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AP collagen peptides (APCPs) promote hair growth by activating the GSK-3β/β-catenin pathway and improve hair condition

Jung Ok Lee[1] | Yujin Kim[1,2] | Jung Min Lee[1] | Jang Mi Suk[3] | Inhee Jung[3] | Sun Young Choi[4] | Kwang Ho Yoo[4] | Joon Seok[1,2] | Beom Joon Kim[1,2]

[1]Department of Dermatology, College of Medicine, Chung-Ang University, Seoul, Korea

[2]Department of Medicine, Graduate School, Chung-Ang University, Seoul, Korea

[3]Global Medical Research Center, Seoul, Korea

[4]Department of Dermatology, Chung-Ang University Gwang-Myeong Hospital, Chung-Ang University College of Medicine, Gyeonggi-do, Korea

Correspondence
Beom Joon Kim and Joon Seok, Department of Dermatology, College of Medicine, Chung-Ang University, Seoul, Korea.
Email: beomjoon74@gmail.com and seokjoon923@gmail.com

Abstract

AP collagen peptides (APCPs) are enzymatically decomposed collagen peptides that contain tri-peptides such as glycine-proline-hydroxyproline. We found that APCPs increased the proliferation of both human dermal papilla cells (hDPCs) and human outer root sheath cells (hORSCs). APCPs also stimulated the secretion of several growth factors, including IGFBP-6, PDGF-AB, PIGF and VEGF in hDPCs. Moreover, APCPs enhanced the phosphorylation of Akt(Ser^{473}), GSK-3β(Ser^{9}) and β-catenin(Ser^{675}), indicating the activation of the GSK-3β/β-catenin signalling pathway. Ex vivo culture of human hair follicles (hHFs) tissue and in vivo patch assay revealed that APCPs promoted the elongation of hHFs and the induction of new hair shafts. In a mouse model, APCPs significantly promoted the transition from telogen to anagen phase and prolonged anagen phase, resulting in increased hair growth. APCPs also improved the thickness, amino acid content (cystine and methionine) and roughness of mouse hair. Taken together, these findings demonstrate that APCPs accelerate hair growth and contribute to overall hair health. Therefore, APCPs have the potential to be utilized as a food supplement and ingredient for preventing hair loss and maintaining hair health.

KEYWORDS
AP collagen peptides (APCPs), dermal papilla cells (DPCs), hair loss, keratin, outer root sheath cells (ORSCs), VEGF

1 | INTRODUCTION

Due to the profound impact of hair loss on social interactions and psychological well-being,[1] there is a growing interest in the prevention and treatment of this condition.[2] Representative drugs approved by the United States Food and Drug Administration (US FDA) include Minoxidil (MNX) and Finasteride.[3,4] The application of topical MNX can potentially result in uncomfortable complications such as contact dermatitis and skin irritation. Finasteride is frequently used to treat male pattern baldness. Unfortunately, these options are not suitable for female patients. Therefore, there is an unmet need for more efficient and safer treatments.[5,6]

Hair loss is often associated with aging, genetic predisposition, hormonal fluctuations and inadequate nutrition.[7–9] Lengg et al.

Yujin Kim and Jung Ok Lee contributed equally to this work.

demonstrated that dietary supplements containing essential nutrients have the potential to increase the number of hair follicles (HFs) in females experiencing telogen effluvium, suggesting that such supplements help mitigate and delay hair loss.[10]

The Wnt/β-catenin signalling pathway plays a critical role in the development of HFs. It acts as a master regulator, governing the proliferation and migration of various cells within the hair, including hair matrix cells, outer root sheath cells (ORSCs) and dermal papilla cells (DPCs). Moreover, it facilitates cell–cell adhesion and angiogenesis, thereby playing a pivotal role in the morphogenesis of HFs.[11–13] Therefore, this pathway has gained recognition as a potential target for preventing and treating hair loss. Li et al. have demonstrated that type II collagen hydrolysates could potentially serve as a novel functional food ingredient for osteoarthritis, as they exert a chondroprotective effect through the Wnt/β-catenin signalling pathway.[14] These findings indicate that collagen can regulate the Wnt/β-catenin signalling pathway.

Collagen is a key component of the extracellular matrix (ECM) found in the skin, hair, nails, joints, bones, tendons and cartilage.[15] The synthesis of collagen diminishes with age, while collagen degradation, facilitated by matrix metalloproteinases (MMPs), increases, resulting in a reduction in both the quantity and length of collagen fibres.[16] Postlethwaite et al. have observed that collagen peptides are transported to the skin through the bloodstream and accumulate there, suggesting a potential mechanism for replenishing collagen in the skin and preserving its structural integrity. This study also indicates that externally supplied collagen peptides may reach and impact the scalp and HFs.[17] Furthermore, collagen represents the richest reservoir of essential amino acids, including proline.[18] Enzymatically degraded collagen peptides, known as AP collagen peptides (APCPs), have already been reported to enhance skin hydration, elasticity and barrier function, as well as reduce wrinkles.[19]

Based on these observations, we formulated a hypothesis positing that APCPs exert a beneficial influence on hair growth and health. Consequently, we embarked on an investigation to assess the hair growth-promoting potential of APCPs. Furthermore, in order to evaluate the impact of APCPs on comprehensive hair health management, we conducted an analysis encompassing parameters such as hair thickness, roughness and total amino acid content in mouse hair.

2 | MATERIALS AND METHODS

2.1 | APCPs preparation and cell culture

APCPs were provided by the AMOREPACIFIC R&I Center (Yongin, Korea). They were manufactured through the enzymatic degradation of collagen extracted from the scales of golden threadfin bream (*Nemipterus virgatus*). The APCPs contained at least 15% Glycine-X-Y tripeptides (X and Y are arbitrary but are often occupied by a proline, hydroxyproline or alanine), including 3% Glycine-Proline-Hydroxyproline. Human DPCs (hDPCs) were purchased from PromoCell (Heidelberg, Germany) and cultured using a follicle dermal papilla cell growth medium kit (PromoCell). Human ORSCs (hORSCs) were obtained from Kyungpook National University College of Medicine (Daegu, Korea) and maintained in EpiLife™ medium with 60 μM calcium (Thermo Fisher Scientific, Waltham, MA, USA) and EpiLife™ defined growth supplements (EDGS) at 37°C with 5% CO_2.

2.2 | Determination of cell viability

Cell proliferation was assessed using the water-soluble tetrazolium salt (WST-8) assay (QuantiMax™, BIOMAX, Gyeonggi-do, Korea). The absorbance was measured at 450 nm using a microplate spectrophotometer (SpectraMax 340; Molecular Devices, Inc., CA, USA).

2.3 | Enzyme Linked Immunosorbent Assay (ELISA)

The hDPCs (1 × 105 cells/well) were treated with APCPs for 48 h, and the cultured medium was clarified by centrifugation at 12,000 rpm for 20 min at 4°C. The supernatant was used to assess the level of VEGF using an ELISA assay kit (R&D Systems, Inc. Corning Inc., MN, USA), following the manufacturer's instructions.

