

# Expression profile analysis of dermal papilla cells mRNA in response to WNT10B treatment

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**Abstract.** Dermal papilla cells (DPCs) are associated with the development of hair follicles (HFs) and the regulation of the hair growth cycle. Previous studies have shown that Wnt family member 10B (WNT10B) plays an important role in the proliferation and survival of DPCs *in vitro*, and promotes the growth of HFs. However, the underlying mechanisms have not been fully elucidated. The present study evaluated the role of WNT10B in regulating HF morphogenesis by characterizing the differential gene expression profiles between WNT10B-treated DPCs and control DPCs using RNA-sequencing (RNA-seq). A total of 1,073 and 451 genes were upregulated and downregulated, respectively. The RNA-seq data was subsequently validated by reverse-transcription quantitative PCR. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that 442 GO terms and 21 KEGG pathways were significantly enriched. Further functional analysis revealed that WNT10B decreased translation initiation, elongation and termination, and RNA metabolic processes in cultured DPCs compared with controls *in vitro*. Human signaling networks were compared using pathway analysis, and treatment of DPCs with WNT10B was revealed to downregulate the ribosome biogenesis pathway and decrease protein synthesis *in vitro*. KEGG pathway analysis showed that WNT10B upregulated the phosphoinositide 3-kinase/protein kinase B signaling pathway. The present study analyzed the expression of mRNA in WNT10B-treated DPCs using next-generation sequencing and uncovered mechanisms regulating the induction of HFs.

## Introduction

In adults, hair follicles (HFs) undergo cycles of growth, quiescence and regeneration (1). Human dermal papilla cells (DPCs) are mesenchymal cells located in the hair bulb of HFs, and are associated with the development and periodic growth of HFs (2-6). Additionally, DPCs serve an important role in the formation of HFs (7,8). Previous studies have demonstrated that the introduction of exogenous DPCs into the follicles induced the formation of new HFs in mice (9-12). Furthermore, DPCs have been investigated as potential cell therapy for hair loss (13,14). However, previous studies demonstrated that DPCs lose their stemness and inductive ability during *in vitro* culture (7,12).

According to previous reports, DPCs rapidly lose their inductive ability when cultured in AmnioMAX™-C100 medium (Thermo Fisher Scientific, Inc.) *in vitro*, and require the addition of chemical factors such as bone morphogenic protein 6 and Wnt3a to maintain their hair-inductive properties (15). The Wnt signaling pathway was demonstrated to be involved in the maintenance of the intrinsic properties in hair follicle morphogenesis and cycling of cultured DPCs *in vitro* (16). The Wnt signaling pathway is associated with the repair of several tissues, including skin and HFs, and was demonstrated to serve a regulatory role in morphogenesis during embryogenesis, the growth of various tissues, the maintenance of stem cells and the occurrence of tumors (17-19). Among the Wnt family, Wnt family member 10B (WNT10B) was demonstrated to be associated with the proliferation and the maintenance of DPCs *in vitro* (20-24). Moreover, adenovirus-mediated WNT10B overexpression was shown to induce HF regeneration *in vivo* (25-29). WNT10B is one of the earliest and most determinate markers during the embryonic stage of HF substrate formation (7,11,12) and promotes the differentiation of epithelial cells and the development of HFs (30). Furthermore, WNT10B promoted the development of HFs during long-term *in vitro* culture (4,7,11-12,29,31-32). However, the mechanisms linking WNT10B, HF formation and the inductive ability of DPCs have not been fully elucidated. As the downstream target genes of the Wnt signaling pathway, the  $\beta$ -catenin/TCF/LEF transcription family are expressed in epithelial and mesenchymal cells at the early morphogenetic stages of HF development and in post-natal HF stem cells (33). Additionally,  $\beta$ -catenin activity regulates the regeneration of hair (15,20,33-37).

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The aim of the current study was to investigate the effect of WNT10B on the DPC transcriptome using mRNA sequencing. Progress in this area of research may facilitate the development of novel and effective therapeutic approaches for HF regeneration dysfunction in alopecia.

