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Original Article

Dendrobium officinale polysaccharide promotes angiogenesis as well as follicle regeneration and hair growth through activation of the WNT signaling pathway

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ABSTRACT

Introduction: Hair loss is one of the common clinical conditions in modern society. Although it is not a serious disease that threatens human life, it brings great mental stress and psychological burden to patients. This study investigated the role of dendrobium officinale polysaccharide (DOP) in hair follicle regeneration and hair growth and its related mechanisms.
Methods: After in vitro culture of mouse antennal hair follicles and mouse dermal papilla cells (DPCs), and mouse vascular endothelial cells (MVECs), the effects of DOP upon hair follicles and cells were evaluated using multiple methods. DOP effects were evaluated by measuring tentacle growth, HE staining, immunofluorescence, Western blot, CCK-8, ALP staining, tube formation, scratch test, and Transwell. LDH levels, WNT signaling proteins, and therapeutic mechanisms were also analyzed.
Results: DOP promoted tentacle hair follicle and DPCs growth in mice and the angiogenic, migratory and invasive capacities of MVECs. Meanwhile, DOP was also capable of enhancing angiogenesis and proliferation-related protein expression. Mechanistically, DOP activated the WNT signaling and promoted the expression level of β-catenin, a pivotal protein of the pathway, and the pathway target proteins Cyclin D1, C-Myc, and LDH activity. The promotional effects of DOP on the biological functions of DPCs and MVECs could be effectively reversed by the WNT signaling pathway inhibitor IWR-1.
Conclusion: DOP advances hair follicle and hair growth via the activation of the WNT signaling. This finding provides a mechanistic reference and theoretical basis for the clinical use of DOP in treating hair loss.

1. Introduction

Hair loss is a disorder of hair regeneration induced by shrinkage or cyclic growth of hair follicles influenced by genetic factors, organ-specific autoimmune disorders, stressful stress, nutritional deficiencies, heavy smoking, and alcohol consumption [1]. In modern society, hair loss groups have shown a significantly lower age and "professionalization". Damage to the external physical appearance caused by abnormal hair loss has caused serious psychological stress and mental burden to the patients, and has emerged as one of the key disorders that substantially disturb the nation's health. Clinical treatments for hair loss are categorized into non-surgical treatments (e.g. Western medicine, traditional Chinese medicine (TCM), combined Chinese and Western medicine, laser and its adjuvant therapy, concentrated growth factor therapy, and botulinum toxin therapy) and surgical treatments (e.g. hair transplantation) [2]. There is no cure for hair loss. Therefore, finding a potent treatment for hair loss is urgently needed.

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TCM has a long history of treating hair loss, with a unique understanding of the etiology and mechanism of the disease and a variety of dialectical treatment modalities, such as internal treatment, external application, and acupuncture, with remarkable efficacy and small side effects. TCM believes that the main cause of hair loss is deficiency of the liver and kidney, damp-heat fumigation, wind heat and blood dryness, and wind and dryness due to blood deficiency, and the treatment is based on nourishing the liver and kidney, strengthening the spleen and dispelling dampness, cooling the blood and dispelling the wind and moistening the dryness, nourishing the blood and dispelling the wind and moistening the dryness [3]. Dendrobium officinale is the dried stem of the orchid plant, which has various pharmacological effects including promoting immunity, antioxidant, regulating blood sugar and lipid metabolism, modulating blood pressure, and suppressing bacteria, and has been utilized as a TCM material to treat diseases for many years in China and some Asian countries [4]. Dendrobium contains polysaccharides, alkaloids, amino acids and trace elements and many other pharmacological components that are beneficial to human health. Dendrobium officinale polysaccharide (DOP) is separated from the stem of Dendrobium, which is the key active compound of Dendrobium officinale, a valuable TCM. The monosaccharide composition of DOP obtained from different varieties, origins and extraction methods is obviously different. However, most DOP consisted mainly of glucose, mannose, and galactose according to different moles [5–7]. It has been reported by modern pharmacological studies that DOP has diverse pharmacological functions, including antioxidant, anti-aging, antifibrotic, and improved immunity [8,9]. Previous literature has reported that DOP promotes hair growth through the upregulation of VEGF mRNA expression [10]. Our previous study also demonstrated that DOP could enhance hair regeneration in testosterone-induced bald mice by upregulating the Wnt signaling pathway expression [11]. This study further investigated the therapeutic effects of DOP upon hair loss and its related mechanisms.

The hair papilla, a structure that induces hair follicle growth and regulates follicle development and regeneration, consists of extracellular matrix and fibroblasts and is entirely surrounded by the epithelial matrix in the lower portion of the hair follicle. Hair papillae exert a dominant effect on the morphogenesis of hair follicles and the regulation of follicular cycle growth. Dermal papilla cells (DPCs) are mesenchymal cells in the hair follicle that accelerate the follicle into the anagen phase by regulating various signaling molecular pathways [12,13]. DPCs exert a pivotal effect on hair follicle morphogenesis and the hair growth cycle, and if the loss of cells and their functions occurs, hair growth will be impeded [14]. Therefore, this paper investigated the novel mechanism of DOP in the treatment of hair loss by exploring the impact of DOP on the cell functions of DPCs.

Perifollicular vascularization is essential for hair growth, which begins within hair follicles and depends on perifollicular vascularization for the nutrients needed for hair growth; evidence has shown that sulfate derivatives of novel hetero-oxygenated glycans in Dendrobium officinale are associated with angiogenesis [15]. However, the effect of DOP on angiogenesis is not clear.

Herein, we examined the effects of DOP upon the biological functions of mouse antennal hair follicles, DPCs, and mouse vascular endothelial cells (MVECs), respectively, by culturing them in vitro. Mechanistically, DOP could activate the WNT signaling to advance tentacle hair follicle and DPC growth and promote neovascularization, which subsequently exerts a therapeutic effect on hair loss. This study delves into the underlying new mechanism of DOP in treating hair loss and provides a theoretical basis for the use of DOP in clinical treatment for hair loss.

2. Materials and methods

2.1. Isolation and culture of mouse tentacle hair follicles

Healthy male SPF grade 7-week-old C57BL/6J mice were procured from SLAC Laboratory Animal Company (Changsha, China). According to the methods of a previous article [16], mice were killed by decapitation after intraperitoneal anesthesia, and the tentacles were clipped bilaterally. Subsequently, the tentacle pads were obtained aseptically, and the skin of which was carefully removed using microscopic scissors. Individual hair follicles in the tentacle pads were isolated under a stereomicroscope, and intact hair follicles of mouse tentacles during the anagen phase were selected, washed twice with phosphate buffer saline (PBS), and cultured in a 24-well plate with Williams E media (A1217601, Thermo Fisher Scientific, Waltham, USA) containing 10 μg/mL insulin (Sigma-Aldrich, St. Louis, USA), 10 ng/mL hydrocortisone (Sigma-Aldrich), 100 IU/mL penicillin (Sigma-Aldrich), 100 mg/mL streptomycin (GIBCO, Grand Island, USA), and 1 mmol/L L-glutamine (GIBCO). DOP was added to the DOP-treated group at a final concentration of 100 μg/ml, and an equivalent amount of PBS was supplemented to the PBS group. Photographs were taken using a microscope on days 0 and 6 of treatment.

