World Rabbit Science

World Rabbit Sci. 2023, 31: 171-178
doi:10.4995/wrs.2023.18171
WRSA, UPV, 2003

THE *FRZB* GENE REGULATES HAIR FOLLICLE DEVELOPMENT IN RABBITS VIA THE WNT/B-CATENIN SIGNALLING PATHWAY

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Abstract: To explore the mechanism of the *FRZB* gene in hair follicle development by regulating the Wnt/β-catenin signalling pathway, Angora rabbits were selected to collect back skin samples for the experiment. The action mechanism is understood by cell culture and transfection, apoptosis and proliferation assays and TOP/FOP Flash Wnt Reporting System methods. The results showed that the interference and overexpression of the *FRZB* gene in rabbit dermal papilla cells indicated that overexpression could inhibit the expression of *SFRP2*, *BMP4*, and *WNT2* genes ($P<0.05$). On the contrary, the expression of Wnt signalling pathway-related genes *LEF1*, *CCND1*, *DKK1*, and *TCF7* was significantly up-regulated ($P<0.05$). Further examination of the luciferase reporter system TOP/FOP revealed that pcDNA3.1-*FRZB* inhibits Wnt activity. PcDNA3.1-*FRZB* was found to promote the level of apoptosis in DP cells, whereas si-*FRZB* inhibited DP cell proliferation. Therefore, it is concluded that *FRZB* inhibits hair follicle development in long-haired rabbits by regulating the Wnt/β-catenin signalling pathway.

Key Words: *FRZB* gene, hair follicle, long-haired rabbit.

INTRODUCTION

The hair follicle is a complex skin organ that regulates the formation of mammalian hair. Hair follicles undergo repeated anagen, catagen and telogen cycles (Maier *et al.*, 2011). Hair follicle morphology is formed by a temporary interaction between dermal and epithelial-stromal cells (O'Shaughnessy *et al.,* 2004), Dermal papilla cells (DP cells) are special mesenchymal components of hair that play a vital role in the morphogenesis and regeneration of hair growth (Saxena *et al.,* 2019). DP cells regulate the development and growth of HF by acting as a reservoir of pleiotropic stem cells, nutrients and growth factors. Hair follicle growth and development are regulated by multiple signalling pathways, such as Wnt, LEF-1and TGF-β, involved in hair follicle development. Among them, the Wnt/β-catenin/Lef-1 signalling pathway plays a key role in the initial stage of hair follicle development (Andl *et al.,* 2002). Our research group has established a synchronisation model of the hair follicle in the early stage of long-haired rabbits through transcriptome sequencing and has screened out the differentially expressed genes linked to the hair follicle cycle, identifying the secreted frizz-related proteins (*SFRPs*) that play an essential regulatory role in the hair follicle growth cycle (Zhao *et al.,* 2017). *FRZB* (also known as *sFRP3*) belongs to a family of secreted Fz-related proteins that was initially identified as a chondrogenic factor during bone morphogenesis (Hoang et al., 1996). Further studies have identified that *FRZB* regulates the activity of Wnt8 during *Xenopus* development (Leyns *et al.,* 1997) inhibiting the canonical Wnt signalling (Person *et al.,* 2005). In addition, the *FRZB* gene is associated with muscle growth and its overexpression can inhibit tumour formation (Guo *et al.,* 2008). However, the molecular mechanism by which *FRZB* regulates the development of the hair follicle in rabbits remains elusive.

Correspondence: Y. Chen, *yangc@yzu.edu.cn*. Received *August 2022* - Accepted *March 2023.*
https://doi.org/10.4995/wrs.2023.18171

Cite as: Wang F., Zhang X., Dai Y., Zhao B., Wu X., Chen Y. 2023. The *FRZB* gene regulates hair follicle development in rabbits via the Wnt/β-catenin signalling pathway. *World Rabbit Sci., 31: 171-178. https://doi.org/10.4995/wrs.2023.18171*

The Wnt family is a class of secreted lipid-modified glycoproteins with an essential regulatory role in the growth cycle of animal hair follicles (Xiang, 2011). When Wnt binds to its receptor, the phosphorylation of β-catenin is inhibited, resulting in changes in the cytoplasm, and the members of the T-cell-specific factor (TCF)/LEF transcription factor family was found to interact with the β-catenin in the nucleus, thereby beginning the transcription of the target gene (Cong *et al.,* 2003). In this study, the *FRZB* gene was cloned to analyse the effect on the activity of the Wnt signalling pathway and the genes concerned, in order to understand the further mechanism of the *FRZB* gene in developing the hair follicles in rabbits.

