

CHARACTERISATION AND FUNCTIONAL ANALYSIS OF THE *WIF1* GENE AND ITS ROLE IN HAIR FOLLICLE GROWTH AND DEVELOPMENT OF THE ANGORA RABBIT

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Abstract: Growth and development of hair follicles (HF) is a complex and dynamic process in most mammals. As HF growth and development regulate rabbit wool yield, exploring the role of genes involved in HF growth and development may be relevant. In this study, the coding sequence of the Angora rabbit (*Oryctolagus cuniculus*) *WIF1* gene was cloned. The length of the coding region sequence was found to be 1140 bp, which encodes 379 amino acids. Bioinformatics analysis indicated that the *WIF1* protein was unstable, hydrophilic and located in the extracellular region, contained a putative signal peptide and exhibited a high homology in different mammals. Moreover, *WIF1* was significantly downregulated in the high wool production in the Angora rabbit group. Overexpression and knockdown studies revealed that *WIF1* regulates HF growth and development-related genes and proteins, such as *LEF1* and *CCND1*. *WIF1* activated β -catenin/TCF transcriptional activity, promoted cell apoptosis and inhibited cellular proliferation. These results indicate that *WIF1* might be important for HF development. This study, therefore, provides a theoretical foundation for investigating *WIF1* in HF growth and development.

Key Words: rabbit, *WIF1*, Angora rabbit, hair follicle, bioinformatics, Wnt signalling pathway.

INTRODUCTION

Hair follicle (HF) growth and development is a complex and dynamic process that occurs across the lifespan of many mammals (Hardy, 1992; Stenn and Paus, 2001). During HF cyclic development, several signalling pathways, bone morphogenetic protein (BMP), Hedgehog, Wnt, and PI3K/Akt signalling pathways critically regulate HF growth and cycling (Wang *et al.*, 2000; Andl *et al.*, 2002; Botchkarev and Sharov, 2010; Chen *et al.*, 2020). The Angora rabbit is suitable for the wool industry due to its excellent hair follicle characteristics, including hair fibre length, which can steadily grow in the prolonged growth phase (anagen) during the HF growth cycle (Oznurlu *et al.*, 2009). The Angora rabbit is commonly used as a model animal to study HF growth and development. The treatment of drug-induced hair loss in Angora rabbits revealed protective mechanisms during the doxorubicin-dependent inhibition of new hair growth (Powis and Kooistra, 1987). An epigenetic mechanism discovered during the secondary HF cycle in the Angora rabbits has also provided a new perspective on DNA methylation and histone acetylation in the HF growth cycle (Bai *et al.*, 2021). In our previous studies, screening HF cycle-related non-coding RNAs (ncRNAs) and mRNAs in the Angora rabbit by RNA sequencing revealed ncRNA mechanisms regulating the HF cycle (Zhao *et al.*, 2019a). Meanwhile, treatment with insulin-like growth factor (IGF) and epidermal growth factor (EGF) in Angora rabbits has provided new insight into potential therapies for hair-related diseases (Zhao *et al.*, 2021).

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Wnt inhibitory factor 1 (WIF1) is a Wnt signalling pathway antagonist with critical roles in biological processes, such as tumorigenesis, nerve development, osteoblastic differentiation and heart development (Wissmann *et al.*, 2003; Hu and Zhao, 2010; Lu *et al.*, 2013; Mashhadikhan *et al.*, 2020). Moreover, WIF1 plays a critical role in HF growth and development. WIF1 is expressed in the whisker HF at the early developmental stage (Kiyozumi *et al.*, 2011). Between early-passage and late-passage dermal papilla cells (DPC), WIF1 is significantly expressed in late-passage DPC (Lin *et al.*, 2014). The expression of WIF1 has been associated with adult dermal papilla maintenance and participates in different stages of the HF cycle (Rendl *et al.*, 2005); however, the molecular functions of WIF1 in the Angora rabbit remain unclear.

This study analysed the biological function of WIF1 using bioinformatics after cloning the coding sequence of the rabbit *WIF1* genes. The overexpression and knockdown of WIF1 in rabbit DPC revealed that WIF1 regulates HF growth and development via the Wnt signalling pathway. These results provide a further understanding of WIF1's role in the HF cycle development in animals, which may solve the problems related to rabbit wool production and hair loss in humans.