2.4 | Quantitative RT-PCR (qRT-PCR) Analysis

hDPCs treated with APCPs were subjected to RNA extraction using TRIzol (Invitrogen, CA, USA). cDNA synthesis was performed using Prime Script™ RT Master Mix (Takara, Tokyo, Japan). Quantitative PCR (qPCR) was performed using 2X qPCR PreMIX SYBR (Enzynomics Seoul, Korea) on a CFX96 Touch RT-PCR Detection System (Bio-Rad, CA, USA). Sequences of the specific primers used for RT-PCR are listed in Table 1. The relative target gene levels were calculated using the ΔCt method and normalized to the expression levels of GAPDH.

2.5 | Western blot analysis

Western blotting was performed as previously described 23. Briefly, protein samples were heated at 95°C for 5-min and analyzed by 10% SDS-PAGE. Immunodetection was performed using an Amersham ECL kit (GE Healthcare, IL, USA) according to the manufacturer's protocol. The protein bands were visualized using a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed using the NIH Image J software (Bethesda, MD, USA). The antibodies used are listed in Table 2.

2.6 | Immunofluorescence (IF)

The cells and paraffin tissue were fixed with 4% paraformaldehyde (PFA) for 30 min, blocked with 3% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS, and incubated with anti-β-catenin antibodies overnight at 4°C. After washing, the cells were incubated with anti-rabbit IgG-FITC secondary antibodies (Santa Cruz Biotechnology, CA, USA) for 1 h, and nuclei were stained with DAPI (Immuno Bioscience Corp., Washington, USA). β-catenin was observed by confocal microscopy (LSM 880, Zeiss, Oberkochen, Germany).

2.7 | Histology and immunohistochemistry (IHC)

Dorsal skin tissues from each mouse were fixed with 10% formalin, embedded in paraffin, and then cut into sections that were stained with hematoxylin and eosin (H&E). For IHC, the sliced sections were incubated with primary antibodies. The stained slides were photographed using a slide scanner (Pannoramic MIDI; 3DHISTECH Ltd, Budapest, Hungary) and observed using Case Viewer software. The number of HFs was counted on a cropped image in a fixed area (1 × 1 mm).

2.8 | Growth factor antibody array

Changes in the profiles of growth factors were evaluated using a human growth factor antibody array membrane kit (Abcam, Cambridge, UK). Briefly, hDPCs (1×10^5 cells/well) were seeded in 6-well plates and incubated with APCPs for 48 h. Then, the medium from the cultured hDPCs was collected for growth factor analysis. This assay was performed following the manufacturer's instructions. The results were analysed under identical conditions using a chemiluminescence EZ-capture (ATTO, New York, USA) and NIH Image J program (Bethesda, MD, USA).

2.9 | Generation of hDPC spheroids

A 30-μL suspension of hDPCs (3×10^5 cells/mL) was dispensed into each well of a clear 96-well round-bottom ultra-low attachment microplate (Nexcelom Bioscience, Lawrence, USA). The unified spheres were treated with APCPs for 24 h. The diameters of the spheres were measured using phase-contrast microscopy (Leica, Wetzlar, Germany).

2.10 | Patch assay

The patch assay was performed as described previously.[19] Briefly, APCPs or MNX were combined with freshly isolated neonatal mouse epidermal and dermal cells. The mixed cells were subcutaneously transplanted into the skin on the backs of nude mice. The dorsal skins of the mice were monitored and photographed using a digital camera for 10 days. To confirm the formation of new HFs, the dorsal skin tissues were fixed with 10% paraffin and stained with haematoxylin and eosin (H&E).

2.11 | Organ culture of human HFs

The hHFs were obtained from Dankook University Hospital (IRB approval number; DKUH 2021-12-025) along with written informed consent from the patients. The isolated anagen follicles were cultured in 500 μL of Williams E medium (Gibco, Grand Island, NY, USA) at 37°C with 5% CO_2. After 24 h, the HFs were cultured with APCPs (1 mg/mL or 3 mg/mL) or MNX (50 μM) for 4 days. Images of the HFs were obtained using a stereo microscope (Zeiss, Oberkochen, Germany). HF elongation was analysed using the ImageJ program (version 1.52a).

2.12 | Hair regeneration model

C57BL/6 mice (6-week-old males) were purchased from Saeron Bio Inc. (Gyeonggi-do, Korea) and allowed a 1-week acclimation period before the start of the experiments. All animal experiments were conducted in accordance with the Principles of Laboratory Animal Care established by the National Institutes of Health (NIH) and approved by the Chung-Ang University Institutional Animal Care and Use Committee (IACUC No. A2022052). Based on previous reports,[20,21] we removed the hair from the dorsal skin of 7-week-old mice using an electronic shaver. Subsequently, we used Niclean cream (Ildong Pharmaceutical, Seoul, Republic of Korea) to eliminate any remaining fine hairs. After a recovery period of 24 h, the mice were randomly assigned to one of five groups: normal control ($n=5$), APCPs 300 mg/kg ($n=5$), APCPs 600 mg/kg ($n=5$), APCPs 900 mg/kg ($n=5$) and MNX 1 mg/kg ($n=5$). APCPs and MNX were administered orally once daily for 16 days. To assess the growth rate, the dorsal skin of the mice was photographed using a digital camera on days 0, 11, 14 and 16 following treatment with APCPs. The growth area to total area ratio was subsequently quantified using the ImageJ program. On day 16, the mice were euthanized, and dorsal skin tissues were excised for histological analysis.

2.13 | Measurement of mouse hair surface roughness

The hair samples were newly grown in the anagen phase after hair depilation. A scanning electron microscope (SEM; JEOL FESEM J-7100) was used to obtain magnified images of the hair samples to study their shape and structure. The roughness of the hair surface was quantified using the Image J 1.52 program (NIH, Bethesda, MD, USA), and the average roughness (Ra) was calculated.

2.14 | Measurement of mouse hair thickness

Following hair depilation, the diameter of newly grown hair in anagen VI was analysed using optical microscope analysis. The mouse pelage consists of four distinct types of hair with varying shapes, lengths and thicknesses: guard, awl, auchene and zigzag hairs.[20,21] Among these, the awl hair type was selected for measuring hair thickness. The measurements were performed on three mice per group. For each mouse, the thickest diameter of eight hairs was measured, and the average value was calculated for statistical analysis. Each hair was examined using an optical microscope (Eclipse Ni-U; Nikon, Tokyo, Japan), photographed and analysed using the Image Studio software (Customized InnerViewTM, Bungdang-gu, Korea). In the hair sample, three points were selected for observation: the top (point 1), the midpoint (point 2) and the bottom (point 3), which was 870 μm away from the top. The thickness was determined by calculating the average value of the widest region and was used to compare the change in diameter.

2.15 | Analysis of amino acid content

The analysis of amino acid content was performed using Shane M. Rutherfurd's Method, as described previously.[22] Briefly, newly grown hairs were collected through depilation and hydrolyzed. Derivatization was conducted by combining a borate buffer (pH 10.2, Agilent), sample solution or standard solution, OPA (Agilent), FMOC (Agilent) and dilution solution. The concentration of each amino acid was determined using the regression equation obtained from the amino acid standard solutions.

2.16 | Statistical analysis

The results are expressed as the mean ± standard deviation (SD) of at least three independent experiments. The data were analysed using one-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test. Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software Inc., CA, USA). Differences with a p-value lower than 0.05 were considered statistically significant and indicated on the graphs with the following symbols: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; and $^{****}p<0.0001$.

