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" Unveiling the Genetic Roots of Gray Hair: The Role of Plexin-A1 in Melanocyte Function and Aging "

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1. Introduction

Gray hair is one of the most visible signs of aging, with significant social and psychological implications. The development of gray hair is primarily attributed to the dysfunction of melanocyte stem cells and melanocytes, leading to a progressive loss of melanin production [1, 2]. However, the detailed molecular mechanisms underlying this process and the external factors that influence it remain largely unknown.

In recent years, advancements in genome analysis have led to the increasing popularity of technologies for predicting physical traits, particularly through genome-wide association studies (GWAS), which identify single nucleotide polymorphisms (SNPs) linked to specific characteristics. GWAS has contributed to the elucidation of genetic factors associated with various cosmetic traits and skin diseases [3, 4]. Recently, SNPs associated with gray hair have also been reported; however, these findings have been limited to Latin American and European populations [5, 6]. These studies have provided valuable insights into the genetic basis of hair aging, but they have primarily focused on non-Asian populations. Given the documented ethnic differences in hair aging characteristics [7], there is a critical need to investigate the genetic factors contributing to gray hair in East Asian populations.

To address this gap, we conducted a GWAS on a Japanese cohort to identify genetic variations associated with gray hair. Through this study, we identified rs891762 as a potential SNP linked to gray hair, and expression quantitative trait locus (eQTL) analysis revealed Plexin-A1 (*PLXNA1*) as a candidate gene. *PLXNA1* encodes a receptor protein involved in semaphorin signaling, which regulates cellular morphology and motility. Its ligand, Semaphorin-3A, is known to inhibit axonal extension in neurons [8]. Since melanocytes originate from neural crest cells, similar to peripheral neurons, it is plausible that *PLXNA1* plays a role in melanocyte function. However, the relationship between *PLXNA1* and melanocytes has not been well explored.

In this study, we investigated the functional role of *PLXNA1* in melanocytes. By elucidating the genetic and molecular mechanisms underlying hair graying, this study provides a foundation for further research into therapeutic interventions for age-related pigmentation disorders.

2. Materials and Methods

Study population

The study participants were recruited from a research project titled "Research for the Realization of a Healthy and Long-Lived Society Using Health Big Data", which utilized samples and information obtained through MYCODE (DeNA Life Science Inc., Japan). An open call for participation in the "Exploratory Research on Genetic Factors Related to Gray Hair" was made to participants of the aforementioned project (recruitment period: November 25, 2022 – December 23, 2022).

A total of 2,201 individuals who provided informed consent and met all the eligibility criteria participated in this study and completed a questionnaire on gray hair. This study was approved by the Ethics Review Committee of DeNA Life Science Inc. and the Head of Research Institution of Rohto Pharmaceutical, registered with the UMIN Clinical Trials Registry (UMIN000049734), and conducted in accordance with the Declaration of Helsinki.

Quality control

Based on the genotyping data obtained from the study "Research for the Realization of a Healthy and Long-Lived Society Using Health Big Data," Sample-QC was performed on 2,201 participants. Specifically, saliva samples were collected from 2,201 Japanese individuals (aged 18–84 years), and single nucleotide polymorphisms (SNPs) of each subject were analyzed. First, genomic DNA was extracted and purified from the samples using the MagaZorb® DNA Mini-Prep Kit (Promega Corporation, USA). Next, SNP analysis was performed using the Infinium HTS Assay Kit (Illumina, Inc., USA). After DNA amplification and fragmentation, hybridization was performed between the fragmented DNA and bead-bound probes on microarrays (Human OmniExpress-24+ BeadChip and Infinium OmniExpress-24+ BeadChip, both from Illumina, Inc., USA), followed by fluorescence labeling and analysis. Samples with sex inconsistencies and low genotyping accuracy (missing call rate > 99.0%) were checked, but none met the exclusion criteria. To exclude related individuals, Identity by Descent (IBD) was evaluated using PI_HAT (IBD estimates) applying the commonly used threshold of 0.1875, which represents the midpoint between second- and third-degree relatives. No pairs exceeding this threshold were identified, and thus, no samples were excluded. Next, principal component analysis (PCA) was conducted using genome-wide SNP data to examine population structure and exclude outliers (PLINK “-indep-pairwise 50 5 0.5”). These outlier samples were removed, and PCA was performed again to confirm that no further outliers remained. Following Sample-QC, genotype imputation—a statistical method that estimates missing genotype information at non-genotyped loci using a reference panel—was performed.