2.2. Cell culture

Mouse DPCs (CP-M312, Procell, Wuhan, China) were cultivated in DMEM/F12 (GIBCO) added with 10% fetal bovine serum (FBS, GIBCO) and penicillin-streptomycin. MVECs (C166, BNCC317467, BeNa, Beijing, China) were cultured in high glucose DMEM (GIBCO) supplemented with 10% FBS. All the cells were cultured under the optimal culture environment (37 °C; saturated humidity; 5% CO_2). The fluid was refreshed every two or three days, and cells were passaged at 1:2.

DPCs from the DOP group were treated for 48 h with 100 μg/ml DOP (SD9310, Solarbio, Beijing, China), and for the PBS group, an equal amount of PBS was added. The WNT pathway inhibitor IWR-1 (S7086, Selleck Chemicals, Houston, USA) was used at a final concentration of 2.5 μM, and the treatment time was 24 h.

2.3. Hematoxylin and eosin (H&E) staining

Paraffin-embedded slices were deparaffinized by xylene, rehydrated with increasing concentrations of ethanol, washed with PBS, and subjected to 10 min immersion within a hematoxylin staining solution. Subsequently, slices were immersed in tap water and washed, and then subjected to 5 s immersion in hydrochloric acid ethanol; slices were subjected to 2 min immersion in eosin staining after tap water washing, which was immersed in 70%, 80%, and 90% alcohols for 10 s after tap water washing, followed by 10 s immersion in anhydrous ethanol. Lastly, slices were cleared using xylene for 10 min and blocked with neutral resin, and next, the pathological changes of the tentacle hair follicle tissues in mice were observed under the microscope.

2.4. Immunofluorescence (IF) staining

DOP and PBS-treated DPCs were made into cell suspension, counted at a cell density of 1×10^5/mL, inoculated onto cell slides in 24-well plates, and IF staining was performed after the cells were attached to the plates for 12 h. In short, cells were subjected to 30 min fixing with 4% paraformaldehyde solution at room temperature (RT), rinsed thrice in PBS, and then added with 0.1% TritonX-100 solution for 20 min at RT. Subsequently, after three

washings with PBS, cells were blocked for 30 min within 10% goat serum at ambient temperature. Next, goat serum was discarded and primary antibody diluted with 10% goat serum (Zhongshan GoldenBridge, Beijing, China) was added: Cytokeratin 8 (17514-1-AP, 1: 200, Proteintech, Wuhan, China), Versican (MA5-27638, 1: 100, Thermo Fisher Scientific), and α-SMA (19,245, 1: 200, Cell Signaling Technology [CST], Danvers, USA), which was incubated at 4 °C overnight devoid of light. Cells were rinsed thrice with PBS, followed by 1 h incubation at 37 °C using Alexa 488-conjugated fluorescent secondary antibody diluted with 10% goat serum. Cells were stained using DAPI staining solution for 1 min at RT, and lastly, blocked with an anti-fluorescence quencher. Pictures were taken under a fluorescent inverted microscope (green light wavelength of 543 nm and blue light wavelength of 458 nm) at 200 x. The hair follicle tissue sections were dewaxed and hydrated. Then, the sections followed the same procedures described above. The primary antibody against CD31 (ab222783, 1:100, Abcam, Cambridge, USA) and Alexa 594-labeled secondary antibody was used.

2.5. Western blot

RIPA lysis solution (Beyotime, Shanghai, China) was employed to lyse cells for obtaining protein specimens. The BCA kit (Beyotime) was applied to measure the protein content. Next, after the addition of the corresponding protein volume into the sampling buffer (Beyotime) and the thorough mixture, a 5 min heating in a boiling water bath was conducted to denature protein. The proteins were subjected to electrophoresis (80 V, 30 min) until the bromophenol blue was migrated into the separation gel; next, a higher voltage (120 V) was used for 1–2 h. The membrane transfer was performed in an ice bath, with a current of 220 mA for 120 min. Subsequently, after 1–2min washing, membranes were blocked with 5% no-fat milk (Bio-Rad, Hercules, USA) in Tris-buffered saline (TBS) containing 0.05% Tween 20 for 60 min at RT, and then incubated overnight at 4 °C with the primary antibody (GAPDH [5174S, 1: 1000, CST], Ki-67 [ab16667, 1: 1000, Abcam], p-S6 [2211, 1: 1000, CST], CD31 [ab222783, 1: 2000, Abcam], VEGFA [ab214424, 1: 1000, Abcam], Cyclin D1 [ab16663, 1: 100, Abcam], MMP-3 [ab52915, 1: 5000, Abcam], β-catenin [9562, 1: 1000, CST], MMP-2 [ab92536, 1: 2000, Abcam], MMP-9 [ab283575, 1: 1000, Abcam], C-Myc [5605, 1: 1000, CST]) and incubated at RT with a secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, 1: 5000, Beijing ComWin Biotech Co., Ltd., Beijing, China). A chemiluminescent imaging system (Bio-rad) was utilized to perform detection after a drop of the developer to the membrane.

2.6. Alkaline phosphatase (ALP) staining

DPCs were stained using ALP staining solution (DE0004, LEAGENE, Beijing, China) according to the protocol of the manufacturer. After PBS washing, the DPCs in 6-well plates were subjected to 15-min fixing at RT with 4% paraformaldehyde. Next, after two washings in PBS, fixed cells were incubated for 15 min within appropriate amount of ALP staining solution at room temperature without light exposure, followed by 2 times of PBS washing and 2-min restaining with Nuclear Fast Red solution. Lastly, after washing with PBS, a microscope was applied to observe cells and capture images.

2.7. Toluidine blue staining

DPCs were stained using Toluidine Blue Staining Solution (G3660, Solarbio) as per the protocol of the manufacturer. After three washings in PBS, cells were dyed with an appropriate amount of toluidine blue staining solution for 5 min. After that, the culture plate was mixed after adding an equal amount of distilled water, and the staining was left to stand for 15 min at RT. The staining solution was removed. Next, after two washings in PBS, cells were submerged by adding the appropriate amount of PBS,then captured under the microscope.

2.8. Cell counting kit (CCK)-8 assay

The CCK-8 kit (Beyotime) was utilized under the manufacturer's protocols to evaluate cell viability. Single-cell suspensions of DPCs were prepared, counted, and then coated in 96-well plates at 5000 cells per well, with 100 μL of culture solution per well. After the cells were all attached to the plates, continue to culture the cells for 0h and 48h, 10 μL of CCK-8 solution was supplemented to each well, and then incubated for 2 h at 37 °C. A microplate reader (Bio-Rad) was then applied to measure the absorbance at 450 nm [17].