## Materials and methods

**Cells and reagents.** HFDPCs isolated from human dermis originating from the lateral scalp were purchased from PromoCell GmbH. Cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.; 10099-14-FBS) and 1% Penicillin-Streptomycin Solution (E607011, Sangon Biotech Co., Ltd.), at 37°C and 5% CO<sub>2</sub>. Human recombinant WNT10B protein was purchased from R&D Systems (cat. no. 7196-WN). The WNT10B treatment conditions used in the present study were as previously described (38). WNT10B protein was reconstituted in PBS containing 0.1% bovine serum albumin (BSA; Sangon Biotech Co., Ltd.; A602440) to form a solution of 10 µg/ml and used at a final concentration of 1 µg/ml. The expression of the Wnt target gene  $\beta$ -catenin in DPCs was increased following treatment with 1 µg/ml WNT10B protein for 3 days at 37°C, which was in agreement with the results of a previous study (25) indicating that the culture conditions were appropriate.

For RNA-sequencing (RNA-seq), the DPCs were divided into two groups: i) The experimental group, in which DPCs were cultured in DMEM supplemented with 10% FBS and 1 µg/ml recombinant WNT10B for 3 days at 37°C; and ii) the control group, in which DPCs were cultured in DMEM containing 10% FBS and 1 µg/ml BSA for 3 days at 37°C.

**RNA extraction and sequencing.** Total RNA from WNT10B-treated and control DPCs was extracted using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The total RNA quantity and purity were analyzed using Bioanalyzer 2100 (Agilent Technologies, Inc.) and RNA 6000 Nano LabChip Kit (Agilent Technologies, Inc.). RNA samples with an RNA integrity number >7.0 were used for subsequent experimentation. The Illumina TruSeq RNA Sample Preparation kit (Illumina, Inc.) was used to construct sequencing libraries. Sequencing was performed using an Illumina HiSeq 2500 Sequencing system (Illumina, Inc.) by Shanghai YingBiotech Co., Ltd. Each group was analyzed in triplicate.

**Mapping and identification of differentially expressed genes (DEGs).** Prior to read mapping, the raw sequencing data were analyzed using FAST-QC, which assessed the nucleotide quality distribution, PCR duplication rate, position-specific sequencing quality, k-mer frequency and GC content. The clean reads were mapped to the human genome (GRCH37). Then the aligned clean read number was further normalized to reads per kilo of per million mapped reads (RPKM) with RSEM software (version 1.2.3). Bioconductor DESeq2 version 1.12.3 (<https://www.rdocumentation.org/packages/DESeq2>) was used to identify DEGs using a fold-change (FC) >2 for significant upregulation or downregulation and a false discovery rate (FDR) <0.05. A volcano plot was drawn according to the analysis of the DEGs.

**Gene Ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.** GO ([www.geneontology.org](http://www.geneontology.org)) analysis was performed to identify the biologic implications of the DEGs. Fisher's exact test was used to identify the significant GO terms with FDR-adjusted P-values. KEGG pathway analysis was performed to identify biologically important pathways associated with the DEGs. Fisher's exact test was used to select the significant pathways based on P-values (P<0.05) and FDR (FDR<0.27).

**Reverse-transcription quantitative PCR (RT-qPCR).** To verify the results obtained from RNA-seq, seven highly expressed and enriched DEGs with high FCs (FC >2 or FC <0.5), including ribosomal protein L17 (RPL17), ribosomal protein L39 (RPL39), Rho associated coiled-coil containing protein kinase 2 (ROCK2), cytoplasmic FMR1 interacting protein 2 (CYFIP2), fibroblast growth factor 14 (FGF14), protein phosphatase 2 regulatory subunit B $\epsilon$  (PPP2R5E) and vascular endothelial growth factor B (VEGFB), were selected for RT-qPCR. Total RNA was extracted from HFDPCs with TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Approximately 5 µg total RNA from each sample was used for RT, which was performed using PrimeScript™ RT Master mix (Takara Biotechnology Co., Ltd.) following the manufacturer's instructions. qPCR was subsequently performed using the One Step TB Green™ PrimeScript™ PLUS RT-PCR kit (Takara Biotechnology Co., Ltd) and the 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR program was: 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. Gene expression was quantified using the 2<sup>- $\Delta\Delta C_q$</sup>  method (39) and normalized to the expression of the internal control GAPDH. Each reaction was performed in triplicate. The primers used for qPCR are presented in Table I.