Genotype imputation

After Sample-QC, phasing of 684,430 autosomal SNPs was conducted using Eagle v2.4.1 software, with reference to the East Asian samples from the 1000 Genomes Project (1KGP) whole-genome reference panel [9, 10].

Following phasing, genotype imputation was conducted using Minimac3 [11]. To ensure imputation accuracy, all SNPs were quality-filtered based on Imputation Rsq values, and those with Rsq < 0.7 were excluded to eliminate low-accuracy imputed variants.

Phenotype data

Participants were asked to complete a questionnaire regarding gray hair. Specifically, they were asked to select the image that most closely resembled their current hair condition from a set of six images depicting hair bundles (Beaulax Co.,Ltd, Japan) with various levels of

gray hair in both the frontal and temporal regions. The questionnaire items related to gray hair were treated as an interval scale. Participants were asked to respond to the question “How much gray hair do you have?” by selecting one of the following six options: 1. No gray hair (all black hair); 2. Almost no gray hairs, but some gray hairs in places; 3. A few gray hairs overall; 4. About half of the hair is gray; 5. Mostly gray hair; 6. Completely gray hair. Participants who used hair dye or coloring were asked about the natural color of the hair at the roots, which is unaffected by dyeing.

Statistical analysis

A GWAS was performed using logistic regression analysis with PLINK version 1.9, targeting 4,347,442 SNPs after Marker-QC. Case/control groups were defined based on gray hair phenotypes, with age, sex, and principal components included as covariates. For statistical significance, genomic control-adjusted *p*-values were calculated using PLINK's adjust option. The genome-wide suggestive significance threshold was set at $p < 1E\text{-}05$. To visualize the results, quantile-quantile plots and Manhattan plots were generated using FUMA version 1.5.3 (https://fuma.ctglab.nl).

eQTL analysis

For SNPs exhibiting significant trends in the GWAS, eQTL analysis was performed using FUMA version 1.5.3. The target tissue was set as GTEx v8 Skin, and biologically relevant genes potentially involved in gray hair were examined.

Cell culture

Adult normal human epidermal melanocytes (NHEMs; Kurabo Industries Ltd., Japan) were used in this study. NHEMs were cultured in DermaLife Basal Medium (Lifeline Cell Technology, USA) supplemented with DermaLife Ma LifeFactors. All cell cultures were maintained at 37°C in 5% CO_2.

Observation of melanocyte dendrites

NHEMs were suspended in medium containing POLARIC™ (Cosmo Bio Co., Ltd., Japan) and seeded into 96-well plates. After 24 hours, recombinant human Semaphorin-3A protein (Abcam plc, UK) was added to the cultures. After an additional 48 hours of incubation, nuclei were stained with Hoechst 33342 solution (Fujifilm Wako Pure Chemical Industries Ltd., Japan), followed by imaging and analysis using the ImageXpress (Molecular Devices, LLC, USA).

Induction of senescent melanocytes

To induce cellular senescence via ionizing radiation (IR), cells at about 70% confluence were exposed to 10 Gy of IR using an MX-160Labo irradiator (MediXtec Inc., Japan). Three days after irradiation, cells were passaged once at a 1:3 ratio and reached full senescence seven days post-irradiation.