2.9. 5-ethynyl-2′-deoxyuridine (EdU) staining

EdU working solution (Ribobio, Guangzhou, China) was added to the 24-well plate at a ratio of 1: 2,000, followed by 2 h continued incubation of the cells. Following EdU labeling of the cells, the culture medium was removed, and 1 ml of 4% paraformaldehyde was supplemented and fixed for 15 min at RT. Subsequently, cells were rinsed thrice using 1 ml of PBS per well (for 3–5 min each time). Afterward, cells were subjected to 5 min incubation with 50 μL 2 mg/mL glycine solution and 15-min incubation with 0.3% Triton X-100 at ambient temperature. Subsequently, 100 μL 1 × Apollo Reaction Buffer was supplemented to the wells, followed by 30 min incubation at RT in the dark. Nuclei were stained with hoechst and protected from light for 10 min at RT, and cells were rinsed thrice with a washing solution, each time for 3–5 min. The proliferating cells were observed to be red fluorescent and the nuclei were blue fluorescent under the fluorescence inverted microscope [18].

2.10. Lactate dehydrogenase (LDH) determination

LDH Activity Assay Kit (E-BC-K046-M, Elabscience, Wuhan, China) was used to detect LDH levels in DPCs after DOP (100 μg/ml) or PBS treatment for 48 h as per the protocol of the manufacturer and previous research [19]. About 1.0×10^6 cells were added with 300 μL PBS (0.01 M, pH 7.4) for mechanical homogenization or sonication. The cell homogenates were subjected to 10 min centrifugation at 4 °C and 10,000×*g*, and the supernatant was put on ice to be measured. A portion of the supernatant was retained for protein concentration determination. The reagents were added and mixed as per the instructions, and left to stand for 5 min at RT, after which, a microplate reader was applied to measure the absorbance of each well at 450 nm. LDH activity (U/gprot) = $(\Delta A^{450} - b) \div a \times f \div C_{pr} \times 1000$*.

2.11. Tube-forming experiment

Matrigel was pre-coated into 96-well plates at 10 mg/ml, 0.05 ml per well, followed by 30 min incubation at 37 °C for solidifying. MVECs treated with DOP (100 μg/ml) or PBS for 48 h were spread in matrigel-coated 96-well plates at 10,000 per well, and were routinely cultured for 4–6 h. The growth and tube formation of MVECs in each well were photographed and counted using a microscope in a bright field [20].

2.12. Scratch test

MVECs after DOP (100 μg/ml) or PBS treatment for 48 h were spread evenly in small wells of six-well plates to guarantee the

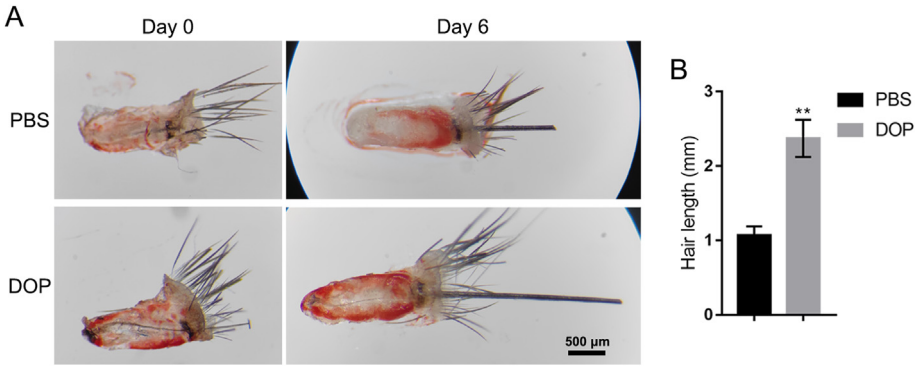

Fig. 1. DOP advances hair follicle growth. A. Freshly isolated tentacle hair follicle tissues of C57BL/6 mice were subjected to 100 μg/ml DOP treatment with the same volume of PBS as a control, and the gross morphological structure of the hair follicles was observed under the microscope on days 0 and 6, respectively. Scale bar = 500 μm. B. Quantitative analysis of tentacle growth length in mice. N = 6 (biological replicates). $^{**}P < 0.01$ compared to PBS group.

same quantity of cells within each well. Upon 100% confluence, a scratch was made smoothly in the middle of the wells with a sterilized 100 μl tip vertical to the plane of the six-well plate. PBS was used to remove the cells scattered by the scratch, and then added with serum-free media and incubated at 37 °C within an incubator with 5% CO_2, photographed, labeled, and recorded. A microscope was employed to observe the scratches at the same location, and then photograph and record at 0 h and 48 h, respectively. The changes in the width of the recorded scratches were statistically analyzed.

2.13. Transwell assay

MVECs treated with DOP (100 μg/ml) or PBS for 48 h were collected, and an FBS-free media was applied to dilute cells in the groups to the same concentration. Sterile Transwells were placed

