 ± 5.4 years) by real-time RT-PCR. Data shown are the mean ± SE ($n = 4$). $^{*}P < 0.05$

Induction of McSC migration by CXCL12

Because CXCL12 is a chemokine that promotes cell migration, we tested the possibility that it affects the location of McSCs. First, cell migration assays using transwells were performed with immature melanocyte precursors (early-passage normal human epidermal melanocytes, NHEMs), an in vitro McSC model [14]. There was a significantly increased number of eNHEMs that migrated to the lower compartment of the transwell when CXCL12 was added, compared to that of the control (Fig. 3a). Knockdown of the CXCL12 receptor CXCR4 or CXCR7 by siRNAs significantly inhibited cell migration to the lower compartment (Fig. 3a–c). One well-known factor that promotes melanocyte migration is stem cell factor (SCF), and SCF is expressed in the epidermis and hair bulb [8]. Therefore, we tested if migration of eNHEM cells towards CXCL12 and SCF compete with one another. When SCF alone was added to the lower transwell chamber, eNHEM migration was promoted significantly (Fig. 3d). However, when SCF was added to the lower compartment and CXCL12 added to the upper compartment at the same time, migration to SCF was significantly inhibited. These results showed that SCF and CXCL12 compete to attract eNHEMs, and suggest that high expression of CXCL12 in the bulge region may be required to attract McSCs.

Maintenance of undifferentiated McSCs by CXCL12

In addition to attracting McSCs in the bulge region, CXCL12 may also be required to maintain McSCs in an undifferentiated state. Therefore, we tested if CXCL12 affects the differentiation state of McSCs. Extrinsic signals, such as a Wnt ligand and endothelin-1, trigger melanocyte differentiation and induce melanocyte-specific gene expression [20], so we cultured eNHEMs in media including Wnt1 and endothelin-1 to induce differentiation of melanocytes with or without CXCL12. Fontana–Masson staining after inducing differentiation revealed that addition of CXCL12 notably suppressed the amount of melanin produced in differentiated eNHEM cells (Fig. 4a). In addition, mRNA expression of *MITF*, a master regulator of melanocyte development and differentiation, was found to be significantly decreased by CXCL12 with real-time RT-PCR analysis (Fig. 4b). *TYR* (tyrosinase) and *DCT* (dopachrome tautomerase), which are downstream target genes of MITF and encode enzymes involved in melanin synthesis, also tended to be down-regulated when CXCL12 was added. Taken together, these results suggest that CXCL12 suppresses McSC differentiation and helps to maintain an undifferentiated state.

Although both Wnt ligands and endothelin-1 are known to be required during the differentiation of McSCs, the first step is thought to be the activation of the canonical Wnt