RNA isolation and real-time PCR analysis

Normal or senescent melanocytes were seeded into 12-well plates, and after 48 hours of incubation, total RNA was extracted using the Maxwell® RSC simplyRNA Cells/Tissue Kit

(Promega Corporation, USA). For real-time PCR analysis, cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix with gDNA remover (Toyobo Co., Ltd., Japan). PCR was then performed using THUNDERBIRD SYBR qPCR/RT Set III (Toyobo Co., Ltd., Japan) and the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). qRT-PCR was performed under the following thermal cycling conditions: 20 s at 94°C, followed by 40 cycles of 2 s at 95°C, 5 s at 60°C, and 30 s at 72°C. Primer sets were purchased from FASMAC Co., Ltd. (Japan) (Table 1). Target mRNA expression levels were normalized to *GAPDH* using the ΔΔCt method and calculated as $2^{-\Delta\Delta Ct}$.

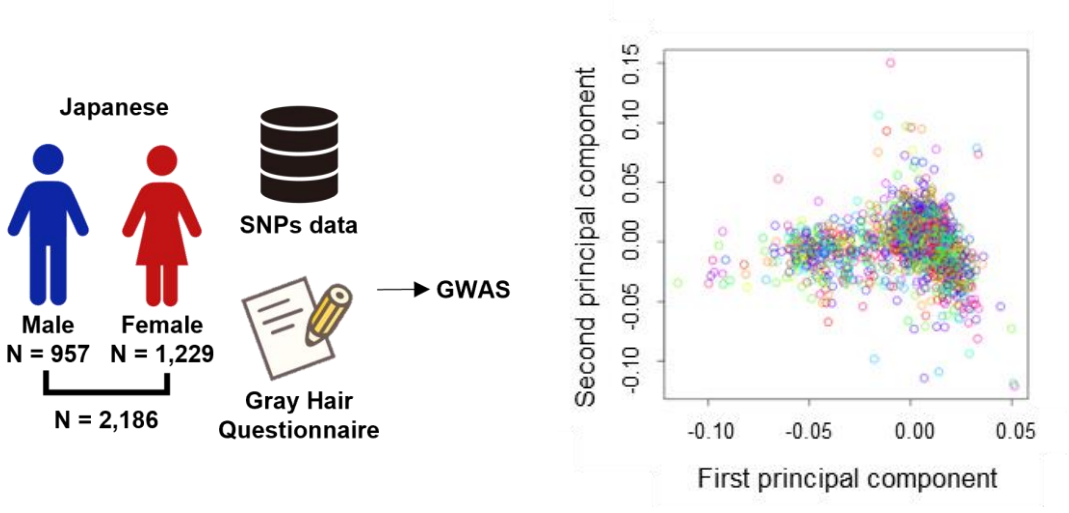
Table 1. PCR Primer Sequences

Gene Symbol	Forward	Reverse
CDKN1A	TGTCCGTCAGAACCCATGC	AAAGTCGAAGTTCCATCGCTC
CDKN2A	GGCAGTAACCATGCCCGCATAG	TACGAAAGCGGGGTGGGTTGT
PLXNA1	ACCCACCTAGTGGTGCATGA	CGGTTAGCGGCATAGTCCA
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

3. Results

3.1. Gray Hair Progression Patterns in the Frontal and Temporal Scalp Regions

To investigate the genetic background of the Japanese population, we conducted a GWAS involving 2,186 participants, including 957 males and 1,229 females (Figure 1a). Self-reported questionnaires on gray hair were administered, and subgroup analyses were conducted based on population structure and questionnaire responses. Specifically, we examined the presence of population clusters using PCA based on SNP data from 2,201 subjects. 15 samples were identified as outliers from the main population cluster, suggesting they were genetically distinct. To minimize false positives, the 15 outlier samples were excluded from the analysis. After outlier removal, PCA was repeated on the remaining 2,186 samples, confirming that no samples deviated significantly from the population cluster (Figure 1b). After quality control for GWAS, 2,186 samples were included in the final analysis, with an average participant age of 42.7 years. By sex, the cohort included 957 males (43.8%, mean age: 44.9 years) and 1,229 females (56.2%, mean age: 48.5 years) (Table 2).