MATERIALS AND METHODS

Animals

Six-month-old Angora rabbits were used for sample collection; twelve 6-month-old male rabbits were divided into two groups according to wool production. Dorsal skin (1 cm²) was collected after anaesthesia using 0.7% pentobarbital sodium (6 mL/kg). To prevent bacterial infection, an iodine solution was applied to the wound. The experimental procedures were approved by the Animal Care and Use Committee of Yangzhou University, China (approval number: 202103358).

Cell culture and transfection

DPC were isolated as previously described and maintained in the Mesenchymal Stem Cell Medium (Sciencell®) (Gledhill *et al.*, 2013). The RAB-9 cell line (ATCC® CRL1414™) was purchased from American Type Culture Collection (ATCC) and cultured in the Minimum Essential Medium (MEM) (Gibco®), containing 10% foetal bovine serum (FBS, One Shot™, Gibco®). Cells were cultured in a humidified incubator at 37°C in the presence of 5% CO₂. Lipofectamine™ 3000 (Invitrogen) was used for cell transfection according to the manufacturer's instructions, where the cells were grown in 24-well plates until they reached 80% confluence.

Cloning and sequencing of the rabbit coding sequence

Total RNA of rabbit skin was extracted using the RNAsimple Total RNA Kit (Tiangen), and the cDNA was obtained using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara). According to the rabbit *WIF1* mRNA sequence (GenBank accession n°. XM_008256742.2), the primers for the *WIF1* CDS sequence were designed by Primer-blast (primer-F: 5'-ATGGCCGGGAGAAGCCCC-3', primer-R: 5'-TCACCAGATGTAATTGGATTCCG-3'). The polymerase chain reaction (PCR) products were generated by Phanta Max Super-Fidelity DNA Polymerase (Vazyme) following the manufacturer's instruction, and the PCR products were gel-purified and cloned to the pMD19-T vector (TaKaRa) after being transformed into the *E. coli*. The positive clones were then identified by PCR and sequencing.

Bioinformatics analysis of WIF1

The DNASTAR software package (DNASStar) was used to analyse the *WIF1* coding sequence. The molecular formula, molecular weight, isoelectric point (pI), and instability coefficient of the WIF1 protein were predicted by ProtParam (<http://web.expasy.org/protparam/>) (Gasteiger *et al.*, 2005). The signal peptide of WIF1 was predicted by SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP-4.1/>) (Petersen *et al.*, 2011). TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) (Möller *et al.*, 2001) online software was used to predict the localisation signal, secretory protein and protein transmembrane region. The WoLF PSORT (<https://www.genscript.com/tools/wolf-psort>) (Horton *et al.*, 2007) online tool was used to predict protein subcellular localisation. Potential threonine, serine, or tyrosine

phosphorylation sites of the protein were predicted by NetPhos 3.1 Server (<http://www.cbs.dtu.dk/services/NetPhos/>) (Blom *et al.*, 1999). Potential O-glycosylation sites and potential N-glycosylation sites were separately predicted by the O-glycosylation sites (<http://www.cbs.dtu.dk/services/NetOGlyc-3.1/>) (Julenius *et al.*, 2005) and NetNGlyc 3.1 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) (Gupta *et al.*, 2004). The phylogenetic tree was constructed using the neighbour-joining method using the MEGA X software (Kumar *et al.*, 2018). The protein secondary structure, the conserved domain and three-dimensional homology of WIF1 were predicted by Hopfield (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) (Deléage, 2017) and SWISS-MODEL (Waterhouse *et al.*, 2018). Finally, the protein-protein interaction (PPI) network of the WIF1 protein was analysed by the STRING database (Horton *et al.*, 2007).

Overexpression and knockdown of WIF1

To obtain the overexpression vector of WIF1, the CDS sequence was subcloned into the *NheI* and *XhoI* digested pcDNA3.1(+) vector (Invitrogen) (primer-F: 5'-gggagaccgaagctggtagcATGGCCGGGAGAAGCCCC-3', primer-R: 5'-aacgggccctctagactcgagTCACCAGATGTAATTGGATTCCG-3'). The interfering RNA (siRNA) (primer-F: 5'-GCAGAGAAUJCGCGCUAUUTT-3', primer-R: 5'-AAUAGCCGGCAUUCUCUGCTT-3') and siRNA-NC (primer-F: 5'-UUCUCCGAACGUGUCACGUTT-3', primer-R: 5'-ACGUGACACGUUCGGAGAATT-3') were designed and purchased from Shanghai GenePharma Co., Ltd.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from skin and cells using the RNAsimple Total RNA Kit (Tiangen). The cDNA was obtained using the HiScript II Q Select RT SuperMix (qPCR; Vazyme). The quantitative real-time polymerase chain reaction (qRT-PCR) used to detect the gene expression levels applied the AceQ qPCR SYBR® Green Master Mix (Vazyme), and the results were analysed using the QuantStudio® 5 (Applied Biosystems, Thermo Fisher Scientific). Relative gene expression level was analysed using the 2^{-ΔΔCt} method (Schmittgen and Livak, 2008), where *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* served as endogenous control. The specific primer sequences are listed in Table 1.