**Statistical analysis.** All data are expressed as mean  $\pm$  standard error of the mean. The statistical analyses were performed using GraphPad Prism software (version 6; GraphPad Software, Inc.). A two-tailed Student's t-test was used to evaluate statistical significant differences. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Analysis of transcription sequencing of WNT10B-treated DPCs.** In order to identify the differential expression of mRNA in DPCs following WNT10B treatment, DPCs were divided into the experimental and control groups. An mRNA library was constructed for each group and subjected to Illumina mRNA deep sequencing. As presented in Table II, 95.37% of 51.5 million reads from the WNT10B-treated group and 93.78% of 52.6 million reads from the control group remained following filtering and quality control.

**Hierarchical clustering of global gene expression.** RPKM analysis was performed to evaluate the differential mRNA expression between WNT10B-treated and control DPCs. Using FC >2 and P<0.05 as the cut-off criteria, a total of 1,525 DEGs were identified, of which 1,074 were upregulated and 451 were downregulated following treatment with WNT10B (Table SI).

Table I. Primer sequence for reverse-transcription quantitative PCR.

Gene	Primer sequences	
	Forward	Reverse
RPL17	5'-AGCCTGAGGTGATCTGTGAAAAT-3'	5'-CGAGTGTTATTTTCGTGGGGTT-3'
RPL39	5'-GCCTTCTAAGCTCGTTCCTCCG-3'	5'-CGAGCAGCGGAGTCAAGAACA-3'
ROCK2	5'-GCAGAAGTGGGTTAGTCGGTTG-3'	5'-GGCAGTTAGCTAGGTTTGTGG-3'
CYFIP2	5'-CCTTAAACCAGCCACTACCTCTC-3'	5'-TCTGTATTCTGCACTCATCCGC-3'
FGF14	5'-TGCTGGATTGCTTTTCGCC	5'-GCTGGGGATCAGTTGGGTTCT-3'
PPP2R5E	5'-TGTCTCAGCACCAACTACTCCT-3'	5'-CAAGATACCTTTTAGCAGCGGC-3'
VEGFB	5'-GATCCGGTACCCGAGCAGT-3'	5'-TTAGGTCTGCATTCACTGGC-3'

RPL17, ribosomal protein L17; RPL39, ribosomal protein L39; ROCK2, Rho associated coiled-coil containing protein kinase 2; CYFIP2, cytoplasmic FMR1 interacting protein 2; FGF14, fibroblast growth factor 14; PPP2R5E, protein phosphatase 2 regulatory subunit B'e ; VEGFB, vascular endothelial growth factor B.

Table II. Analysis of the data generated.