(**a**) (**b**)

Figure 1. Overview of the Study Design and Phenotype Assessment in a Gray Hair GWAS of a Japanese Cohort

(a) Workflow of gray hair GWAS. A total of 2,186 Japanese individuals (957 males and 1,229 females) with available SNP data completed a self-reported assessment of their degree of hair graying.; (b) PCA plot based on the genotype data from samples included in the GWAS.

Table 2. Age and Sex Distribution of the 2,186 Participants Included in the Study

Age group (years)	Male	Female	Total
18 ->19	1	0	1
20 ->29	20	35	55
30 ->39	149	198	347
40 ->49	283	396	679
50 ->59	332	448	780
60 ->69	139	138	277
70 ->79	29	14	43
80 ->89	4	0	4
Total	957	1,229	2,186

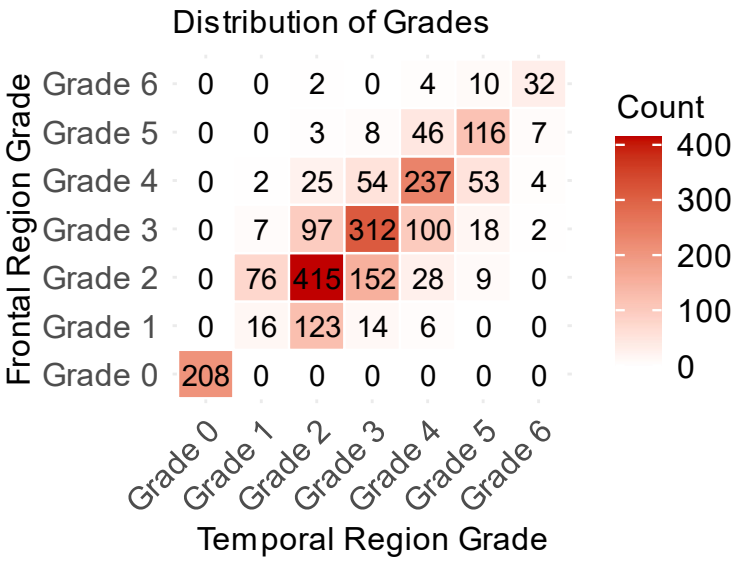
3.2. PLXNA1 identified as a gray hair-associated factor from GWAS

To investigate the contribution of genetic factors to gray hair, we administered a questionnaire using a color scale grading system (Figure 2a). In response to the question “Do you have any gray hair?”, 1,978 individuals (90.5%) answered “yes” and 208 people (9.5%) answered “no”. Participants who answered “no” were categorized as Grade 0 for further subsequent analyses. By sex, 871 males (91.0%) answered “yes” and 86 (9.0%) answered “no”; 1,107 females (90.1%) answered “yes” and 122 (9.9%) answered “no”. These results indicate no significant difference in the prevalence of gray hair between sexes. Among respondents, the number of individuals decreased with increasing gray hair severity from Grade 2 to Grade 6 (Figure 2b). Additionally, analysis of gray hair grades in the frontal and temporal regions showed parallel progression patterns in both areas. These results confirm that samples with extremely advanced gray hair in either region were not included in the present analysis.