3 | RESULTS

3.1 | APCPs increase the proliferation of hDPCs and hORSCs

Human DPCs and hORSCs play significant roles in increasing hair growth. We treated these cells with various concentrations of APCPs for 24 h and evaluated cell proliferation using a WST-8 assay. Treatment with APCPs (10 or 30 μg/mL) induced an increase in the proliferation of hDPCs (Figure 1A). In addition, the proliferation of hORSCs was significantly increased upon treatment with APCPs (Figure 1B). The growth and function of hORSCs are closely influenced by growth factors secreted from hDPCs.[23] Therefore, we investigated whether conditioned medium (CM) obtained from APCP-treated hDPCs affects the proliferation of hORSCs. As shown in Figure 1C, the CM obtained from hDPCs not treated with APCPs also stimulated the proliferation of hORSCs. This result is consistent with previous reports.[23] Interestingly, CM obtained from APCP-treated hDPCs enhanced the proliferation of hORSCs even more than CM obtained from untreated hDPCs. Additionally, to confirm the effect of APCPs on the change of growth factors secreted from hDPCs, we performed a human growth factor antibody array analysis using the CM. When comparing the CM obtained from APCP-treated hDPCs with that obtained from vehicle-treated hDPCs, we observed an altered secretome profile. Specifically, the levels of insulin-like growth factor binding protein 6 (IGFBP-6), platelet-derived growth factor-AB (PDGF-AB), placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) were upregulated (Figure 1D). Furthermore, we validated that APCPs elevated the level of VEGF in the CM obtained from APCP-treated hDPCs through an ELISA assay (Figure 1E). Collectively, these findings indicate that APCPs promote hair growth by enhancing the interplay between hDPCs and hORSCs.

3.2 | APCPs increase new hair formation in BALB/c nude mice

To assess the hair growth-inducing properties of APCPs on hDPCs, a three-dimensional (3D) spheroid culture system was utilized. As shown in Figure 2A,B, APCPs promoted the aggregation of spheroids and increased the mRNA levels of trichogenic genes, such as *HEY1*, *ALPL*, *β-catenin*, *BMP2* and *SHH*, in a dose-dependent manner. Next, a patch assay was performed to confirm the ability of APCPs to induce new hair formation. On day 10 after transplantation, a clump of black hair shafts emerged in the hypodermis across all groups (Figure 2C,D). Remarkably, APCPs significantly increased the number of HFs compared to both the vehicle and MNX (Figure 2E,F). These results suggest that APCPs facilitate the induction of new hair formation (Tables 1 and 2).

3.3 | APCPs enhance the GSK-3β/β-catenin signalling pathway

The levels of p-Akt(Ser^{473}), p-GSK-3β(Ser^{9}) and p-β-catenin(Ser^{675}) increased in hDPCs after 1 h of treatment with APCPs (Figure 3A). β-catenin interacts with TCF/LEF1 transcription factors, resulting in the transcriptional activation of Wnt target genes.[12] Figure 3B,C

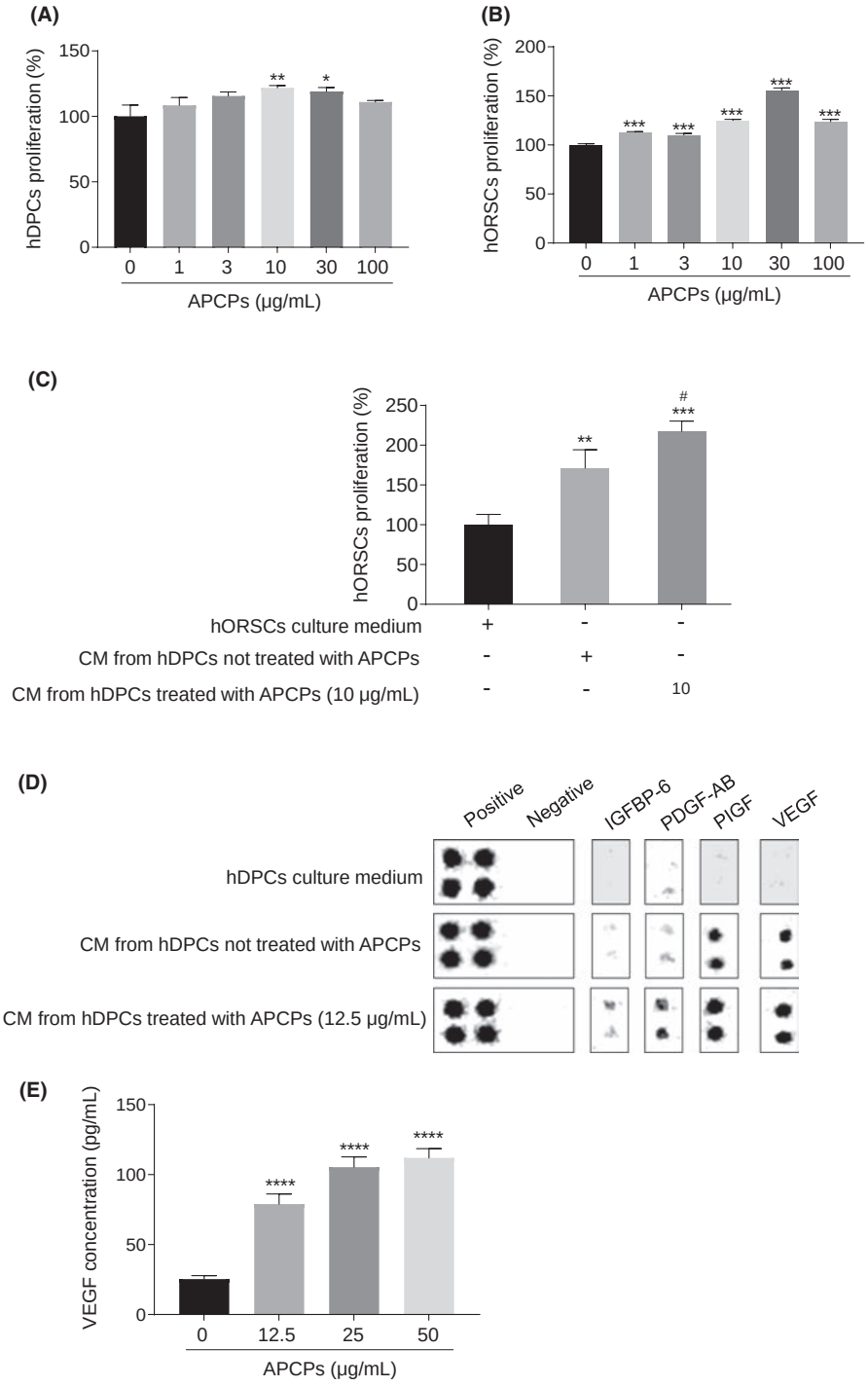


FIGURE 1 Effect of APCPs on the proliferation of hDPCs and hORSCs. (A) The proliferation of hDPCs was measured after treatment with APCPs (0, 1, 3, 10, 30 or 100 μg/mL) using a WST-8 assay. (B) The proliferation of hORSCs was measured after treatment with APCPs (0, 1, 3, 10, 30 or 100 μg/mL) using a WST-8 assay. (C) The hORSCs were treated with the hORSCs culture medium or CM obtained from the hDPCs treated with APCPs (0 or 10 μg/mL) for 1 day. Then, the proliferation of hORSCs was analysed using a WST-8 assay. (D) A growth factor antibody array analysis was performed using PromoCell Follicle Dermal Papilla Cell Growth Medium and two types of CM obtained from hDPCs treated with either the vehicle control or APCPs (12.5 μg/mL) for 48 h. (E) The level of VEGF in CM obtained from hDPCs treated with APCPs (0, 12.5, 25 or 50 μg/mL) for 48 h was measured using ELISA assay. The results are expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and $^{****}p < 0.0001$ compared with the vehicle control group. $^{\#}p < 0.05$ compared with the CM obtained from hDPCs treated with APCPs (10 μg/mL).