Sample	Total reads	High quality	Reads filter %	Clean reads	Mapped reads	Mapped rate %
DPC-WNT10B-1	5.74x10 <sup>7</sup>	5.44x10 <sup>7</sup>	9.49x10 <sup>-1</sup>	4.05x10 <sup>7</sup>	3.86x10 <sup>7</sup>	9.52x10 <sup>-1</sup>
DPC-WNT10B-2	5.21x10 <sup>7</sup>	5.00x10 <sup>7</sup>	9.58x10 <sup>-1</sup>	7.51x10 <sup>7</sup>	7.16x10 <sup>7</sup>	9.54x10 <sup>-1</sup>
DPC-WNT10B-3	4.5 x10 <sup>7</sup>	4.30x10 <sup>7</sup>	9.54x10 <sup>-1</sup>	4.13x10 <sup>7</sup>	3.95x10 <sup>7</sup>	9.54x10 <sup>-1</sup>
DPC-NC-1	4.08x10 <sup>7</sup>	3.83x10 <sup>7</sup>	9.39x10 <sup>-1</sup>	5.64x10 <sup>7</sup>	5.45x10 <sup>7</sup>	9.65x10 <sup>-1</sup>
DPC-NC-2	7.52x10 <sup>7</sup>	7.10x10 <sup>7</sup>	9.44x10 <sup>-1</sup>	5.18x10 <sup>7</sup>	5.00x10 <sup>7</sup>	9.64x10 <sup>-1</sup>
DPC-NC-3	4.20x10 <sup>7</sup>	3.93x10 <sup>7</sup>	9.38x10 <sup>-1</sup>	4.45x10 <sup>7</sup>	4.31x10 <sup>7</sup>	9.69x10 <sup>-1</sup>

DPC, dermal papilla cells; WNT10B, Wnt family member 10B; NC, negative control. Total reads: All sequencing data getting from next generation sequence. High quality: Reads with high quality of sequencing identity. Reads filter%: The ratio of High quality in Total reads. Clean reads: Remained reads after detecting with FASTQC. Mapped reads: Reads that could be unique mapped to the reference genome. Mapped rate %: The ratio of matched genome in clean reads.

The 3 WNT10B treatment and 3 control samples were used for hierarchical clustering and to construct a volcano plot. The WNT10B-treated DPCs were easily distinguished from the control cells, suggesting that there was a significant difference in gene expression between the two groups (Fig. 1).

**GO analysis.** GO enrichment includes biological process, cellular component (CC) and molecular function (MF). Significantly enriched GO terms were associated with translational initiation, elongation and termination (Fig. 2). The 'structural constituent of ribosome' in the MF analysis and the 'cytosolic large ribosomal subunit' in the CC analysis indicated that WNT10B treatment may influence RNA translation and protein synthesis in DPCs, which subsequently affect HF induction. Several upregulated genes were enriched in the term 'stem cell maintenance'. These included dicer 1 ribonuclease III, APC regulator of WNT signaling pathway, NIPBL cohesin loading factor, notch receptor 2, cell division cycle 73, replication timing regulatory factor 1, mediator complex subunit 12 and bone morphogenetic protein receptor type 1A (Table SIII).

**KEGG pathway analysis.** KEGG pathway analysis revealed that DEGs were enriched in a total of 21 pathways following

WNT10B treatment. Among those pathways, the 'ribosome' was identified as the most enriched (Fig. 3), suggesting that WNT10B may influence protein synthesis, which was consistent with the GO analysis.

In addition, KEGG pathway analysis revealed that the upregulated DEGs were significantly enriched in the 'PI3K-Akt signaling pathway' (Table SII). Therefore, WNT10B may activate the signaling PI3K/Akt pathway and maintain the HF inductive properties of DPCs.

**Validation of RNA-seq results.** RT-qPCR was performed to verify the expression levels of the DEGs. DEGs with high FPKM and high FCs (FC>2 or FC<0.5) were selected, including RPL17, RPL39, ROCK2, CYFIP2, FGF14, PPP2R5E and VEGFB. The RT-qPCR results were highly consistent with RNA-seq data (Fig. 4).

## Discussion

Human HFDPCs are a type of mesenchymal cell isolated from the DPCs of HFs (5,6,40-41). According to reports, DPCs serve important roles in the dermal-epidermal interactions regulating hair regeneration and in the hair growth cycle (5,42-43).