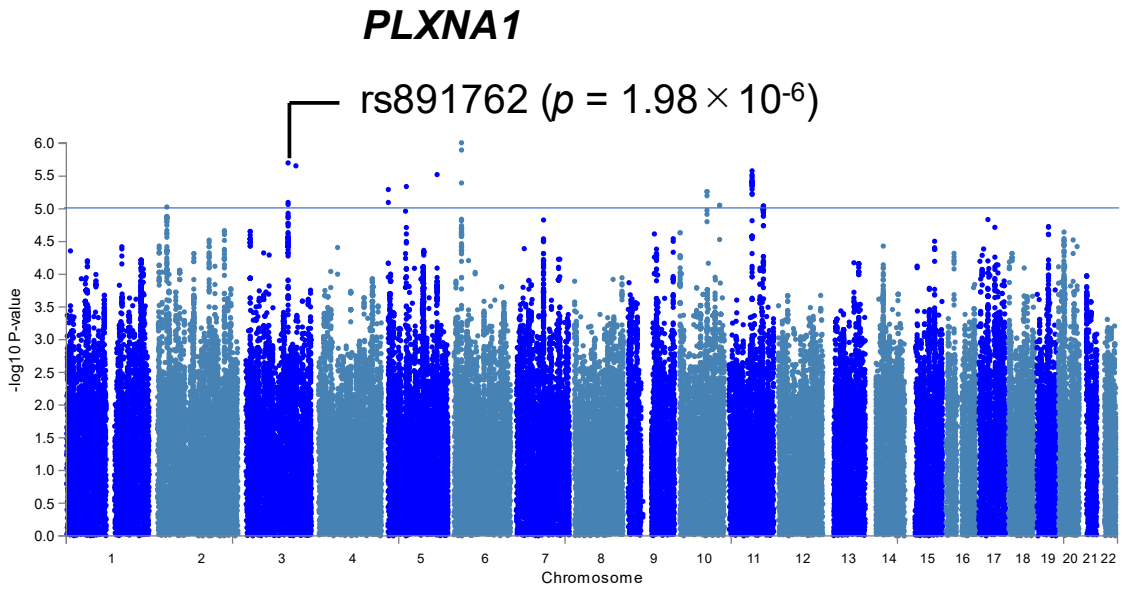
For GWAS analysis, participants reporting more than Grade 3 gray hair in either the frontal or temporal region were assigned to the case group (N = 1,348), while the remaining participants were classified as controls (N = 838). As a result, the GWAS identified 51 SNPs meeting the suggestive significance threshold ($p < 1.0 \times 10^{-5}$) for gray hair compared to no gray hair (all black hair) and almost no gray hair (Figure 2c). Among them, rs891762 located on 3q21.3 ($p = 1.98 \times 10^{-6}$), was identified as an associated SNP. A LocusZoom plot confirmed that rs891762 is located in an intronic region of the *PLXNA1* gene (Figure 2d). Furthermore, eQTL analysis using FUMA identified *PLXNA1*, *CHCHD6*, and *TXNRD3* as candidate genes associated with rs891762 in GTEx v8 skin tissue. Among these associated genes, we focused on *PLXNA1*. Plexin-A1, a receptor in the plexin family, mediates signaling pathways influencing cellular morphology and motility through interaction with ligands such as Semaphorin-3A [12]. This interaction is known to inhibit axonal extension in neurons [8]. Therefore, we investigated whether *PLXNA1* influences the dendritic outgrowth in melanocytes.