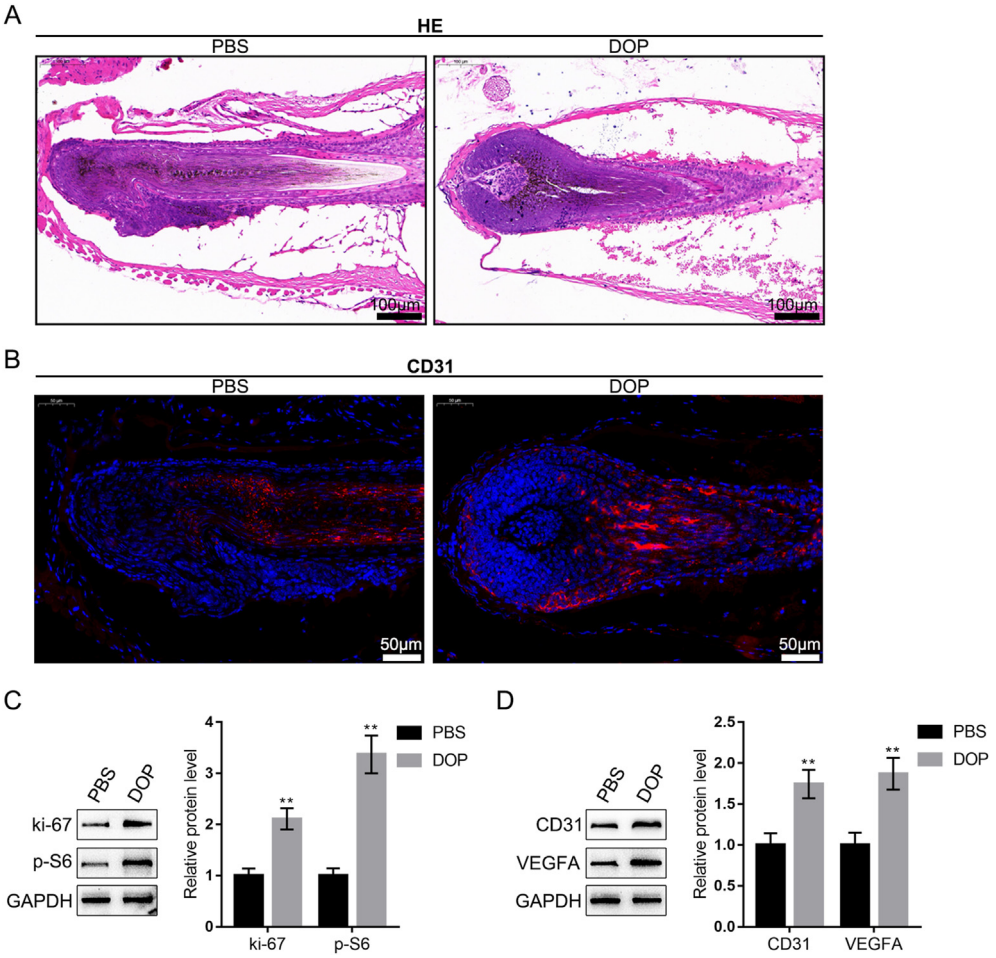

Fig. 2. DOP enhances the expression of vascularization and proliferation factors in tentacle follicles. The tentacle hair follicles of mice were treated for 6 days with DOP and its control PBS. A. The pathological structural changes of hair follicles were observed by HE staining. Scale bar = 100 μm. B. CD31 expression within hair follicles was evaluated using Immunofluorescence staining. Scale bar = 50 μm. C-D. Western blot was conducted to determine the protein expression levels of proliferation factors Ki-67 and p-S6 (C) and vascular growth factors CD31 and VEGFA (D) in hair follicles. N = 6 (biological replicates). $^{**}P < 0.01$ compared to PBS group.

into 24-well plate wells and the chambers were coated using Matrigel (1:8, CLS356234, Corning Incorporated, Corning, USA). Afterward, 100 μl of cell suspension of equal concentration was supplemented to the top chamber and 800 μl of complete media containing 10% FBS was supplemented to the bottom chamber and kept in the cell culture incubator. 24 h later, the Transwell was taken out, followed by 20 min staining with a crystal violet staining solution. Five randomly -selected fields of view were photographed, and Image J was utilized to statistically analyze cell numbers [21].

2.14. Statistical analysis

The cell experiments were performed for three biological replicates. The animal experiments were performed for six biological replicates. All data were expressed as mean ± standard deviation, and GraphPad Prism 8.0 software was employed to process data. Comparisons among groups were assessed using one-way ANOVA followed Tukey's post hoc test. Comparisons between two groups were assessed using the *t*-test. The threshold for statistical significance was $P < 0.05$.

3. Results

3.1. DOP promotes hair follicle growth

Tentacle skin tissues of C57BL/6 mice were first bluntly isolated, and hair follicles within the anagen phase and structurally intact were screened from the isolated tissues for observation. After removing the connective and dermal tissues, the hair follicle could be observed with the naked eye as a long oval shape with a transparent connective tissue sheath wrapped around the outer

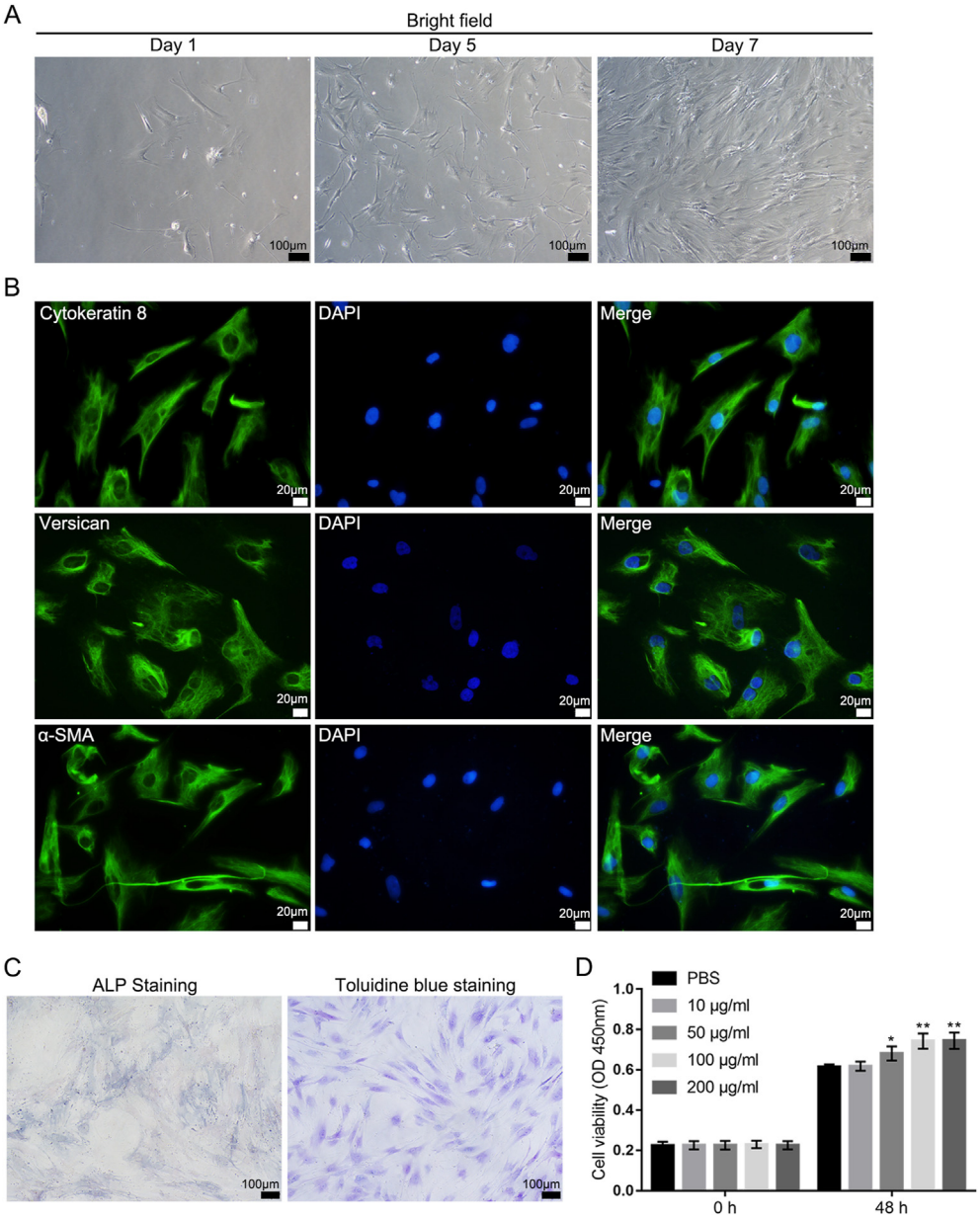

Fig. 3. DOP strengthens the viability of DPCs. A. Cell morphology of DPCs cultured at day 1, 5, and 7 days was observed under the microscope. Scale bar = 100 μm. B. Cytokeratin 8, Versican and α-SMA expression levels within DPCs were determined by immunofluorescence staining. Scale bar = 20 μm. C. ALP levels and the active level of cell proliferation in DPCs were evaluated by ALP staining and toluidine blue staining. Scale bar = 100 μm. D. CCK-8 assay was performed to measure the viability of DPCs subjected to treatments with various concentrations of DOP (10, 50, 100, and 200 μg/ml). N = 3 (biological replicates). *$P < 0.05$, **$P < 0.01$ compared to PBS group.