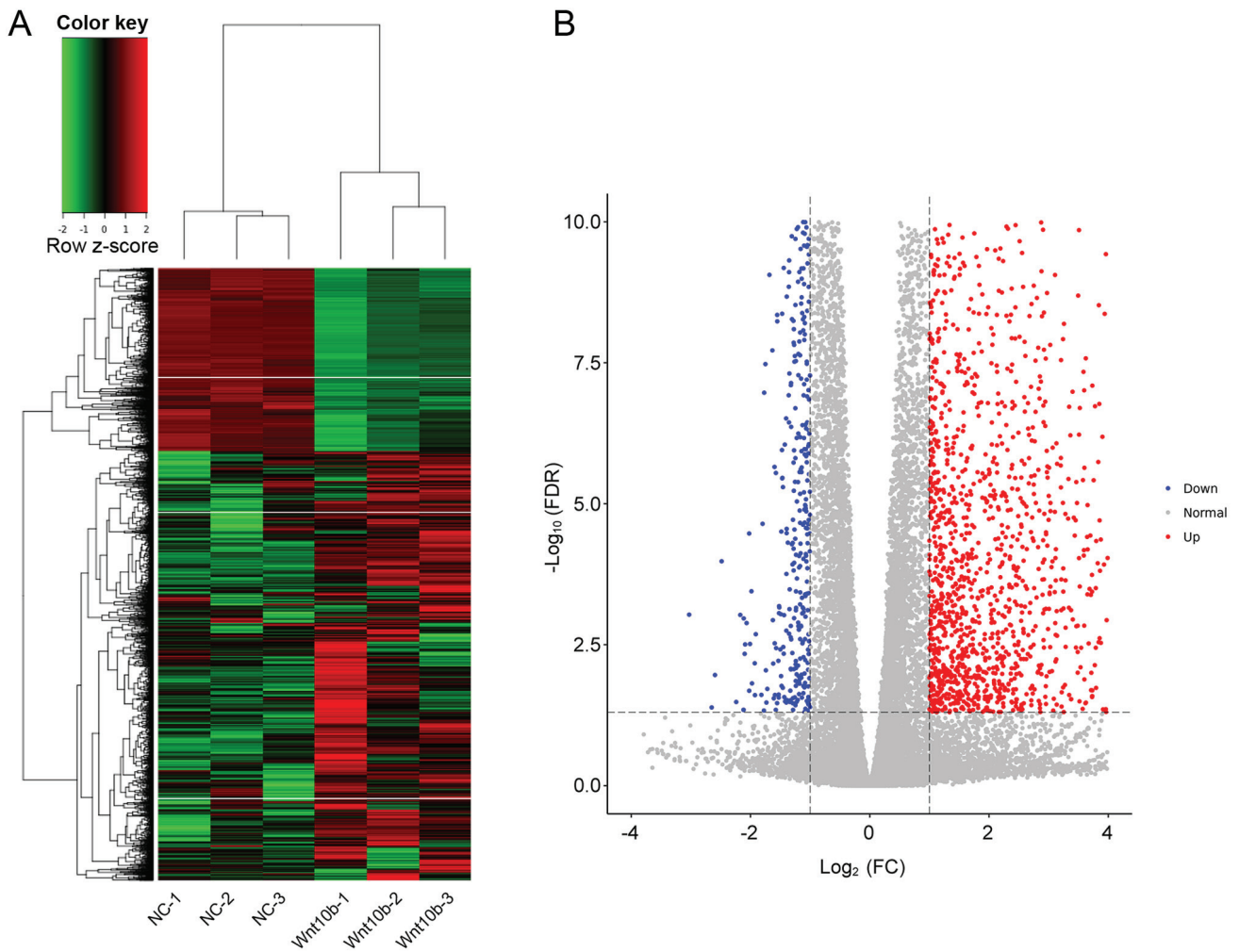


Figure 1. Gene expression in WNT10B-treated and control DPCs. (A) The heat map displays gene expression changes in the 3 WNT10B-treated and 3 control DPCs samples. Red, black, and green represent increased, unchanged, and decreases expression, respectively. (B) Volcano plot of upregulated and downregulated differentially expressed genes between WNT10B-treated and control DPCs. WNT10B, Wnt family member 10B; DPCs, dermal papilla cells; FC, fold-change; FDR, false discovery rate; NC, negative control.