Grade 1 2 3 4 5 6

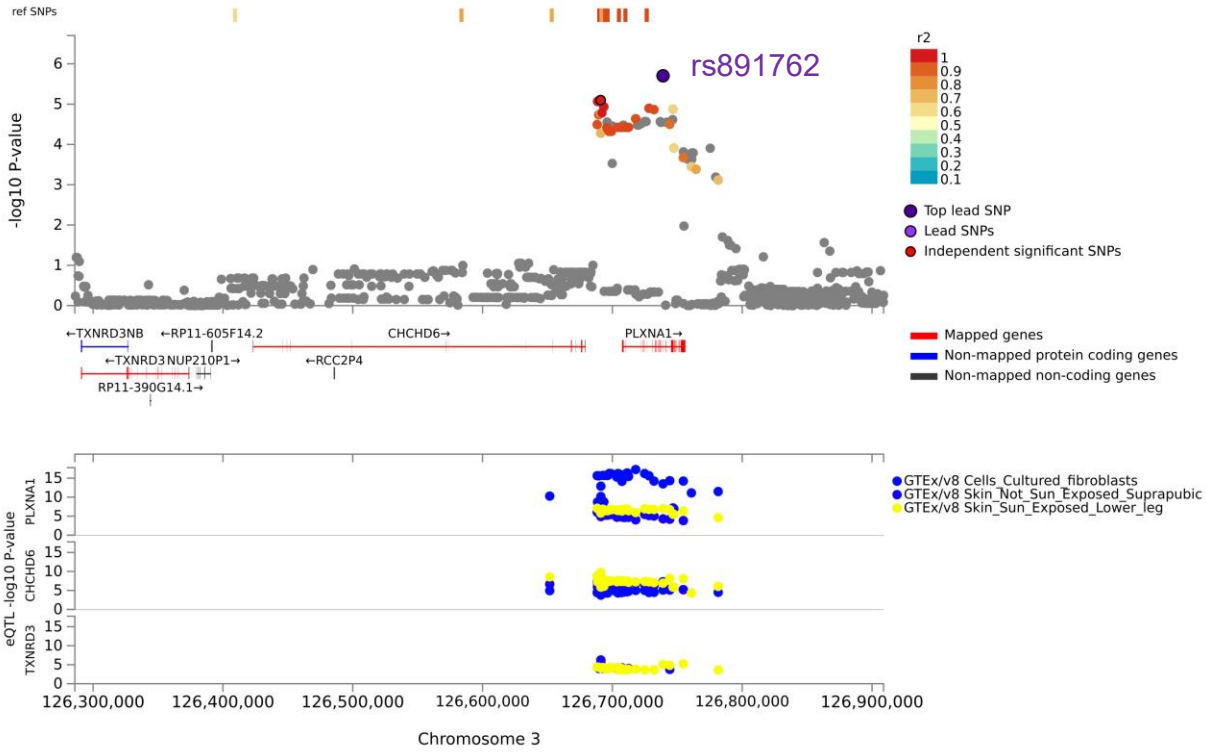
(**a**)


Distribution of Grades
Frontal Region Grade
Grade 6 0 0 2 0 4 10 32
Grade 5 0 0 3 8 46 116 7
Grade 4 0 2 25 54 237 53 4
Grade 3 0 7 97 312 100 18 2
Grade 2 0 76 415 152 28 9 0
Grade 1 0 16 123 14 6 0 0
Grade 0 208 0 0 0 0 0 0
Grade 0 Grade 1 Grade 2 Grade 3 Grade 4 Grade 5 Grade 6
Temporal Region Grade
Count
400
300
200
100
0

(**b**)


PLXNA1
rs891762 (p = 1.98 × 10-6)
-log10 P-value
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Chromosome

(**c**)



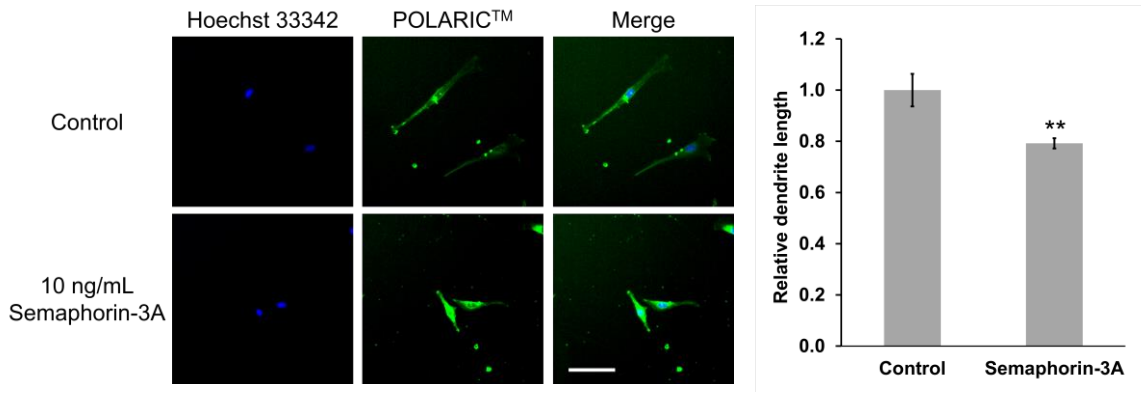
(**d**)