Wes automated Western blotting analysis

Cell and skin samples were collected for protein extraction using RIPA Lysis Buffer (PPLYGEN, Beijing, China). The Enhanced BCA Protein Kit (Beyotime, Shanghai, China) was used to evaluate protein concentrations. Next, the protein expression level of WIF1, lymphoid enhancer binding factor 1 (LEF1), cyclin D1 (CCND1) and GAPDH was determined by Wes automated Western blotting system (Protein Simple), according to the manufacturer's instruction.

Table 1: Primer sequences used in quantitative real-time polymerase chain reaction.

Gene	Primers
<i>GAPDH</i>	Forward primer: 5'-CACCAGGGCTGCTTTAACTCT-3' Reverse primer: 5'-CTTCCCGTTCTCAGCCTTGACC-3'
<i>WIF1</i>	Forward primer: 5'-ACCTGCCATGAACCAACAA-3' Reverse primer: 5'-TGCTGTCCCCGACCTTTTT-3'
<i>CCND1</i>	Forward primer: 5'-GAACGCTACCTCCCCAGTGTC-3' Reverse primer: 5'-CCTCACAGACCTCCAGCATCCAG-3'
<i>EGF</i>	Forward primer: 5'-GTGCGATGCCGAGAAGT-3' Reverse primer: 5'-AGCCCAATCTGAGAACCAC-3'
<i>LEF1</i>	Forward primer: 5'-CATCTGGGTGGATTGAGG-3' Reverse primer: 5'-ATGAGGGATGCCAGTTGTG-3'
<i>SFRP2</i>	Forward primer: 5'-CCAGCCGACTTCTCCTACAAGC-3' Reverse primer: 5'-TCCAGCACCTTTTCATGGTCT-3'

The following antibodies were used: 1:50 Anti-WIF1 mouse monoclonal antibody (Sangon Biotech), 1:100 Anti-LEF1 rabbit polyclonal antibody (Proteintech, China), 1:100 Anti-CCND1 mouse monoclonal antibody (Proteintech, China), 1:100 Anti-GAPDH mouse monoclonal antibody (Proteintech, China).

TOP/FOP-flash Wnt reporter assays

The TOP-flash and FOP-flash (TOP-flash mutant) Wnt reporter plasmids were acquired from the MiaoLingPlasmid Sharing Platform. To evaluate β -catenin/TCF transcriptional activity, pcDNA3.1-WIF1 and siRNA-WIF1 were co-transfected with TOP/FOP-flash plasmid and pRL-TK into RAB-9 cells using Lipofectamine™ 3000 (Invitrogen). The FOP-flash reporter plasmid was used as a negative control. The Dual-Luciferase Reporter Assay System (Promega) was used to analyse the luciferase activity according to the manufacturer's protocol.

Cell apoptosis and proliferation assays

Apoptosis was determined using the Annexin V-FITC Apoptosis Detection Kit (Vazyme) according to the manufacturer's instructions and fluorescence-activated cell sorting (FACS) was performed using the FACS Aria SORP flow cytometer (Becton Dickinson). Cell proliferation was analysed using the Cell Counting Kit-8 (Vazyme) according to the manufacturer's instructions. The optical densities of the 96-well plates were determined after 0, 24, 48, and 72 h at 450 nm using Infinite M200 Pro (Tecan).

Statistical analysis

SPSS 22.0 (SPSS, USA) software was used for statistical analyses and relative gene expression was analysed using a paired sample *t*-test. All the error bars represent the mean \pm standard deviation and each analysis contained three biological replicates (n=3).