show that APCPs increased the mRNA levels of *β-catenin* and *LEF1*, as well as the protein levels of β-catenin and LEF1, in a dose-dependent manner. APCPs also stimulated the translocation of β-catenin from the cytosol to the nucleus compared with the control, as shown through the IF analysis (Figure 3D). To further evaluate the role of the GSK-3β/β-catenin pathway in the hair growth-promoting effect of APCPs, we used XAV939, a Wnt/β-catenin pathway inhibitor. XAV939 blocked the APCPs-induced upregulation of β-catenin and LEF1 (Figure 3E). These results suggest that APCPs promote hair growth by activating the AKT-GSK-3β/β-catenin signalling pathway.

3.4 | APCPs enhance the growth of human HFs in an ex vivo model

The hHFs treated with APCPs (25 and 50 μg/mL) exhibited enhanced growth compared to the control hHFs after 4 days, similar to the

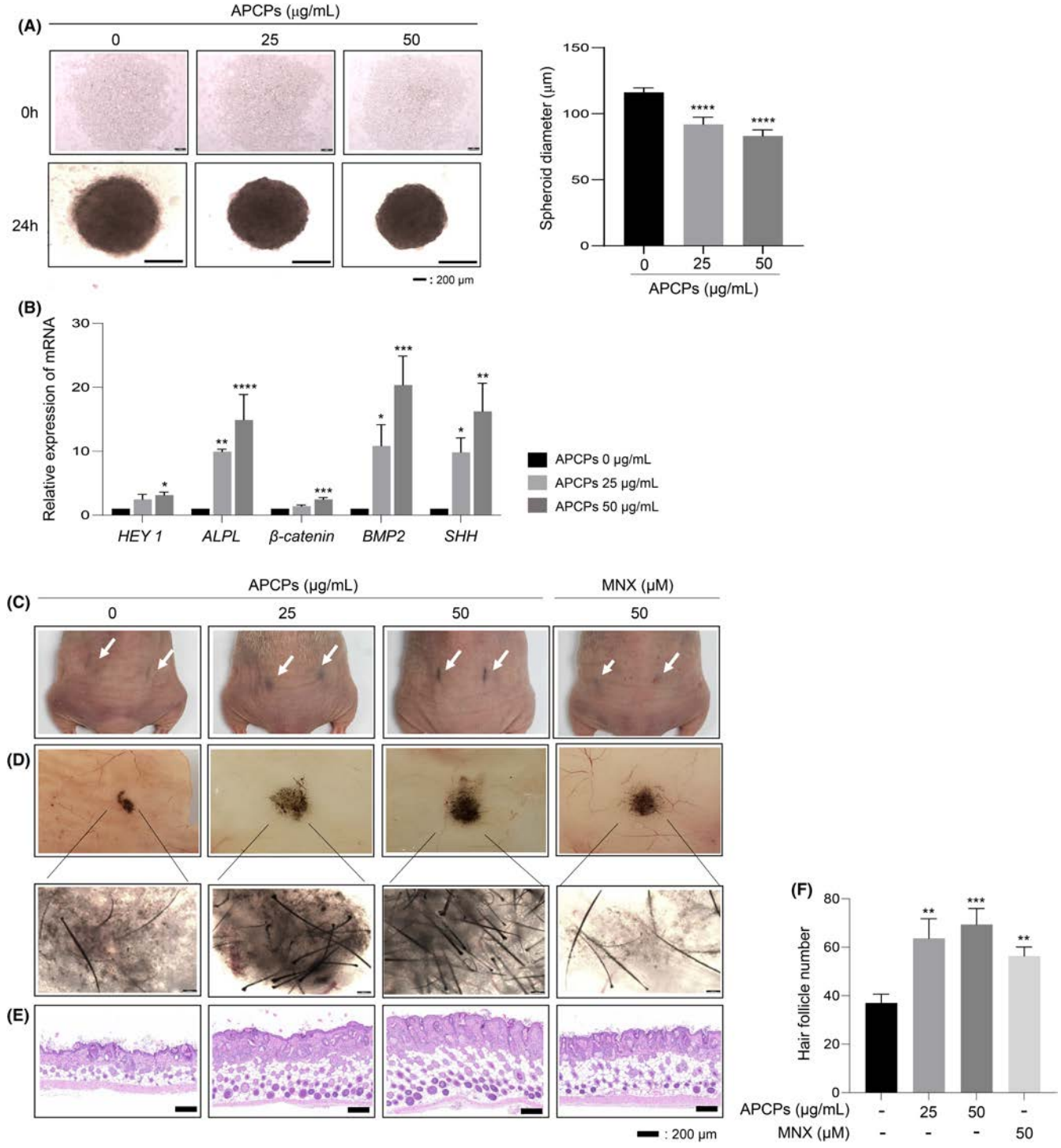


FIGURE 2 Effect of APCPs on sphere formation by hDPCs. (A) Comparison of spheroid formation in the control and APCP-treated (25 or 50 μg/mL) hDPCs. Phase images of the spheroids are shown. The images were analysed 0 and 24 h after the treatment with APCPs, and the diameter of each spheroid was determined. (B) hDPCs treated with APCPs for 24 h were lysed, and the mRNA expression levels of *HEY1*, *ALPL*, *β-catenin*, *BMP-2* and *SHH* were analysed using qPCR ($n = 3$). (C, D) The patch assays assessing in vivo hHFs regeneration. (E) Representative images of the H&E-stained hair follicle regeneration area. Scale bar, 200 μm. (F) The number of hHFs. The results are expressed as the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$ compared with the vehicle control group.

growth observed in hHFs treated with MNX (50 μM) (Figure 4A). According to a previous report, VEGF-mediated angiogenesis promotes hair growth and increases HF size.[24] As shown in Figure 4B, APCPs increased the size of the dermal papilla (DP) compared to the control. Moreover, APCPs upregulated the expression of β-catenin and VEGF in hHFs compared to the control (Figure 4C,D), suggesting

TABLE 1 Primer sequences used for qPCR.

Gene	Primer sequence (5′ to 3′)	
Human HEY 1	F	TGGATCACCTGAAAATGCTG
	R	CGAAATCCCAAACTCCGATA
Human ALP	F	ATTGACCACGGGCACCAT
	R	CTCCACCGCCTCATGCA
Human β-catenin	F	GCCAAGTGGGTGGTATAGAGG
	R	GCGGGACAAAGGGCAAGA
Human BMP2	F	GAGGTCCTGAGCGAGTTCGA
	R	TCTCTGTTTCAGGCCGAACA
Human SHH	F	GCGCCAGCGGAAGGTAT
	R	CCGGTGTTTTCTTCATCCTTAAA

TABLE 2 Antibodies used for Western blot analysis.