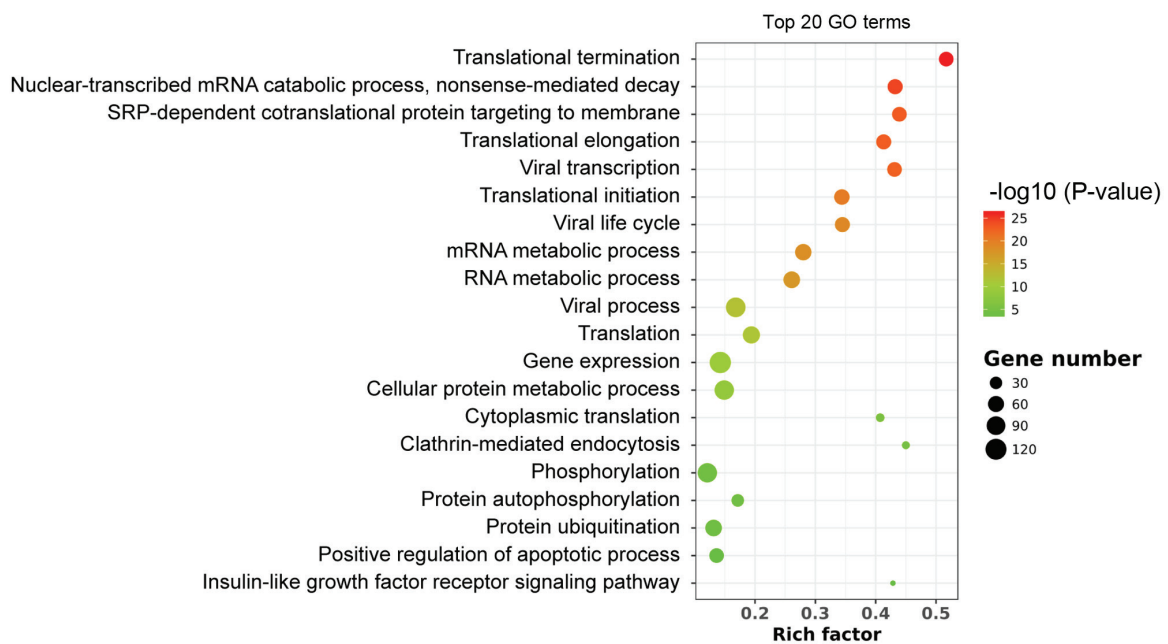


Figure 2. Scatter plot of the top enriched 20 GO terms. The size of the circle indicates the number of enriched genes in each pathway. Each color indicates a different P-value as indicated. GO, Gene Ontology. Rich factor is the ratio of enriched genes in selected pathway to the total genes of the pathway.