RESULTS

Cloning and bioinformatics analysis of WIF1

The open reading frame (ORF) sequence of WIF1 was cloned, obtaining a 1140 bp sequence, which encodes 379 amino acids, and provides a molecular weight of around 41.4 kDa. The ProtParam software revealed the molecular weight of 41395.61 Da and a molecular formula of $C_{1802}H_{2792}N_{518}O_{523}S_{41}$. The total number of atoms was 5676, the theoretical pI was 8.22, the total number of negatively charged residues (Asp+Glu) was 31 and positively-charged residues (Arg+Lys) was 37. The instability index (II) estimated WIF1 to be an unstable protein with an index of 51.26, an aliphatic index of 62.06, and a value of the grand average of hydropathicity (GRAVY) of -0.286 . WIF1 was presumed to have a putative signal using the peptide SignalP 4.1 server (Figure 1A), while the highest cleavage site was located at the 24th amino acid residue where the value was 0.195, and the 4th amino acid residue exhibited the highest value of 0.950; the signal peptide scores of 1st to 23rd amino acid residues were 0.894. According to the prediction by the TMHMM server, WIF1 did not contain a transmembrane domain, while the total probability of the N-terminal in the cytoplasm was 0.00952 (Figure 1B). The NetPhos server predicted that 16 threonine, 18 serine and 8 tyrosine amino acids of the WIF1 protein had putative phosphorylation sites. The WIF1 protein had no O-glycosylation sites according to the prediction of the NetOGlyc server, while the results of the NetNGlyc server found the WIF1 protein to have two N-glycosylation sites, located at the 88th amino acid (potential rate=0.5926) and 245th amino acid (potential rate=0.4494). The online tool WoLF PSORT was used to analyse the subcellular localisation of the WIF1 protein, and predicted a localisation coefficient of the extracellular space of 14. In conclusion, the WIF1 protein is a hydrophilic and unstable protein, with an extracellular localisation. The secondary structure of the WIF1 protein was predicted, with results showing that 245 (64.64%) amino acids formed a random coil, 79 (20.84%) amino acids formed an α -helix, and 55 (14.51%) amino acids formed an extended strand (Figure 1C). The tertiary structure of WIF1 was predicted using the SWISS-MODEL software (Figure 1D). The protein-protein interaction network for WIF1 was constructed using the STRING database to explore protein interactions, according to co-expression, co-occurrence, text mining, experiment databases, neighbourhood and gene fusion. Ten genes were identified as related to WIF1 (Figure 1E). The