Antibodies	Product code	Company
Anti-p-GSK-3β (Ser^{9})	#93365	Cell Signaling Technology
Anti-total GSK-3β	#12456	Cell Signaling Technology
Anti-p-β-catenin (Ser^{675})	#4176	Cell Signaling Technology
Anti-total β-catenin	#8480	Cell Signaling Technology
Anti-LEF	#2230	Cell Signaling Technology
Anti-p-AKT (Ser^{473})	#4060	Cell Signaling Technology
Anti-AKT	#4691	Cell Signaling Technology
Anti-VEGF	#50661	Cell Signaling Technology
Anti-β-Actin	#3700	Cell Signaling Technology
Anti- type I + II hair keratin	GP-PANHK	PROGEN Biotechnical GmbH
Horse Anti-Mouse IgG	PI-2000-1	Vector Laboratories
Goat Anti-Rabbit IgG	PI-1000-1	Vector Laboratories
Anti-rabbit IgG-FITC	sc-2359	Santa Cruz Biotechnology

that APCP-mediated HF growth is associated with increased expression of β-catenin and VEGF.

3.5 | APCPs accelerate hair growth in C57BL/6 mice

After shaving their dorsal skins, the mice were orally injected with either the vehicle (saline), APCPs at various concentrations (300, 600 or 900 mg/kg; referred to as APCPs300, APCPs600 and APCPs900, respectively), or MNX (1 μM) every day for 16 days (Figure 5A). As previously reported, a new round of synchronous growth was observed in the mouse dorsal skin after depilation. During this time, the mice's skin changed colour as it accumulated melanin, gradually altering from pink to pinkish white, white, greyish white, grey,and dark grey. This change in skin colour is a sensitive indication of hair regrowth after depilation, as the melanogenic activity of HFs is closely related to the hair cycle.[25] According to the mouse skin colour index (Figure 5B), we determined the activity of APCPs on hair regrowth on day 11. The MNX group exhibited the highest score, followed by the APCPs900, APCPs600 and APCPs300 groups (Figure 5C,D). After 16 days, the area of hair regrowth was measured, and the highest regrowth relative to the vehicle control group ($p < 0.001$) was noted in the APCPs600 and APCPs900 groups, along with the MNX group (Figure 5C,E). The thickness of the hypodermal fat layer was higher in the APCPs600, APCPs900 and MNX groups ($p < 0.0001$; Figure 5F,G). Moreover, by analysing the transverse (HF number) and longitudinal (hair growth phase) sections, it was confirmed that the total number of HFs and the number of anagen HFs were increased in the APCP-treated groups compared with the control group (Figure 5H,I). Additionally, the levels of β-catenin and VEGF were significantly elevated in the dorsal skins of the APCPs600, APCPs900 and MNX groups (Figure 5J,K). These results indicate that APCPs promote anagen transition and induce hair growth.

3.6 | APCPs improve the thickness, amino acid content and roughness of hair

Phototrichogram studies have confirmed that the density and diameter of hair decrease with chronological aging.[26] To investigate the effect of APCPs on hair thickness, the diameter of hair in mice was measured. The thickness of hair from the APCPs600 and APCPs900 groups of mice was increased compared with the vehicle group (Figure 6A,C). The hair cuticle layer, which is located at the outermost part of the hair, protects the hair shaft from external mechanical stress.[27] SEM imaging revealed that the APCPs group had a decrease in the average roughness (Ra) of the hair cuticle compared to the vehicle group, indicating that the APCPs smoothed and lubricated the cuticles (Figure 6B,D). In addition, the total amino acid content of mouse hair increased in a dose-dependent manner in the APCPs300, APCPs600 and APCPs900 groups (Figure 6E). Interestingly, the abundance of the main amino acids found in hair, cystine and methionine, also increased (Figure 6F). Furthermore, we confirmed that oral injection of APCPs upregulated the expression levels of type I and type II keratin in the back skin of mice (Figure 6G). These results demonstrate that APCPs can increase hair thickness and improve hair roughness.

4 | DISCUSSION

APCPs contain essential amino acids, which are key components of the ECM in hair. To verify the impact of APCPs on hair growth, we investigated their hair-growth-promoting effect using in vitro

models with ORSCs and DPCs, as well as an in vivo model with telogenic C57BL/6 mice. Our findings demonstrate that APCPs possess the capability to improve both hair growth and hair health.

Human DPCs secrete paracrine factors that induce the migration of stem cells from the bulge region to the bulb of the hHFs and stimulate the proliferation and differentiation of hDPCs and hORSCs.[28–31] Jong-Hyuk et al. reported that the CM of Keratinocyte Stem/Progenitor Cells (KSCs) not only promotes the proliferation of hDPCs and hORSCs, but also enhances hair growth in C3H/HeN mice.[32] They additionally confirmed an increase in VEGF and IGFBP-6 in KSC-CM. Interestingly, as shown in Figure 1D, VEGF and IGFBP-6 also increased in the CM from hDPCs treated with APCPs. These results strongly support the function of APCPs in inducing hair growth.

ECM is a network consisting of extracellular macromolecules and minerals, such as collagen, enzymes and glycoproteins, that provide structural and biochemical support to surrounding cells.[33] Specifically, the ECM participates in the interaction and communication between cells. Cells within the hair follicle exchange signals through the ECM, influencing hair growth and maintenance.[34,35] Furthermore, the ECM facilitates the supply of nutrients through blood vessels and eases the exchange between cells.[36] Proper nutrition is essential for hair health, and the ECM contributes to the delivery of these nutrients. APCPs are collagen peptides derived from the scales of golden threadfin bream (Nemipterus virgatus). In summary, collagen and the ECM enhance the structural stability of hair and intercellular communication, playing a crucial role in hair health. Adequate collagen and a healthy ECM can support the strength and elasticity of hair. In this study, we confirmed that APCPs induce hair growth, and our findings suggest that APCPs may serve as a component of the ECM.

The formation of new blood vessels from existing capillaries is achieved through a combination of angiogenic factors, ECM and protease.[37] Among these factors, VEGF plays a crucial role in increasing vascular permeability and stimulating capillary formation in vivo, which are core angiogenic functions.[24,38,39] Back et al. have shown that VEGF reduces hair loss by promoting blood circulation and the formation of new blood vessels around the hHF.[40] As shown in Figure 1E, we confirmed that APCPs enhance the secretion of VEGF from hDPCs, suggesting that APCPs stimulate hair growth by facilitating the supply of nutrients to hHFs.