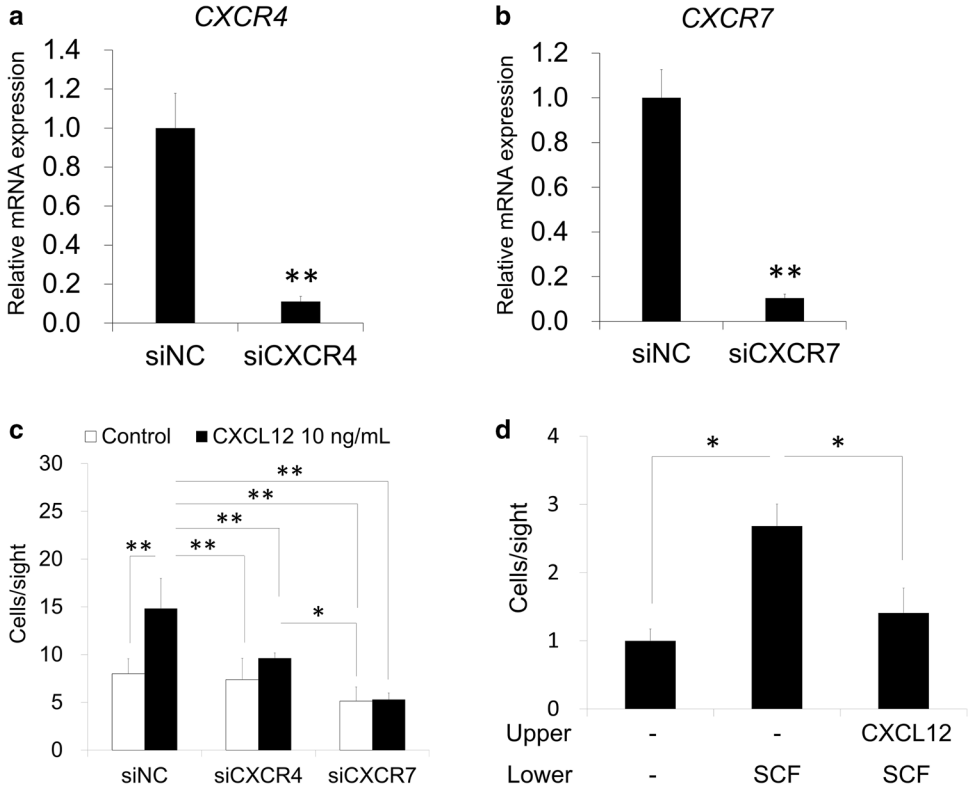

Fig. 3 Induction of eNHEM migration by CXCL12. **a, b** Expression of *CXCR4* (**a**) and *CXCR7* (**b**) was examined by real-time RT-PCR to analyze knockdown efficiency of siRNAs against CXCR4 and CXCR7 (siCXCR4 and siCXCR7, respectively) or a negative control (siNC) in eNHEMs, an in vitro human McSC model. **c** Cell migration assay was performed with recombinant human CXCL12 protein. siNC, siCXCR4 or siCXCR7 was added to eNHEMs, and cells were seeded onto transwell inserts with CXCL12 in the lower compartment. For a control, only PBS, the solvent for CXCL12, was added. **d** A cell migration assay for eNHEM seeded on transwell inserts was performed with SCF in the lower compartment, and with or without CXCL12 in the upper compartment. Data shown are the mean ± SE ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$

pathway (i.e., Wnt/β-catenin signaling; [21]). The activation of canonical Wnt signaling pathway leads to the translocation of β-catenin into the nucleus, and then β-catenin upregulates MITF expression as a transcriptional coactivator in McSCs to differentiate into melanocytes. Wnt/β-catenin signaling is suppressed by Wnt ligands in the non-canonical Wnt pathway [17], and CXCL12 elevates Wnt5a expression, which activates the non-canonical Wnt pathway [7]. These findings prompted us to examine the effect of CXCL12 on the activation of the Wnt/β-catenin signaling pathway and on Wnt5a expression. We found that CXCL12 reduced the nuclear translocation of β-catenin in eNHEMs (Fig. 4c) and induced a significantly higher gene expression of *WNT5A* (Fig. 4d). These results suggest that CXCL12 functions to maintain McSCs in an undifferentiated state in addition to attract them in the bulge region.

Discussion

In this study, we focused on CXCL12, a chemokine that regulates cell migration, as a candidate niche factor for maintaining McSCs. The involvement of CXCL12 in the melanocyte lineage had been reported by a few groups, and Belmadani et al. revealed the importance of the CXCL12–CXCR4 axis for proper positioning of immature melanocyte precursors in mouse hair follicles [2]. They studied the signaling pathway in the migration of immature melanocyte precursors, which occurs during embryogenesis and continues until around 7 days after birth, but the role of CXCL12 at later stages, such as in mature adults, as well as that in human hair follicles has not been reported. Although there are in vitro studies showing that CXCL12 attracts human cultured cells, NHEMs [11, 22], whether CXCL12 functions in immature melanocyte precursors remained unknown.