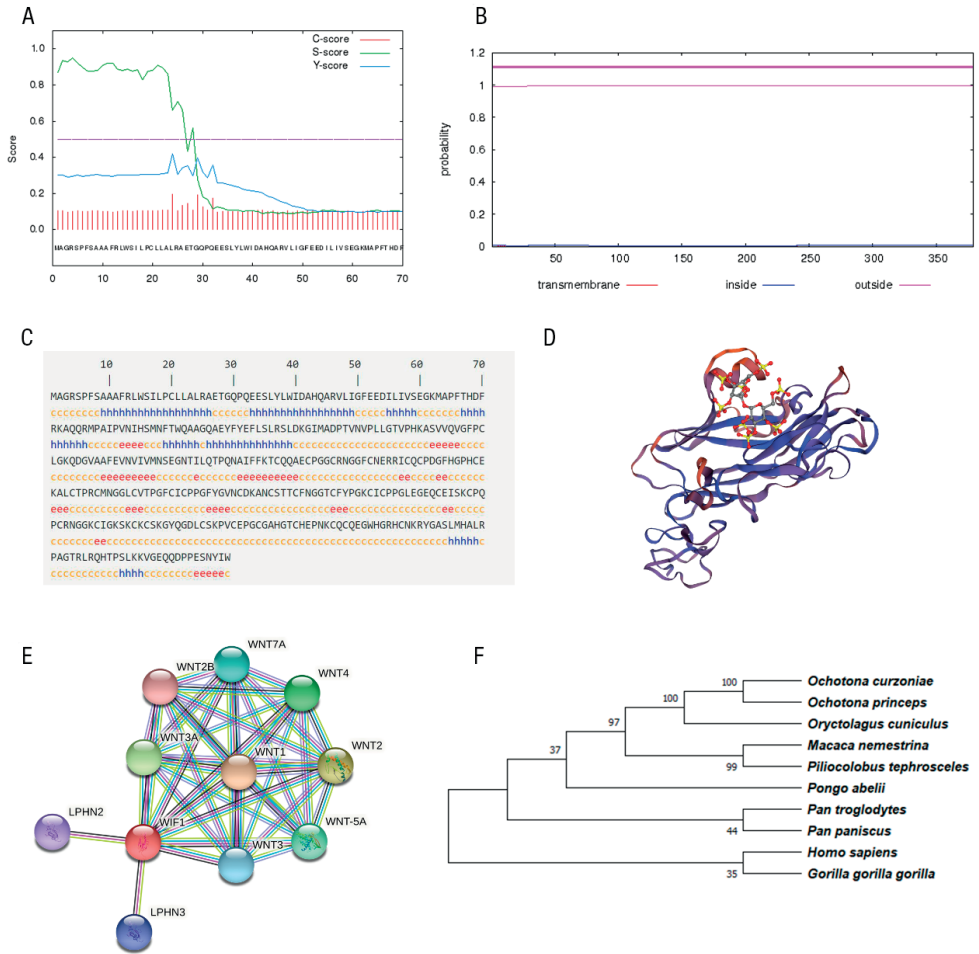


Figure 1: Bioinformatics analysis of WIF1. (A) Signal peptide prediction of the WIF1 protein. (B) Transmembrane domain prediction of the WIF1 protein. (C) Secondary structure prediction of the WIF1 protein. Note: h represents α -helix, e represents extended strand and c represents random coil. (D) Tertiary structure prediction of the WIF1 protein. (E) Protein interaction network of WIF1. (F) Phylogenetic tree of WIF1 and other homologous sequences.

evolutionary relationship of the *WIF1* gene among the different animal species was analysed using MEGA X software. The construction of a phylogenetic tree map (Figure 1F) revealed that *Ochotona princeps* and *Ochotona curzoniae* formed a branch suggesting that the three species exhibit the highest homology.

Expression of WIF1 in the Angora rabbit

Wool was collected from the Angora rabbits to analyse wool production. A significant difference was found between the high and low wool-producing groups ($P < 0.01$, Figure 2A). Gene expression analysis revealed high *WIF1* expression in low wool-producing Angora rabbit ($P < 0.01$, Figure 2B), and the correlation analysis found a *correlation coefficient* between wool production and *WIF1* mRNA expression of -0.988 ($P < 0.01$), which indicated a significant negative correlation between the *WIF1* gene expression and wool production.

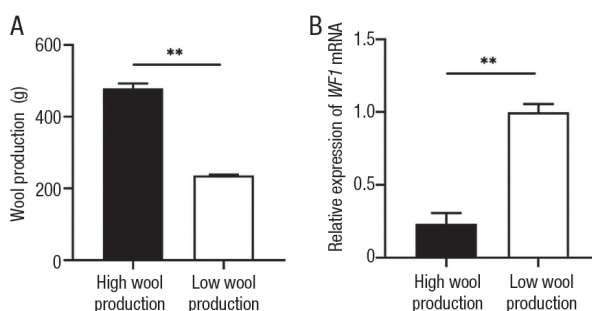


Figure 2: Wool production and *WIF1* mRNA expression in the Angora rabbit. (A) Differences in the wool production of Angora rabbit between the high and low production groups. (B) The *WIF1* mRNA expression in the Angora rabbit. ** $P < 0.01$.

Regulation of *WIF1* overexpression and knockdown

Next, *WIF1* mRNA expression was detected by qRT-PCR following the transfection of the overexpression vector (pcDNA3.1-*WIF1*) and the knockdown vector (siRNA-*WIF1*) in cells. The results indicated that pcDNA3.1-*WIF1* significantly increased *WIF1* expression ($P < 0.01$), while siRNA-*WIF1* significantly decreased *WIF1* expression ($P < 0.01$, Figure 3A). *WIF1* overexpression significantly upregulated *secreted frizzled related protein 2* (*SFRP2*) expression and downregulated *LEF1* and *CCND1* expression ($P < 0.01$); meanwhile, *WIF1* knockdown Connecticut Lee increased *LEF1* and *CCND1* expression and downregulated *SFRP2* expression ($P < 0.05$, Figure 3B). Western blotting results showed that the overexpression and knockdown of *WIF1* regulated *WIF1* protein expression, and *WIF1* downregulated *CCND1* and *LEF1* protein levels (Figure 3C). To determine the role of *WIF1* as a Wnt antagonist in the Wnt/ β -catenin signalling pathway, the activity of the β -catenin/TCF transcription was evaluated using the TOP/FOP reporter assay. The results demonstrated that *WIF1* overexpression inhibited β -catenin/TCF transcription activity ($P < 0.05$), while *WIF1* knockdown promoted β -catenin/TCF transcriptional activity (Figure 3D).