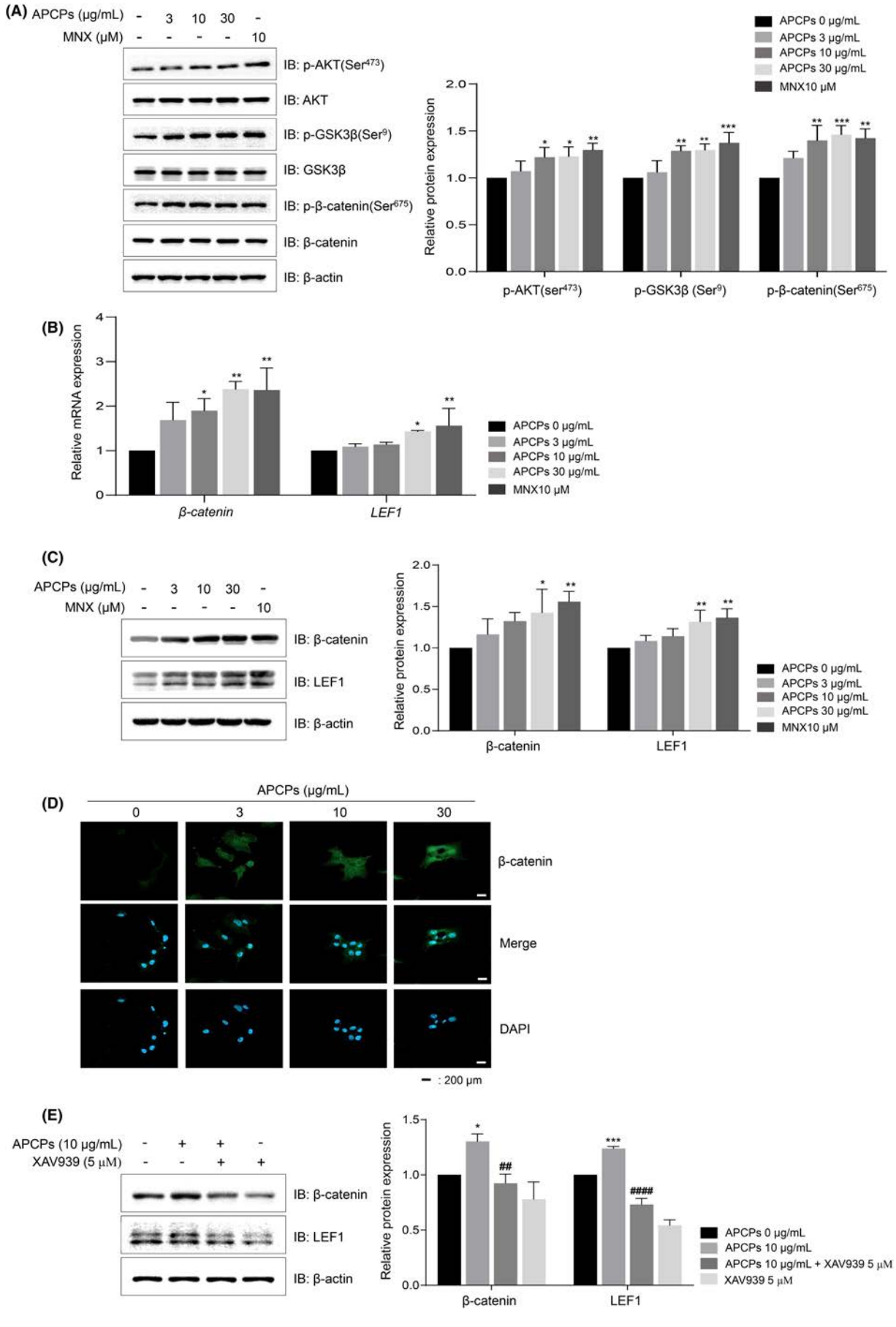
In hHFs, most cells are well-assembled into a 3D structure, where cell–cell and cell–ECM interactions play an indispensable role in the cycling and morphogenesis of HFs.[31,41] Zhang et al. have reported that the human placenta extracellular matrix (HPECM) hydrogel successfully reinstates the hair-inductive capacity of high-passaged hDPCs, suggesting that ECM has a crucial function in the activity of hDPCs.[42] In addition, other extracellular components such as synthesized ECM, gelatin-sponge and artificial scaffold stimulate the trichogenic activity of hDPCs.[43–45] Taken together, these results demonstrate that APCPs have a positive impact on hair growth as a key ingredient in the HF.

Damage to the cuticle layer can accelerate the process of hair aging.[46] Therefore, it is important to protect the cuticle in order to maintain healthy hair. Ceramide, a component of hair, plays a crucial role as a barrier in the hair cuticle.[47–49] Moreover, ceramides help to keep the hair cuticle smooth and flat.[46] Interestingly, a clinical trial showed that oral treatment with APCPs increases the ceramide content in the skin.[50] In a mouse experiment, oral administration of APCPs improved the roughness of the cuticle in mouse hair (Figure 6B,D), suggesting that APCPs may have the potential to enhance hair gloss and maintain the health of aging hair.

Hair is primarily composed of keratin (65%–95%).[51] Keratin consists of 21 amino acids and provides hair with a stable structure.[52] Cystine residues of keratin are particularly important, as they form the disulfide bonds.[53] The amino acid profile can serve as an indicator of hair health conditions.[54] Oral administration of APCPs increased hair thickness and the total amino acid content, including cystine and methionine (Figure 6C,E,F), as well as the expression levels of type I and type II keratin in mice (Figure 6G). Interestingly, high-quality protein supplements significantly increase not only wool production rates but also hair fibre diameter in Angora rabbits.[55] These findings confirm the positive effect of amino acid supplementation on hair thickness. Furthermore, it can be inferred that APCPs are responsible for the observed increase in hair thickness in mice.

Collectively, these observations suggest that APCPs supplementation could be a promising functional food to improve hair growth and overall hair health.

FIGURE 3 Effect of APCPs on the GSK-3β/β-catenin signalling pathway. (A) hDPCs were treated with APCPs (0, 3, 10 or 30 μg/mL) or MNX (10 μM) for 1 h and analysed using western blotting with primary antibodies against p-AKT (Ser^{473}), AKT, p-GSK (Ser^{9}), GSK, p-β-catenin (Ser^{675}), β-catenin and β-Actin. (B) hDPCs were treated with APCPs (0, 3, 10 or 30 μg/mL) or MNX (10 μM) for 24 h, and the mRNA expression levels of *β-catenin* and *LEF1* were analysed using qPCR ($n = 3$). (C) hDPCs were treated with APCPs (0, 3, 10 or 30 μg/mL) or MNX (10 μM) for 24 h and analysed using Western blotting with specific antibodies against β-catenin, LEF1 and β-Actin. (D) Expression of β-catenin was analysed through ICC. (E) After treatment with APCPs with or without XAV939 for 24 h, the protein samples were analysed through Western blotting using specific antibodies against β-catenin, LEF1 and β-Actin. The results are expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and $p <$ compared with the control group. $^{\#\#}p < 0.01$; $^{\#\#\#\#}p < 0.0001$ compared with the APCPs-only treatment group.