To our knowledge, this is the first study to focus on the role of CXCL12 to regulate human immature melanocyte precursors. We analyzed CXCL12 expression pattern in human hair follicles and its function in human immature melanocyte precursors in vitro. It was found that CXCL12 was highly expressed specifically in the bulge region of hair follicles, and that expression was decreased in white hair (Figs. 1, 2). Avniel et al. reported that CXCL12 expression was observed in the fibrous sheath of the hair follicle [1], while we could not observe any expression of CXCL12 in the fibrous sheath (Fig. 1e). The difference in the two observation results may be explained by differences in hair cycle stages at which the samples were or in the donors' age and health condition. Zhang et al. isolated DP cells from the human scalp, cultured them, and identified secreted CXCL12 in the supernatant [23], which seems contradictory to our result. To interpret the data, the following needs to be taken into account: (1) cell profiles are altered by culture conditions, (2) DP cells express different factors depending on hair cycle, and (3) Zhang et al. did not observe directly CXCL12 expression in vivo, i.e. in the human scalp. Given

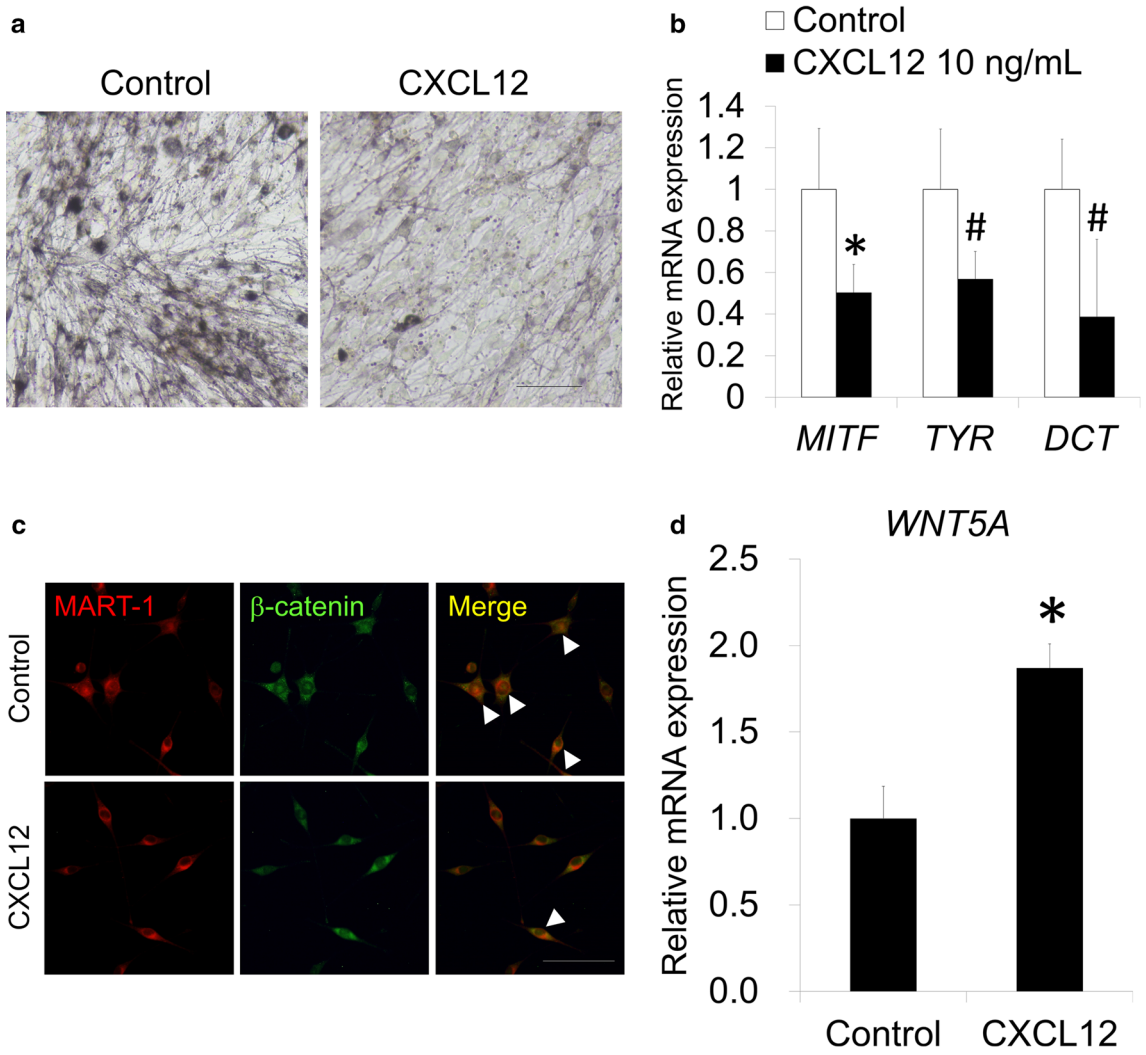

Fig. 4 Maintenance of the eNHEM undifferentiated state by CXCL12. a–d eNHEMs were cultured for 6 days in differentiation–induction media with or without 10 ng/mL CXCL12, and the effect of CXCL12 on melanocyte differentiation was examined. **a** Microscopic image of melanin staining by Fontana–Masson method. **b** Expression analysis of genes related to melanocyte differentiation and melanin synthesis by real-time RT-PCR. **c** Analysis of β-catenin location in MART1-expressing melanocyte lineage cells by immunohistochemistry. Arrowheads indicate cells with β-catenin in the nuclei. **d** Expression analysis of *WNT5A* gene by real-time RT-PCR. Scale bar 50 µm. Data shown are the mean ± SE ($n = 3$). $^{\#}P < 0.1$; $^{*}P < 0.05$

that the distinct approaches were taken, it would be reasonable that there is a discrepancy between the result of Zhang et al. and this study. To elucidate the function of CXCL12 in McSCs, we considered the expression in the bulge region, where McSCs reside, most important.