WIF1 promotes apoptosis and inhibits the proliferation of DPC

WIF1 inhibits cell growth and induces cell apoptosis in many cell lines (Ramachandran *et al.*, 2012; Ng *et al.*, 2014; Huang *et al.*, 2016). Our study determined any role for *WIF1* in regulating cell apoptosis and cell proliferation in DPC. The results showed that *WIF1* overexpression upregulated DPC apoptosis rate of DPC ($P < 0.01$), while the *WIF1* knockdown inhibited DPC apoptosis ($P < 0.01$, Figure 4A). Moreover, *WIF1* reduced the DPC viability after 48 h ($P < 0.01$), while *WIF1* knockdown enhanced cell proliferation after 48 h ($P < 0.01$, Figure 4B).

DISCUSSION

Hair follicles play crucial roles in protection, sensory activity and social functions in mammals and humans (Schneider *et al.*, 2009). *WIF1* can regulate HF growth and development; however, no previous studies have revealed how *WIF1* regulates HF growth and cycling in rabbits. In our study, the coding sequence of rabbit *WIF1* was cloned and the bioinformatics analysis indicated that the *WIF1* protein was unstable and hydrophilic. *WIF1* was found to have a putative signal peptide and did not contain a transmembrane domain. *WIF1* may be located in the extracellular region, with most amino acids forming an α -helix. Furthermore, the protein interactions analysis demonstrated that Wnt family members (including Wnt1, Wnt2, Wnt2B, Wnt3, Wnt3A, Wnt4, Wnt5A and Wnt7A) interacted with the *WIF1* protein, suggesting an interactive relationship between *WIF1* and the Wnt signalling pathway. The phosphorylation and glycosylation of proteins essentially affect various cellular processes such as immune responses, transcriptional control and signal transduction (Hunter and Karin, 1992; Ohtsubo and Marth, 2006; Ubersax and Ferrell Jr, 2007).

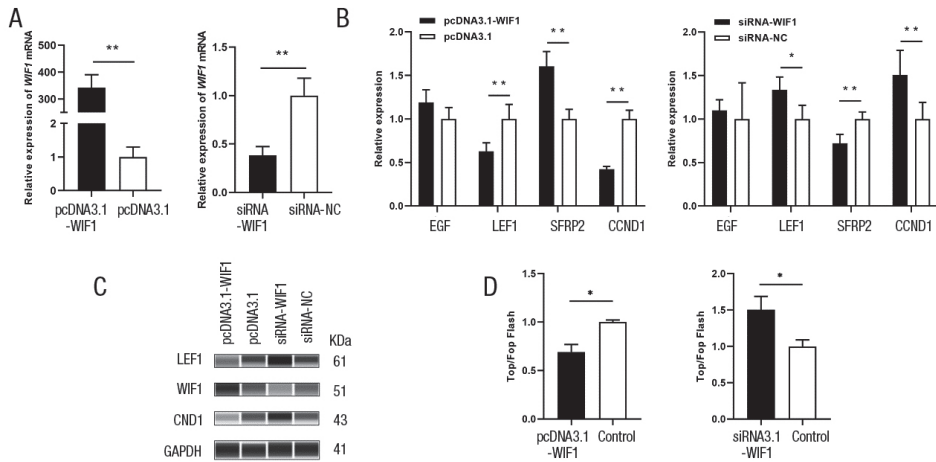


Figure 3: Overexpression and knockdown of WIF1 regulate the HF growth and development-related genes and proteins. (A) Overexpression and knockdown of WIF1 regulated the expression of the *WIF1* mRNA levels using pcDNA3.1-WIF1 and siRNA-WIF1 in rabbit DPC. (B) Overexpression and knockdown of WIF1 regulated the HF development-related genes. (C) Overexpression and knockdown of WIF1 regulated the HF development-related proteins. (D) Overexpression and knockdown of WIF1 regulated the β -catenin/TCF transcriptional activity. * $P < 0.05$, ** $P < 0.01$.

Although predictions indicated that WIF1 contains putative phosphorylation and N-glycosylation sites, the role of phosphorylation and glycosylation of WIF1 warrants further investigation.