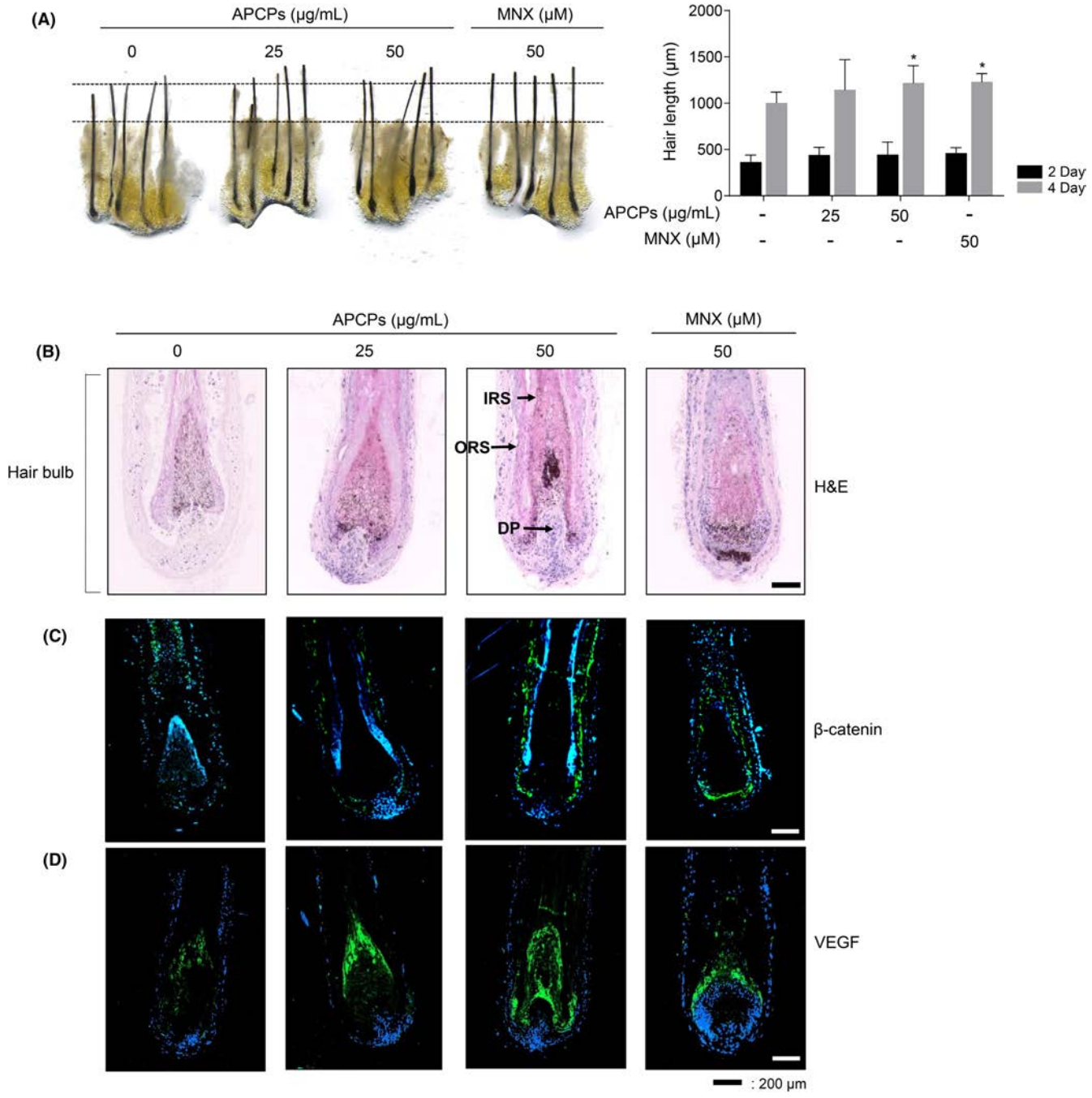


FIGURE 4 Effect of APCPs on hair growth in the hHFs organ culture model. The hHFs (five hair follicles/group) were treated with APCPs (0, 25 or 50 μg/mL) or MNX (50 μM) for 4 days. (A) The HF length was analysed using a stereomicroscope on days 2 and 4. The relative length of each hair shaft was determined using ImageJ. (B) The H&E staining of hHFs on day 4. (C, D) The IF staining of HFs for β-catenin and VEGF at day 4. The results are expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$ compared with the control group receiving no APCPs (0 μg/mL). DP, dermal papilla; IRS, inner root sheath; ORS, outer root sheath.

FIGURE 5 Effect of APCPs on anagen induction in C57BL/6 mice. The vehicle, APCPs (300, 600 or 900 mg/kg; $n = 5$), and MNX (1 mg/kg; $n = 5$) were orally administrated seven times per week for 16 days. (A) The timetable of experimental treatments and sample collections. (B) Mouse skin colour score index. (C) The mice were photographed on days 0, 11, 14 and 16. (D) Representative photographs and quantification of the skin colour score for 11 days. (E) Representative photographs and the hair growth area of the back skin for 16 days. (F) Representative images of the H&E-stained longitudinal and transverse sections of skin tissues on day 16. Scale bar, 200 μm. (G) Hair dermis thickness, (H) Anagen/Telogen ratios and (I) the number of HFs on day 16. (J) The expression of β-catenin and (K) VEGF on the dorsal skin at day 16. Representative images of the IHC analysis. Scale bar, 200 μm. The results are expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$; $^{***}p < 0.001$; and $^{****}p < 0.0001$ compared with the vehicle control group.