layer, which was not easy to separate and destroy. There were typically 2 hairs growing in each follicle, with the hair shaft being relatively thin and short and the pestle hairs being thick and long in shape (Fig. 1A, left). After 6 days of treatment with 100 μg/ml DOP, treatment with DOP significantly promoted tentacle growth than those with PBS treatment (Fig. 1A, right, and Fig. 1B). This result suggests that DOP significantly enhances hair follicle growth in mice.

3.2. DOP enhances the expression of vascularization and proliferation factors in tentacle follicles

Further observation of mouse tentacle hair follicle tissues using HE staining enabled clear visualization of the organizational structures of the hair bulb, dermal sheath, external root sheath, inner root sheath, and hair shaft. Compared with the PBS-treated hair follicles, those treated with DOP entered the anagen phase faster, the bulbous part was round-like and increased in size, and its hair papilla was significantly larger (Fig. 2A). CD31 and VEGFA expressed in vascular endothelial cells are commonly used to assess the status of angiogenesis, which is closely related to hair growth [22]. Meanwhile, the hair follicle transition from the resting phase to the anagen phase of the cyclic cycle is accompanied by a large amount of cell proliferation [23]. Besides, Ribosomal S6 protein is one of the important regulators of downstream signaling of the mTOR pathway, and activation of the mTOR signaling can activate p-S6 which in turn promotes cell proliferation and provides an anti-apoptotic environment for cells [24]. Thus, we examined the effect of DOP upon the expression of vascularization and proliferation factors within mouse tentacle hair follicles. IF staining and Western blot demonstrated DOP treatment markedly upregulated the protein expression levels of angiogenesis-related factors CD31 and VEGFA (Fig. 2B–D) and proliferation factors Ki-67 and p-S6 (Fig. 2C).

3.3. DOP strengthens the viability of DPCs

DPCs are mesenchymal cells within hair follicles and contribute to modulating hair follicle cycle growth. Therefore, we further investigated the impact of DOP on the function of DPCs. After DPCs were cultured for 1, 5, and 7 days, microscopic observation showed that the cell morphology was long shuttle or polygonal, with abundant cytoplasm, swirling agglutinative growth of cell aggregates, and tight intercellular junctions (Fig. 3A). IF staining demonstrated strong positivity for DPCs-associated markers, including Cytokeratin 8, Versican and α-SMA (Fig. 3B). Meanwhile, the results of ALP staining and toluidine blue staining were positive, indicating that the DPCs were in good growth status and active proliferation (Fig. 3C). DPCs were subjected to treatments with various concentrations of DOP (10, 50, 100, and 200 μg/ml), and CCK-8 assay was carried out after 0 h and 48 h of culture to measure cell viability, which revealed that the gradient concentration of DOP significantly promoted the viability of DPCs. This facilitation was no longer significant at concentrations above 100 μg/ml, and therefore, 100 μg/ml of DOP was selected for subsequent experiments (Fig. 3D).

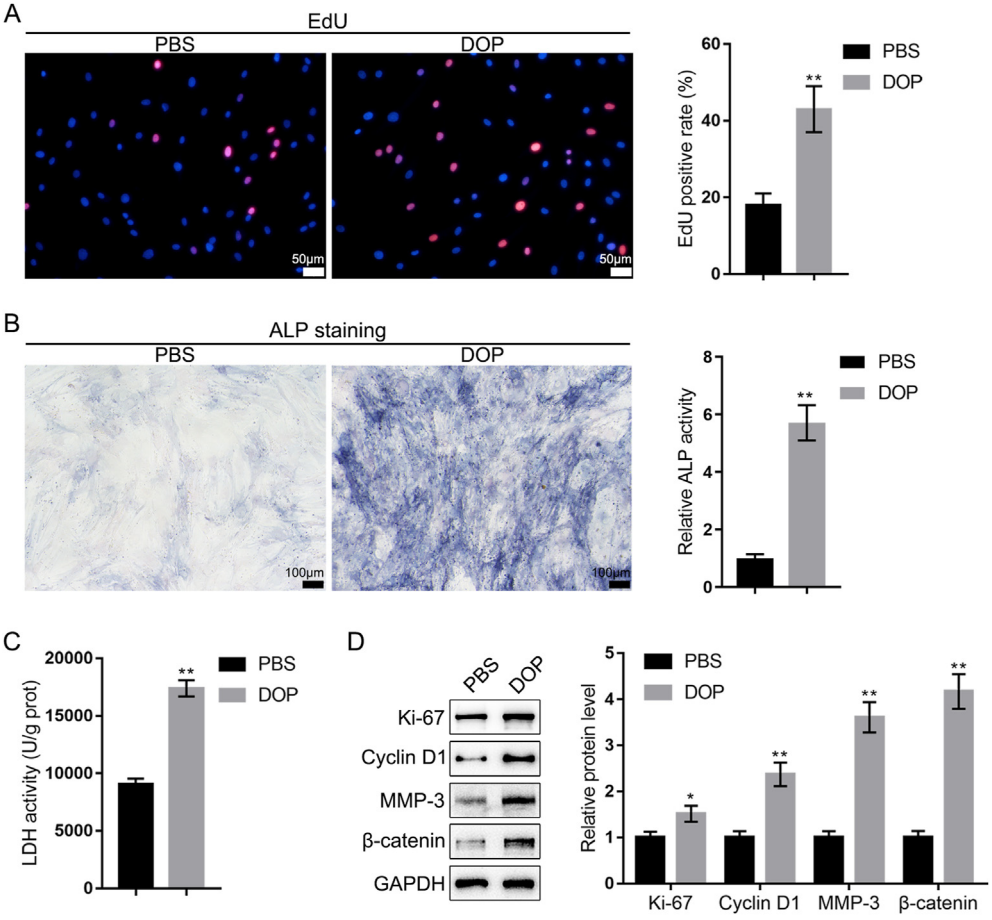

Fig. 4. DOP promotes DPC cell proliferation by activating the WNT signaling pathway. DPCs were subjected to 48-h treatment with 100 μg/ml of DOP. A. DPC proliferation was assessed using EdU staining. Scale bar = 50 μm. B. ALP expression levels in DPCs were measured by ALP staining. Scale bar = 100 μm. C. LDH activity in DPCs was evaluated by LDH kit. D. Ki-67, Cyclin D1, MMP-3, and β-catenin proteins within DPCs were detected using Western blot. N = 3 (biological replicates). *$P < 0.05$, **$P < 0.01$ compared to PBS group.