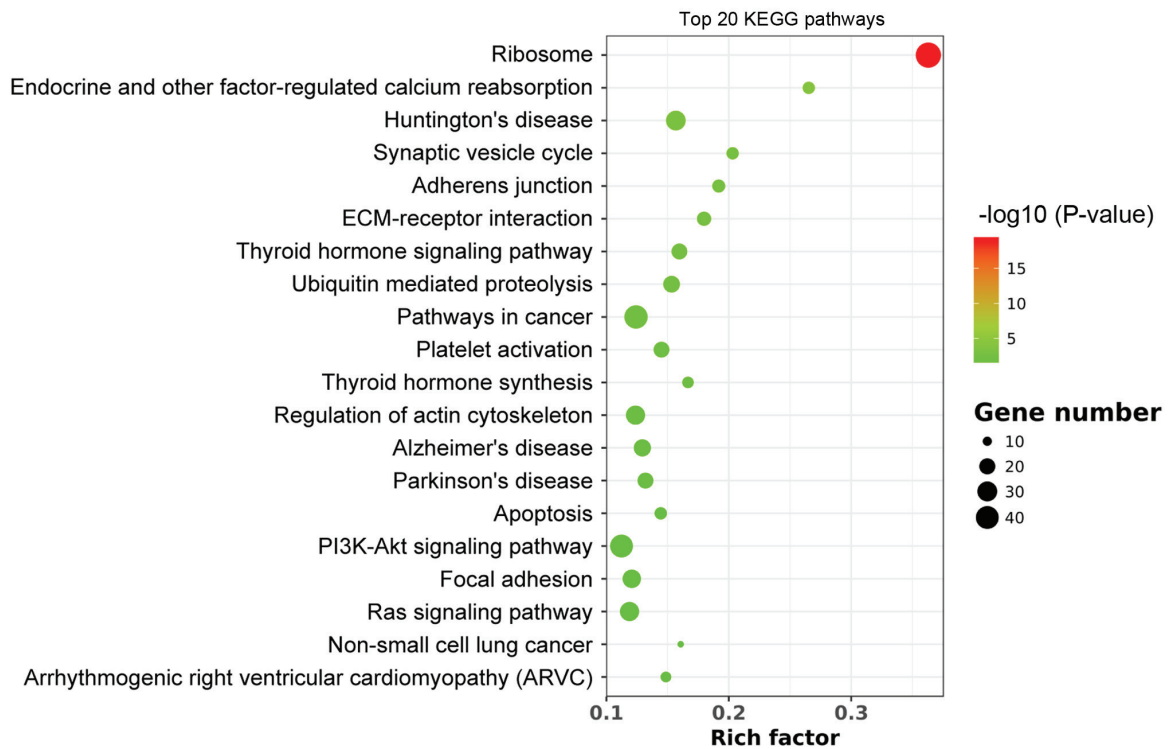


Figure 3. Scatter plot of the top enriched 20 KEGG pathways. The rich factor represents the proportion of differentially expressed genes to all the genes that are annotated in a specific pathway term. The size of the black circle indicates the unigene number. Each color indicates a different P-value as indicated. KEGG, Kyoto Encyclopedia of Genes and Genomes.

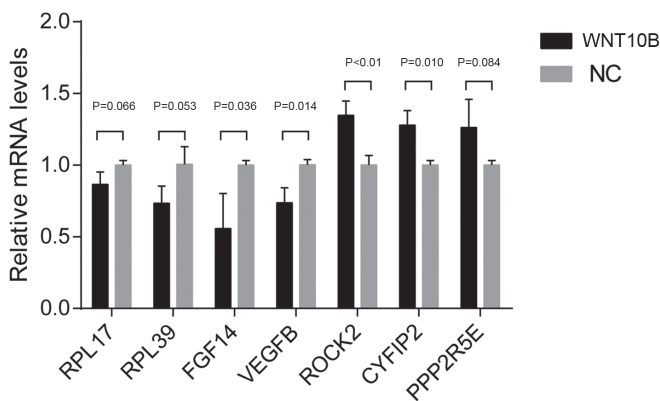


Figure 4. RT-qPCR to confirm RNA sequencing results from WNT10B-treated and control dermal papilla cells. A total of 7 genes were selected for the qRT-PCR analysis, including RPL17, RPL39, ROCK2, CYFIP2, FGF14, PPP2R5E and VEGFB. The data are presented as the mean  $\pm$  SEM of three independent experiments. RT-qPCR, reverse-transcription quantitative PCR; WNT10B, Wnt family member 10B; RPL17, ribosomal protein L17; RPL39, ribosomal protein L39; ROCK2, Rho associated coiled-coil containing protein kinase 2; CYFIP2, cytoplasmic FMR1 interacting protein 2; FGF14, fibroblast growth factor 14; PPP2R5E, protein phosphatase 2 regulatory subunit B'e; VEGFB, vascular endothelial growth factor B; NC, negative control.

DPCs have been investigated as potential cell therapy due to their HF inductive ability (44,45). However, this ability rapidly diminishes when DPCs are cultured *in vitro* (8,12,46), which limits the potential for application in alopecia therapy.