The in vitro experiment using immature melanocyte precursors (eNHEMs), a McSC model, demonstrated that CXCL12 attracts them and maintains their undifferentiated state (Figs. 3, 4). We also showed that CXCL12 and SCF compete to attract eNHEMs (Fig. 3d). It was suggested that the function of CXCL12 in vivo is to prevent McSCs from migrating out from the bulge region and from differentiating ectopically. Growth factors and chemokines, such as CXCL12, function through a concentration gradient. At telogen, the dermal papillae (DP) and the bulge region are close together. At the onset of anagen, DP cells secrete factors that activate HFSCs and McSCs to proliferate and induce differentiation. At the onset of early anagen, the concentration of SCF, Wnt ligands, and endothelin-1 may temporally exceed that of CXCL12 so that McSCs differentiate and their progenies migrate to the hair bulb. From mid-to-late anagen, the DP and bulge are further apart, which may lower the concentration of DP cell-derived factors than that of CXCL12 and maintain McSCs at an undifferentiated state in the bulge.

Taken all together, these results raised a strong possibility that CXCL12 attracts and positions human McSCs in the bulge region and maintains their undifferentiated state, and

when CXCL12 expression is decreased, McSCs may be unable to stay in the bulge and migrate out to the epidermis or hair bulbs, which causes McSC depletion in the bulge region and results in hair graying. Actually, CXCL12 expression was decreased in the old subjects compared to the young subjects (Fig. 2d), which we think is a part of the mechanism involving the phenomenon that graying hair increases with age. Further histological analysis would provide more robust evidence on the correlation between the localization of McSCs and the CXCL12 expression.

Understanding the mechanisms of regulating McSCs may make it possible to prevent hair graying. Generally, stem cell niches are comprised of various factors, and the mechanisms that regulate stem cells are complicated [3, 4, 6]. In this study, we showed the possibility that CXCL12 in the bulge region of hair follicles in humans is involved in the localization of McSCs and maintenance in their undifferentiated state. It is likely that there are other unidentified niche factors. Further elucidation of the mechanisms, such as (1) how CXCL12 and unidentified niche factors cooperate to regulate the McSC niche, and (2) what causes impaired expression of McSC-regulating factors, especially CXCL12, is essential for development of the technology to prevent hair graying.

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Author Contributions TY and SH designed the research study. TY, MA, YI, TK, SN and NY performed the research. TY, SH, SN, KS and HA carried out the data analysis and wrote the manuscript. All authors critically revised the manuscript and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors state no conflict of interest.

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