3.4. DOP promotes DPC cell proliferation by activating the WNT signaling pathway

The effects of DOP upon DPC proliferation and viability and its underlying mechanism were further examined. When DPCs were subjected to 48-h treatment with 100 μg/ml of DOP, the changes in cell proliferation were detected by EdU assay, which showed that DOP treatment notably increased the number of EdU-positive cells, demonstrating that DOP can significantly advance cell proliferation (Fig. 4A). ALP is constantly expressed in DPCs during the hair growth cycle and is considered as a viability marker for DPCs [25]. DOP was found to significantly enhance the ALP activity in DPCs, as revealed in ALP staining (Fig. 4B). LDH is an important enzyme involved in glycolysis and gluconeogenesis, and as a key enzyme in glycolysis, it is able to promote hair follicle stem cell activity and induce hair follicle growth [26]. LDH levels in DPCs, determined by the LDH kit, unveiled that DOP treatment could upregulate LDH levels in DPCs (Fig. 4C). This suggests that DOP may promote the growth of hair follicles by affecting glucose metabolism.

In addition, the influence of DOP upon the expression levels of proliferation-associated proteins and proteins involved within the WNT signaling pathway was examined. It was revealed by Western blot that DOP treatment could markedly elevate the protein levels of Ki-67, MMP-3, β-catenin, and Cyclin D1. These results collectively suggest that DOP may subsequently trigger cell proliferation in DPCs by activating the WNT signaling pathway and its downstream LDH and Cyclin D1.

3.5. DOP enhances the angiogenesis, migration, and invasion ability of MVECs

Hair renewal involves changes in microvessel formation, and hair loss is strongly associated with reduced microvascular formation in hair follicles [27]. Based on this, we examined the effect of DOP on the biological functions of MVECs at the cellular level. MVECs were treated for 48 h with 100 μg/ml of DOP, and the capabilities of tubule formation and cell migration and invasion were detected. The results were consistent with the expectation

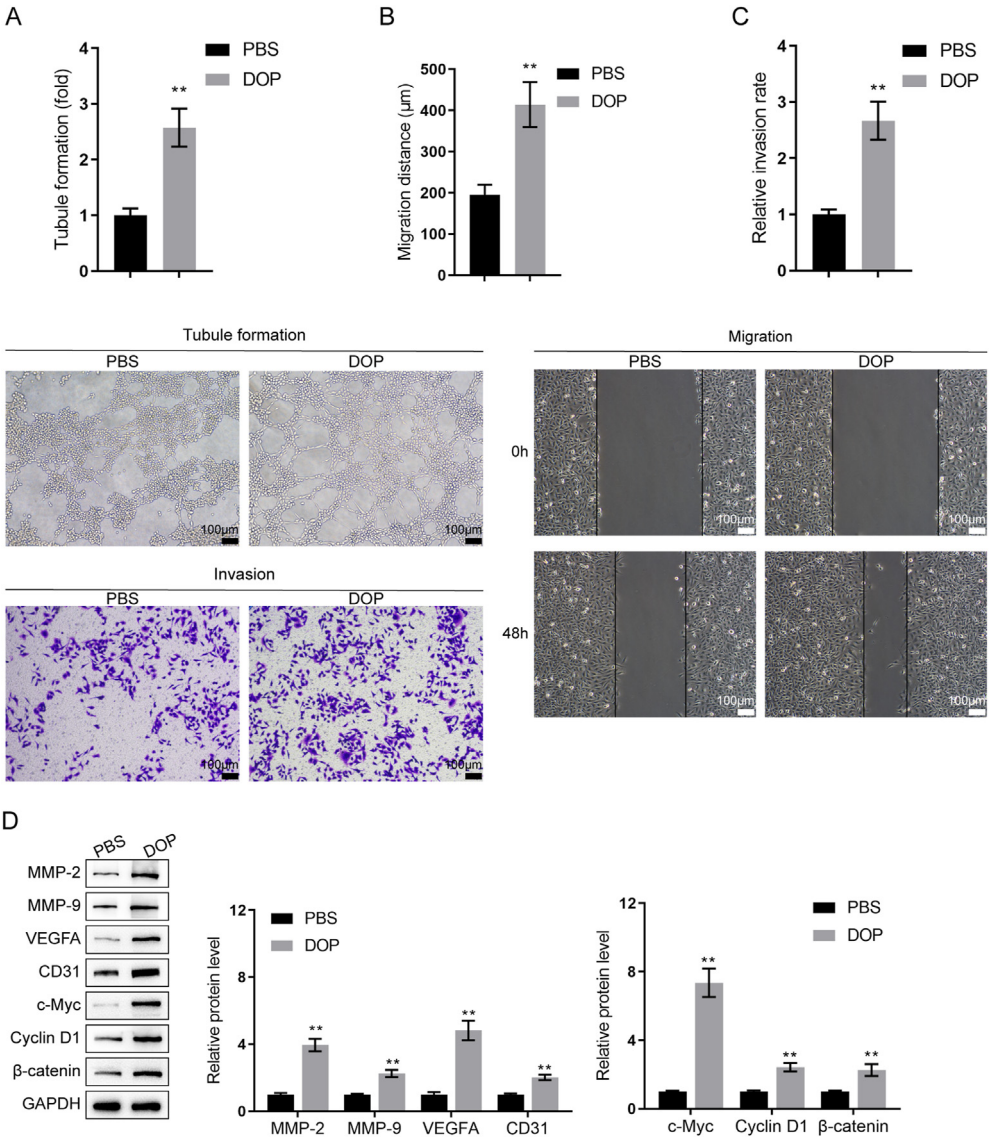

Fig. 5. DOP enhances the angiogenesis, migration, and invasion ability of MVECs. MVECs were subjected to 48-h treatment with 100 μg/ml of DOP. A. The tubule formation assay was carried out to examine MVECs vascularization level. B. The migration ability of MVECs was detected using Scratch test. C. MVECs invasion was examined using Transwell assay. D. Western blot was implemented to detect the expression levels of key proteins of angiogenesis (MMP-2, MMP-9, VEGFA, and CD31) and proteins associated with the WNT pathway (C-Myc, Cyclin D1, and β-catenin). All scale bar = 100 μm. N = 3 (biological replicates). *$P < 0.05$, **$P < 0.01$ compared to PBS group.