The Wnt signaling pathway is the most important regulator of DPC behavior (47,48). Previous studies have revealed that

WNT10B serves a vital role in the proliferation and maintenance of DPCs and promotes the growth of HFs and the differentiation of mouse HF melanocytes (7,11,18,20). However, the mechanisms underlying these processes remain unclear. The present study used GO term and KEGG pathway analyses to explore the potential mechanisms based on mRNA-seq data. Results revealed 1,073 upregulated and 451 downregulated DEGs between WNT10B-treated and control DPCs *in vitro*. GO and KEGG results suggested that WNT10B significantly downregulated all the three steps (initiation, elongation and termination) of the translation process and as well as the ribosome pathway. In the majority of adults, progenitor cells such as hematopoietic progenitor cell are quiescent and undergo lower levels of protein synthesis (49), suggesting that the level of protein synthesis may be related to stemness and properties of HFDPCs. Additionally, KEGG pathway analysis revealed that WNT10B upregulated the PI3K/Akt signaling pathway, which is known to regulate various cellular processes, including proliferation, metabolism, transcription, protein synthesis, growth and survival (49). A previous study revealed that deletion of the PI3K/Akt signaling pathway antagonist phosphatase and tensin homolog increased protein synthesis and depleted hematopoietic stem cells (50), suggesting that translational regulation and lower rates of ribosome biogenesis may maintain the properties of HFDPCs. The results of the present study suggested that WNT10B treatment may downregulate the protein synthesis rate.

The PI3K/Akt signaling pathway promotes the development, proliferation and differentiation of adult stem cells, particularly neural stem cells (51). As the downstream effector of Wnt signaling pathway,  $\beta$ -catenin regulates the proliferation of HFSCs through the PI3K/Akt signaling pathway (52,53).

Furthermore, the activation of the PI3K/Akt signaling pathway triggers the expression of growth factors and promotes DPC-mediated hair growth (54-56). The activation of the PI3K/Akt signaling pathway may therefore be essential for DPCs to perform their normal functions.

It has previously been reported that Wnt signaling increases mTOR complex 1 (mTORC1) activity. mTORC1 plays a key role in protein synthesis and positively regulates cellular metabolism, ATP production and lipid synthesis (57-59). Wnt signaling may therefore promote protein synthesis by upregulating mTORC1. However, the regulation of the Wnt signaling pathway is an area requiring further study and may involve other signaling pathways (60,61). Moreover, the Wnt signaling pathway maintains stem cell pluripotency and balances progenitor self-renewal and differentiation (62). A previous study identified several Wnt proteins that were expressed in mouse embryonic and postnatal skin, including WNT3, WNT3A, WNT4, WNT5A, WNT6, WNT7A, WNT7B, WNT10A, WNT10B and WNT16. The earliest and most highly expressed Wnt ligand in mouse hair follicle development and hair cycle induction was shown to be WNT10B (47). In agreement with the GO term enrichment results in the present study, WNT10B is involved in signaling pathways controlling stemness, pluripotency and cell fate (63). Recent studies have shown that embryonic and somatic stem cells rely on low translation rates to maintain an undifferentiated state. By contrast, differentiation requires increased protein synthesis. The transition from self-renewal to differentiation relies on enhanced ribosome biogenesis accompanied by increased protein synthesis (64). Therefore, the regulation of protein synthesis is important for cellular differentiation. WNT10B may maintain the stemness of human dermal papilla cells via decreasing the rate of protein synthesis.

The results obtained in the current study may aid in the elucidation of mechanisms of DPC activation and contribute to the development of therapies to treat dysfunction of HF regeneration in diseases such as alopecia.

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#### Authors' contributions

QZ, YS and HC designed the experiments. QZ, YS, QLZ, and RH carried out the experiments and interpreted the data. QZ and YS prepared the sequenced samples and performed the qPCR. HC supervised all experiments. All the authors contributed to the manuscript.

#### Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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