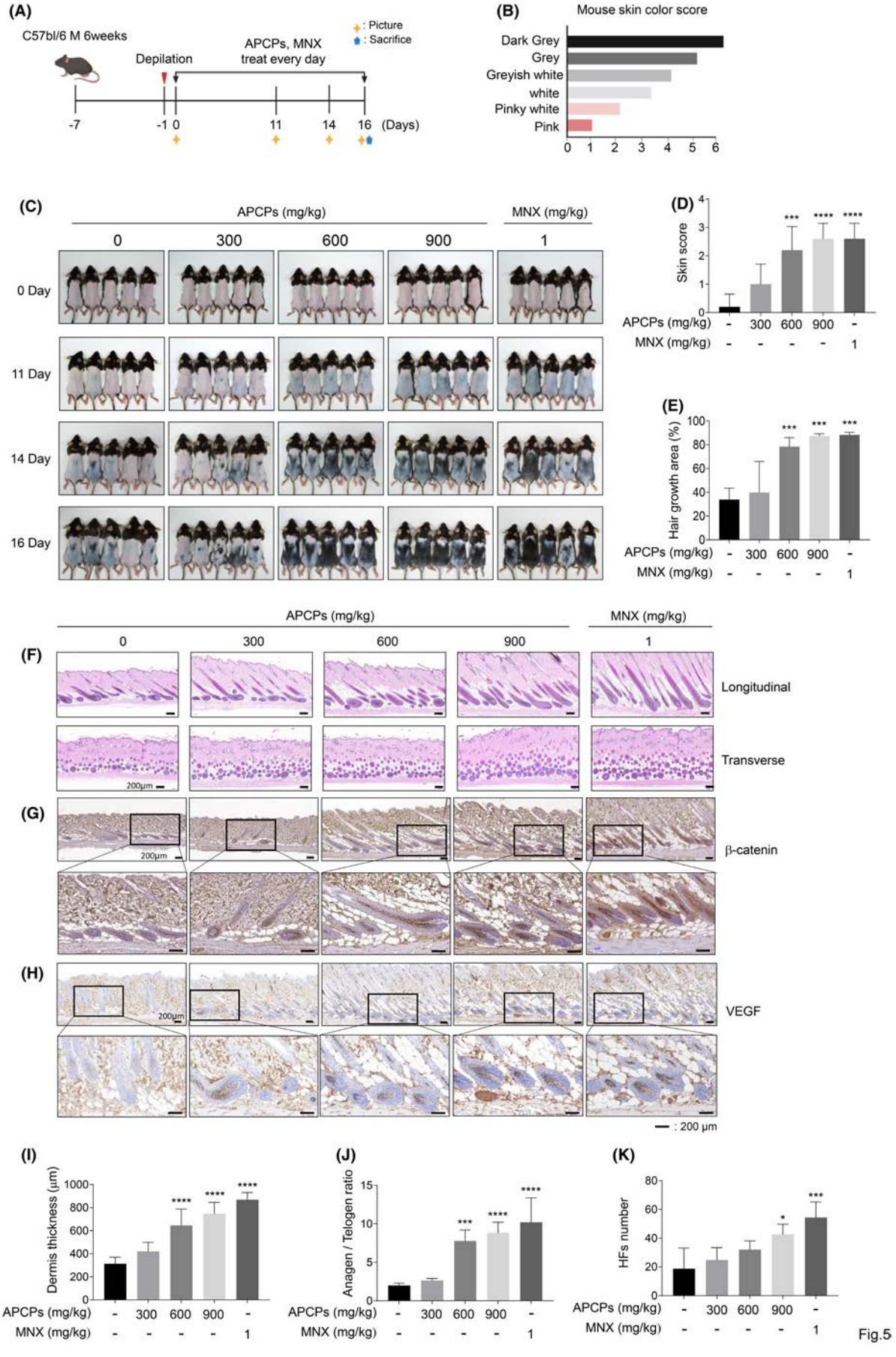


Fig.5

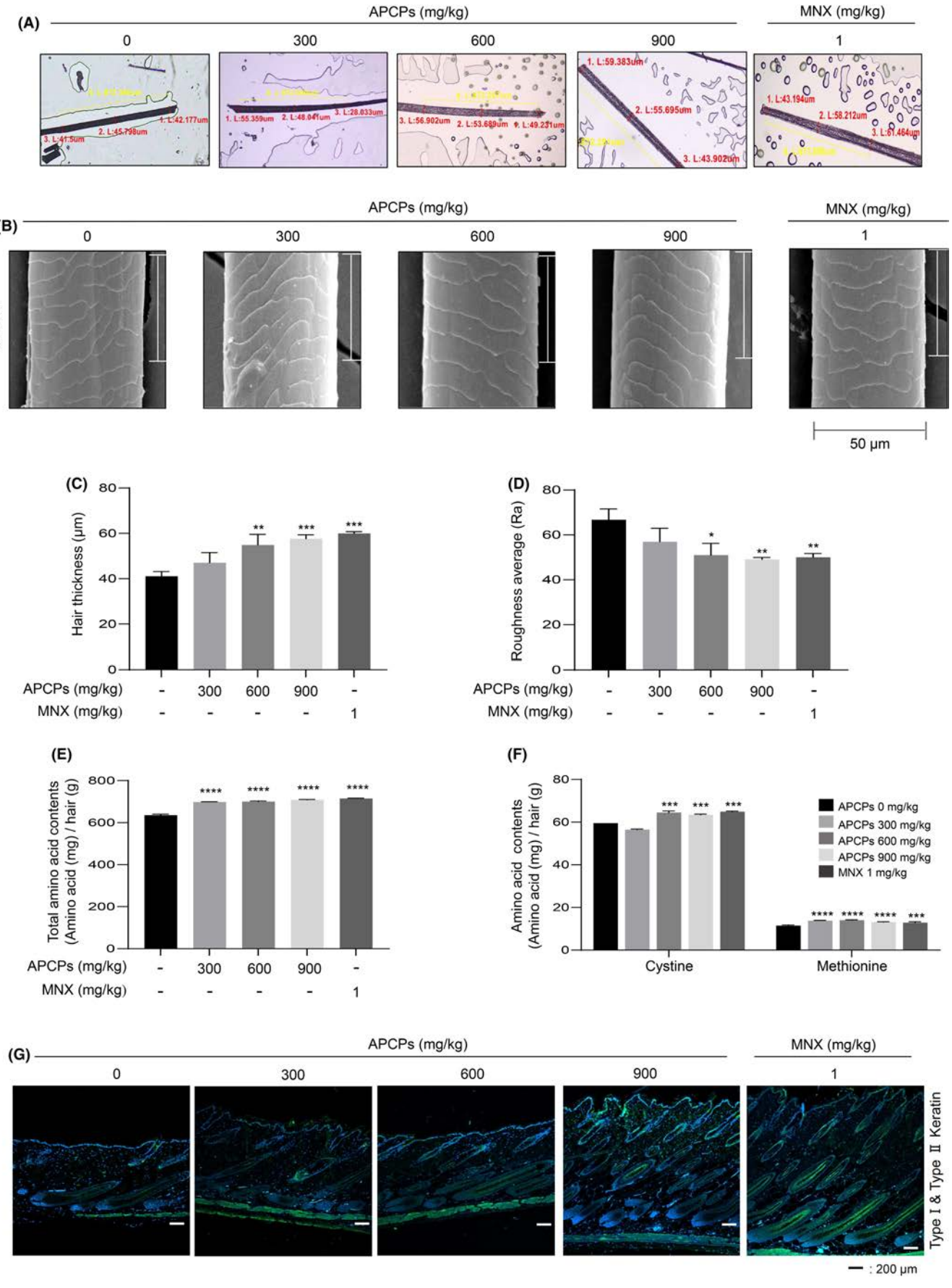


FIGURE 6 Effect of APCPs on hair condition. (A) Mouse hair thickness on day 16. The images were captured using a reflecting microscope and analysed using Image Studio. (B) The SEM image of mouse hairs on day 16. (C) Diameter and (D) Ra of mouse hairs on day 16. (E) Total amino acid, (F) cystine and methionine content of the mouse hairs on day 16. (G) The expression of Type I and Type II keratin on the dorsal skin at day 16. Representative images of the IF analysis. Scale bar, 200 μm. The results are expressed as the mean ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$ compared with the vehicle control group.

5 | CONCLUSION

The results of this study provide the first evidence that APCPs promote hair growth by activating the GSK-3β/β-catenin signalling pathway. APCPs stimulated the growth of both hDPCs and hORSCs, as well as the formation of new hair. Moreover, the administration of APCPs accelerated the onset of the anagen phase, increased the number of HFs and enhanced the ratio of the anagen phase to the telogen phase in mice. Furthermore, APCPs improved the roughness of the cuticles, increased hair diameter and elevated the levels of total amino acids and main components of keratin (cystine and methionine) in mouse hair. Taken together, our findings suggest that APCPs could be a potential functional food for improving hair loss and hair health.

AUTHOR CONTRIBUTIONS

Jung Ok Lee was involved in methodology, investigation and writing. Yujin Kim was involved in data curation, formal analysis, writing, investigation and editing. Jung Min Lee and Joon Seok were involved in investigation and data curation. Jang Mi Suk and Inhee Jung were involved in methodology. Beom Joon Kim was involved in reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

DATA AVAILABILITY STATEMENT

All data generated and analysed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1.

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