Figure 2. Assessment of Gray Hair Distribution and Associated Genomic Variants in a Japanese Cohort

(a) Gray hair grading scale used in the questionnaire. Participants selected one of the following six options in response to the question “How much gray hair do you have?”: 1. No gray hair (all black hair); 2. Almost no gray hairs, but some gray hairs in places; 3. A few gray hairs overall; 4. About half of the hair is gray; 5. Mostly gray hair; 6. Completely gray hair; (b) Sample distribution of gray hair grade in this study; (c) Manhattan plot of GWAS in the Japanese cohort (N = 2,186). The blue line indicates the suggestive significance threshold at a *p*-value of 1.0×10^{-5}.; (d) LocusZoom plot for rs891762. (Top) SNP association results are shown as $-\log_{10}$ (*p*-values) plotted against chromosomal position. The top SNPs are shown in purple, while other SNPs are colored according to their degree of linkage disequilibrium with the top SNPs, as indicated by the r^2 legend. (Bottom) Zoomed-in regional plot of the *PLXNA1*, *CHCHD6*, and *TXNRD3* loci with eQTL *p*-values.

3.3. Semaphorin-3A inhibits dendritic extension of melanocytes

Although melanocytes, which are responsible for hair pigmentation, are derived from neural crest cells like various peripheral neurons, the relationship between *PLXNA1* and melanocytes remains unclear. To evaluate the effects of Semaphorin-3A on NHEMs, the recombinant protein was added to the culture, and changes in cell morphology were observed (Figure 3a). Dendritic morphology of melanocytes was visualized using NHEMs stained with POLARIC™. POLARIC™ staining highlights intracellular polarity through color variation, while the cell membrane fluoresces green. As a result, Semaphorin-3A treatment inhibited dendritic elongation in melanocytes (Figure 3b). These findings suggest that semaphorin–plexin signaling may regulate dendritic outgrowth in melanocytes.



(**a**) (**b**)

Figure 3. Semaphorin-3A Inhibits Dendritic Extension in NHEMs

(a) NHEMs treated with or without 10 ng/mL Semaphorin-3A, stained with Hoechst 33342 and POLARIC™. Scale bar: 100 μm; (b) Relative dendrite length (N = 3; mean ± SD. ***p* < 0.01; Student's *t*-test)

3.4. The expression of PLXNA1 is upregulated in X-ray-irradiated melanocytes

To investigate the relationship between *PLXNA1* expression and aging, NHEMs were irradiated with X-rays to induce senescence. The method for inducing senescence was based on the approach described by Jianhui Chang *et al* [13]. Real-time PCR analysis revealed that the expression of senescence markers *CDKN1A* and *CDKN2A* was upregulated in melanocytes irradiated with 10 Gy X-rays compared to non-irradiated controls (Figure 4). Furthermore, *PLXNA1* expression was also upregulated in X-ray-irradiated melanocytes. These results indicate that *PLXNA1* is upregulated in senescent melanocytes, suggesting that semaphorin-plexin signaling may be enhanced during aging and contribute to the suppression of dendritic outgrowth in melanocytes.