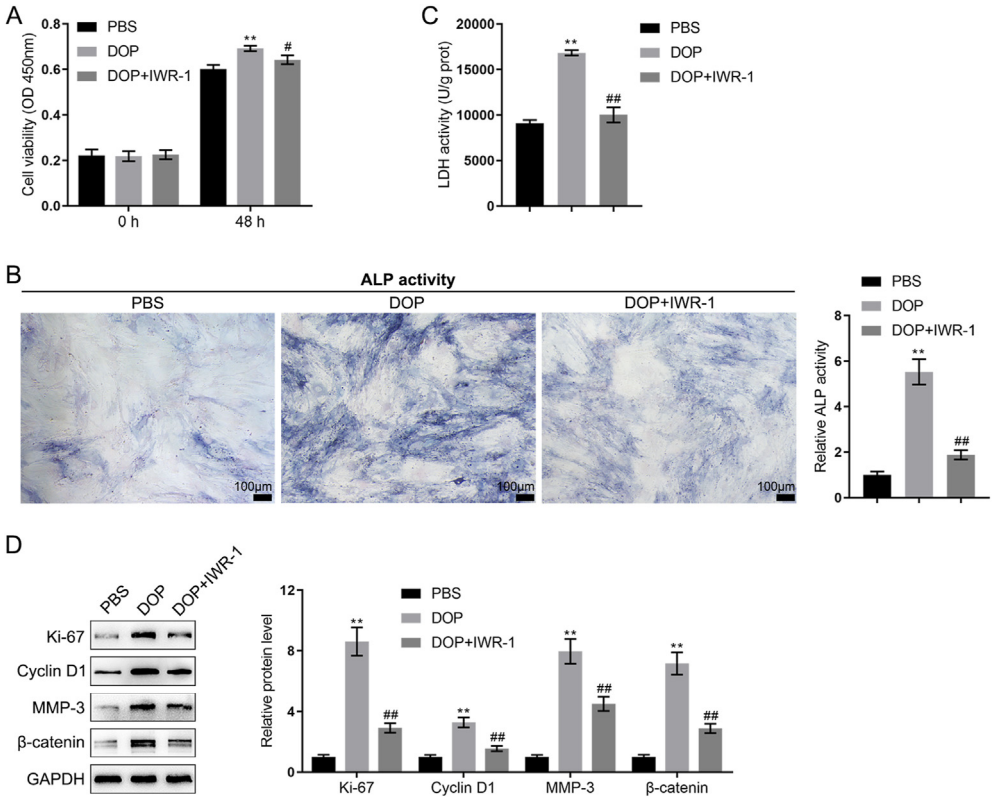

Fig. 6. WNT pathway inhibitor reverses the effects of DOP in DPCs. After treating DPCs with 100 μg/ml DOP for 24 h, 2.5 μM IWR-1 was given for a total of 24 h. A. CCK-8 assay was conducted to assess DPC viability. B. ALP expression levels in DPCs were measured by ALP staining. Scale bar = 100 μm. C. LDH activity in DPCs was evaluated by LDH kit. D. Ki-67, Cyclin D1, MMP-3, and β-catenin protein levels within DPCs were detected using Western blot. N = 3 (biological replicates). $^{**}P < 0.01$ compared to PBS group; $\#P < 0.05$; $\#\#P < 0.01$ compared to DOP group.

that DOP treatment significantly promoted the tubule-forming (Fig. 5A), migratory (Fig. 5B), and invasive (Fig. 5C) abilities of MVECs. Further exploration of the regulatory mechanism demonstrated that DOP treatment could notably upregulate the expression levels of the key angiogenic proteins MMP-2, MMP-9, VEGFA, and CD31 within MVECs. Consistent with DPCs, DOP also upregulated the levels of β-catenin, a key WNT signaling protein, and WNT target proteins Cyclin D1 and C-Myc in MVECs (Fig. 5D). These results imply that DOP may advance the angiogenesis, migratory and invasive properties of MVECs via activating WNT signaling pathway.

3.6. WNT pathway inhibitor reverses the effects of DOP in DPCs

Based on the aforementioned experimental results, we hypothesized that DOP may induce DPC proliferation and viability through activating the WNT signaling; therefore, DPCs were then subjected to treatment with the WNT pathway inhibitor IWR-1 to verify this conjecture. After treating DPCs with 100 μg/ml DOP for 24 h, 2.5 μM IWR-1 was given for a total of 24 h. The results disclosed that, as expected, IWR-1 significantly reversed the promotional effects of DOP treatment on DPC cell viability (Fig. 6A), ALP activity (Fig. 6B) and LDH levels (Fig. 6C). Additionally, as shown by Western blot, IWR also attenuated the enhancing effect of DOP treatment upon cell proliferation-related proteins Ki-67, MMP-3, and the key protein of WNT pathway β-catenin and WNT target protein Cyclin D1 (Fig. 6D). To conclude, DOP can exert a promotional effect on DPC proliferation and viability by activating the WNT signaling, which can be effectively reversed by the WNT pathway inhibitor IWR-1.

3.7. WNT pathway inhibitor reverses the effects of DOP in MVECs

Furthermore, we examined the influences of IWR-1 on the biological functions of MVECs using IWR-1. DOP and IWR-1 treatment concentrations and times were as before. The results of tubule formation, scratch test and Transwell assays showed that, as expected, IWR-1 significantly reversed the promotional effects of DOP on the tube-forming ability, migration and invasion of MVECs (Fig. 7A–C). In the meantime, IWR-1 notably reversed the upregulation of DOP upon the levels of key angiogenic proteins MMP-2, MMP-9, VEGFA, and CD31; IWR-1 also downregulated the levels of β-catenin, a crucial protein of the WNT signaling, and the WNT target proteins Cyclin D1 and C-Myc in MVECs (Fig. 7D). These results reveal that DOP can exert a promotional effect on MVECs tube-forming capacity, migration and invasion through activation of the WNT signaling pathway, an effect that can be effectively reversed by WNT pathway inhibitors.