Verifying that WIF1 influences HF growth and development via the Wnt signalling, WIF1 was found to play a role in regulating the expression of Wnt signalling pathway-related genes such as *SFRP2*, *LEF1*, and *CCND1*. As a Wnt inhibitor, *SFRP2* is highly expressed during catagen and inhibits keratinocyte proliferation (Kim and Yoon, 2014). Studies have also indicated that SFRP2 negatively affects skin and HF development (Zhao *et al.*, 2019b). LEF1 activates hair development by directing HF patterning and cell fate (Zhou *et al.*, 1995; Kratochwil *et al.*, 1996), while CCND1 is differentially expressed during the HF cycle and affects HF morphogenesis (Xu *et al.*, 2003). WIF1 was found to upregulate *SFRP2* expression and downright *LEF1* and *CCND1* expression, indicating that WIF1 might regulate HF growth and development by regulating genes such as these. The Wnt/ β -catenin signalling pathway represents one of the most important signalling pathways that explain the cyclic development of HF (Millar *et al.*, 1999). The Wnt/ β -catenin activates the LEF/TCF complex to regulate downstream genes, which play important roles in HF cycle and development (DasGupta and Fuchs, 1999). Further experiments revealed that WIF1 inhibited the Wnt/ β -catenin signalling pathway-related genes and decreased β -catenin/TCF transcription activity, indicating that WIF1 can regulate HF growth and development through the Wnt signalling pathway. WIF1 is known to be crucial for tumorigenesis, as studies have shown that WIF1 controls the metastasis, proliferation, and apoptosis of gall bladder tumour cells, and can induce cellular apoptosis in cervical and non-small-cell lung cancer (Liu *et al.*, 2011; Ramachandran *et al.*, 2012; Huang *et al.*, 2016). In this study, WIF1 was found to promote apoptosis and inhibit the proliferation of DPC, suggesting that WIF1 can inhibit HF growth and development.

CONCLUSION

The coding sequence of WIF1 was obtained and the physicochemical properties and molecular functions of WIF1 were analysed. The overexpression and knockdown of WIF1 revealed the biological functions of WIF1 in HF growth and development. As a Wnt antagonist, WIF1 induced apoptosis and inhibited proliferation of rabbit DPC. This study

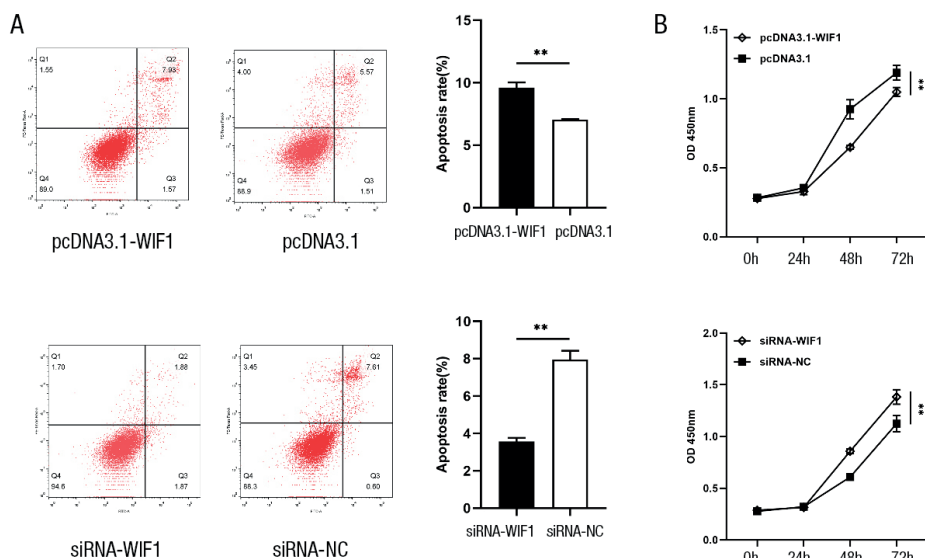


Figure 4: WIF1 regulates the apoptosis and proliferation of rabbit DPC. (A) The cell apoptosis rate of DPC was detected after the overexpression and knockdown of WIF1. (B) The cell proliferation was determined after the overexpression and knockdown of WIF1. ** $P < 0.01$.

provides a theoretical foundation for further research on WIF1 and helps to improve functional research into HF growth and development.

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Conflict of interest: The authors declare that they have no conflict of interest.

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