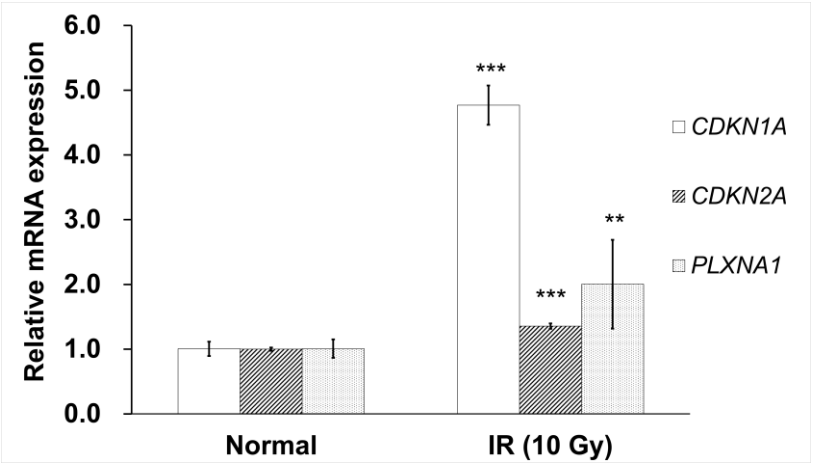


Figure 4. Gene Expression Changes in X-ray-Irradiated Senescent Melanocytes

Real-time PCR analysis of the senescence markers *CDKN1A* (blank bars), *CDKN2A* (diagonal-striped bars), and *PLXNA1* (dotted bars) in melanocytes under normal and 10 Gy IR conditions (N = 6; mean ± SD. ***p* < 0.01, ****p* < 0.001; Student's *t*-test). Target mRNA expression levels were normalized to *GAPDH*.

4. Discussion

In this study, we investigated the changes in *PLXNA1* expression in human graying hair follicles and its effect on melanocyte dendricity, thereby proposing a novel molecular mechanism underlying hair graying. The Plexin family, which functions as receptors for semaphorins (neural guidance molecules), plays key roles in cytoskeletal remodeling and cell migration [14]. Notably, Plexin-C1 suppresses dendrite formation in melanoma cells through R-Ras inactivation and modulation of the actin cytoskeleton, suggesting potential involvement in melanocyte morphology. Peters *et al*. [15] compared black, gray, and white hair follicles from the same donors and reported a significant downregulation of melanocyte-related genes in white hair follicles. They also showed that Plexin-C1 expression was localized to pigmentary units in pigmented follicles but was markedly reduced in white follicles, suggesting a role in melanocyte localization and function. Furthermore, exogenous Plexin-C1 administration suppressed melanocyte proliferation and pigmentation, further reinforcing the role of Plexin signaling in melanocyte regulation. In a more recent study by Dai *et al*. [16], an X-ray-irradiated model of human graying hair follicles revealed that radiation induces oxidative stress and DNA damage, leading to melanocyte dysfunction and reduced dendrite formation. Importantly, they found that both X-ray–irradiated and naturally aged white follicles exhibited rounded and shortened melanocyte dendrites, likely impairing melanin transfer to keratinocytes. RNA-seq analysis supported these observations, revealing alterations in axon guidance pathways, including Plexin family members, which may be implicated in the suppression of dendricity and melanin transfer.

Based on these findings, we hypothesize that upregulated *PLXNA1* expression in graying hair follicles suppresses melanocyte dendricity, thereby inhibiting melanosome transfer to matrix keratinocytes and contributing to hair depigmentation. This mechanism provides a novel perspective on hair graying, complementing existing theories such as the “melanocyte stem cell depletion theory” [1] and the “oxidative stress theory” [17], by highlighting structural disruption of melanocytes as a contributing factor.

To further validate *PLXNA1* as a gray hair-associated gene, functional studies using knockdown or overexpression in melanocytes could confirm its role in dendritic extension and melanin transfer. Investigating *PLXNA1* expression in human hair follicle melanocytes from young and aged individuals would provide additional insight. Future studies targeting *PLXNA1* expression and signaling may lead to novel therapeutic strategies for the prevention or reversal of hair graying.

5. Conclusion

This study identifies *PLXNA1* as a candidate gene associated with gray hair, supported by genetic, molecular, and functional evidence linking it to melanocyte dendritic morphology. Our findings suggest that age-related upregulation of *PLXNA1* may enhance semaphorin-plexin signaling, resulting in reduced dendritic outgrowth and melanin transfer by melanocytes. These insights contribute to the understanding of the mechanisms underlying hair graying and may inform future research on pigmentation-related aging processes.

6. References

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