4. Discussion

The mouse antennae are rich in blood sinus cavities and nerve endings inside, and are able to connect precisely to the cerebral cortex, which is an important tactile organ in mice. Tentacles are thick in shape and hard in texture, and are widely used in hair research because of their features such as large size and ease of separation, making them a typical model for studying hair follicle function, molecular influences, and hair follicle regeneration. The most important function of the hair follicle is to grow hair, and it has a unique development mechanism and growth pattern. In this paper, we observed that DOP treatment was effective in boosting

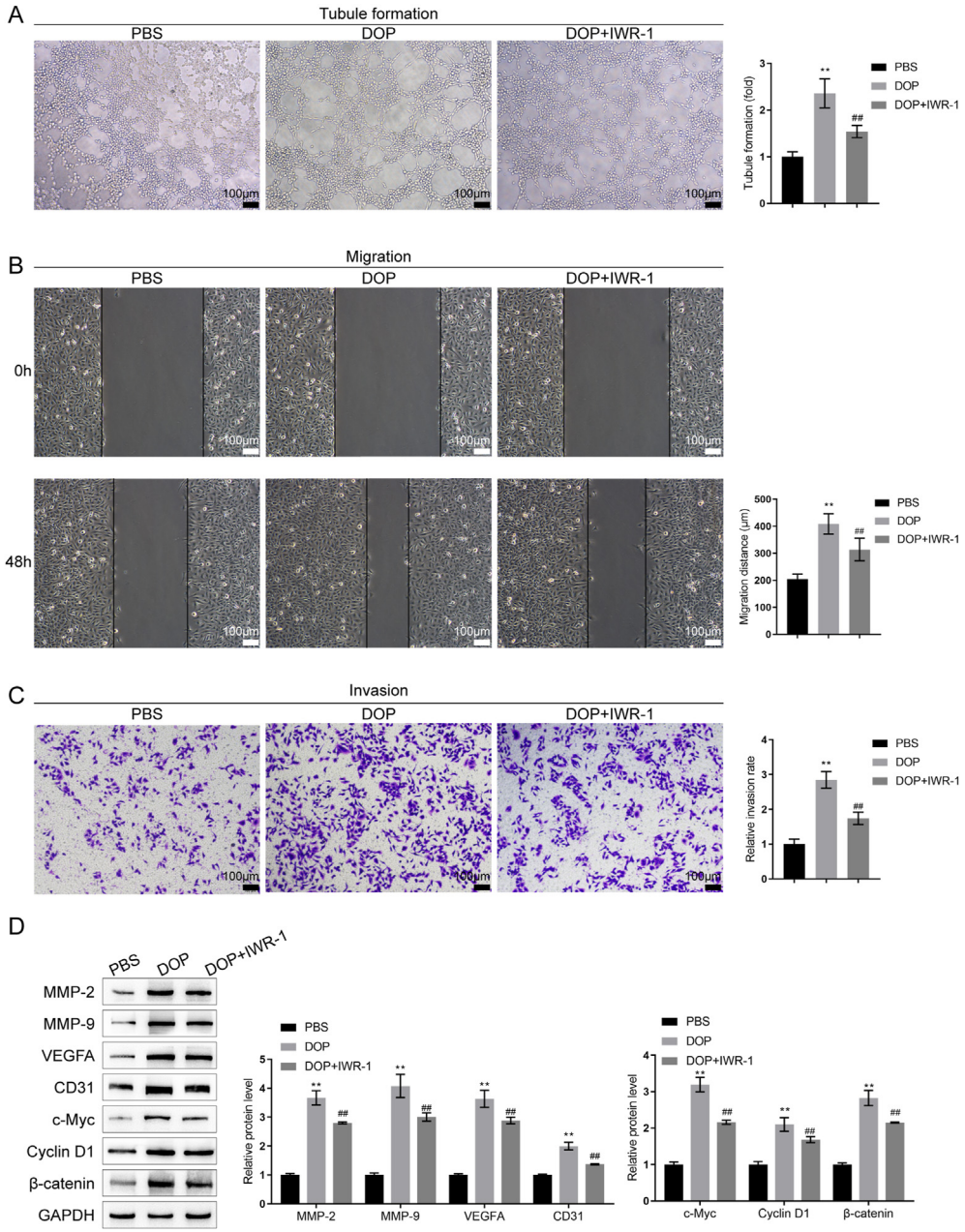

Fig. 7. WNT pathway inhibitor reverses the effects of DOP in MVECs. A. The level of vascularization of MVECs was examined by tubule formation assay. B. Scratch test was utilized to detect the migration ability of MVECs. C. MVECs invasion was examined using Transwell assay. D. The expression levels of key proteins of angiogenesis (MMP-2, MMP-9, VEGFA, and CD31) and proteins related to the WNT signaling pathway (C-Myc, Cyclin D1, and β-catenin) were determined using Western blot. All scale bar = 100 μm. N = 3 (biological replicates). $^{**}P < 0.01$ compared to PBS group; $^{\#\#}P < 0.01$ compared to DOP group.

the growth of hair follicles in mouse antennae, which further validated and complemented the function and potential mechanism of DOP's ability to induce hair growth in mice [10], suggesting that DOP has the potential to be an effective drug for promoting hair follicle growth.

In this research, we found that DOP treatment advanced the growth of hair follicles in mouse antennae, further validating the ability of DOP to promote hair growth in mice. Hair growth is associated with perifollicular angiogenesis, and perifollicular angiogenesis is very active during the growth phase [28]. The interaction of VEGFA with its receptor, MMP-2/MMP-9, exerts crucial effect on angiogenesis [29,30]. This study, for the first time, demonstrated that DOP dramatically increased the expression levels of vascularization factors CD31 and VEGFA in mouse tentacle hair follicles. Meanwhile, DOP markedly elevated MMP-2 and MMP-9 protein contents, and also promoted angiogenesis, migration and invasion of MVECs, signifying its potential to enhance angiogenesis.

Hair growth regulation is a complex process that involves a variety of signaling pathways in the skin and hair follicles, but the Wnt/β-catenin signaling exerts a critical effect on hair follicle development as well as hair growth during the embryonic stage and adulthood [31]. As previously reported, the removal of β-catenin leads to a significant reduction in its target genes, which leads to reduced cell proliferation, rapid decline in the anagen phase of hair follicles, and ultimately, hair thinning [32]. As the mesenchymal component of the hair follicle, DPCs have the most fundamental role in promoting anagen and hair growth. In this study, we

treated DPCs with DOP to investigate the potential mechanism via which DOP promoted hair follicle development. Consistent with expectations, DOP was able to notably induce the proliferation and viability of DPCs via activating the WNT signaling.

LDH, a pivotal enzyme in glycolysis, induces hair follicle development [26]. Based on this, we examined the impact of DOP on LDH levels and, as expected, DOP significantly upregulated LDH levels, implying that DOP may promote hair follicle growth by modulating LDH. The c-Myc gene, as an upstream regulatory gene of LDH, can modulate the expression of LDH, thereby inducing the hair follicle cycle and triggering hair follicle regeneration [26]. Besides, c-Myc is also a downstream target gene of the β-catenin-TCF/LEF complex within the WNT signaling. Thus, herein, DOP was hypothesized to activate the WNT signaling and upregulate the target genes of this pathway, thereby elevating the expression of LDH and ultimately, playing a promotional role in hair follicle growth.

5. Conclusion

In conclusion, the effect of DOP on hair follicle development and its potential mechanism is deeply investigated at the in vitro level in this study, and DOP can promote angiogenesis and hair follicle development via activating the WNT signaling, which provides new perspectives and theoretical basis for the clinical use of DOP in treating hair loss.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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