MATERIAL AND METHODS

Ethical statement

This study was approved by the Animal Care and Use Committee of Yangzhou University (Yangzhou, China, Nov. 5, 2020, No. 202010045) and conducted in strict accordance with the regulations for the management of laboratory animals.

Sample collection

This experiment used 6-month-old male Angora rabbits and the rabbit fur was removed from the back using a razor to reveal the pink skin. The hair follicle structure was found to be damaged, which prompted the hair follicle to enter a new growth cycle. The laboratory supplies required for tissue extraction were prepared in advance. The rabbits were anaesthetised by injecting XYLAZINE (0.35 mL/kg) through the ear vein. A 1 cm^2 skin tissue sample was collected from the back and the skin was cleaned with iodophor to avoid infection.

Analysis of FRZB expression using real-time quantititative polymerase chain reaction (RT-qPCR)

RNA was isolated from the collected tissues using the RNA Simple total RNA kit (Tiangen, China) according to the instructions of the manufacturer and the quality of the RNA sample was checked by 1.0% agarose gel and the RNA purity was determined by the Nanodrop ND-2000 (Implen, CA, USA). The cDNA was synthesised using the HiScript reverse transcriptase (Vazyme, China). The *FRZB* RT-qPCR experiments were performed using the ChamQSYBR qPCR Master Mix according to the instructions (Vazyme, China). Using *GAPDH* as a control, the relative expression of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and the experimental data were processed using the QuantStudio®5 software (Thermo FisherScientific, Foster City, CA). The primer was designed using Primer5, synthesised by Tsingke Biotechnology (Nanjing, China). Primer sequences are shown in Table 1.

Table 1: Primer sequences for real-time quantititative polymerase chain reaction (RT-qPCR).

Gene	Primer sequence	
LEF1	F:	5′-CATCTCGGGTGGATTCAGG-3′
	R:	5′-ATGAGGGATGCCACATACCAG-3′
GAPDH	F:	5′-CACCAGGGCTGCTTTTAACTCT-3′
	R:	5′-CTTCCCGTTCTCAGCCTTGACC-3′
CCND1	F:	5′-GAACGCTACCTTCCCCAGTGCTC-3′
	R:	5′-CCTCACAGACCTCCAGCATCCAG-3′
SFRP2	F:	5′-TCGAGTACCAGAACATGCGG-3′
	R:	5′-GAAGAGCGAGCACAGGAACT-3′
DKK1	F:	5′-CACAGAGGACGAGGAGTGTG-3′
	R:	5′-CTTCCTGCAAGCCAGACAGA-3′
WNT2	F:	5′-AGCCATCCAGGTCGTCATGAACCAG-3′
	R:	5′-TGCACACACGACCTGCTGTACCC-3′
TCF7	F:	5′-TGTGAGCTGGAAGACACAGGG-3′
	R:	5′-GGGGTTTCTTGATGGTTGGC-3′
BMP4	F:	5′-AGCCCTAAGCATCACCCAC-3′
	R:	5′-TCCAGTCATTCCAGCCCAC-3′

Cloning the FRZB coding sequence and vector construction

The long-haired rabbit *FRZB* coding sequence (NCBI sequence: NC_013675.1) was obtained from the GenBank database and the Primer5 software was used (forward: 5'-TAGTCCAGTGTGGTGGAATTCTCAGTTGCGGGCTTGCCG-3'; reverse: 5'-GGGAGACCCAAGCTGGCTAGCATGGCCTGCGACAG-3') to design the gene specificity which comprised the primers for the *NheI* and *EcoRI* restriction sites. The PCR reaction conditions were: 95°C for 3 min, 95°C for 15 s, 61°C for 30 s and 72°C for 2 min (35 cycles), and finally with a complete extension at 72°C for 5 min. The PCR result was detected using 1% agarose gel and then recovered by cutting the gel. The product was then purified and sequenced to verify the amplified sequence. The obtained product was cloned into the *pMD19-T* vector, transformed into *DHa5 E. coli* competent, and *FRZB* was cloned into the *pcDNA3.1* vector expressing the *EcoRI* and *NheI* recombinant enzymes.

FRZB sequence analysis

The *FRZB* coding sequence was analysed using DNAStar software. The physicochemical properties of proteins (https://web.expasy.org/cgi-bin/protpAram/protipAram) were predicted by ProtParam (UniProt, 2009. The Universal Protein Resource (UniProt) 2009, *Nucleic Acids Res., 37:* D169–D174, *https://doi.org/10.1093/nar/gkn664*

2009) while SignalP4.1 was used for predicting the signal peptide cleavage points (http://www.cbs.dtu.dk/services/SignalP-4.1/)(Petersen *et al.,* 2011). The protein transmembrane regions were analysed using the software TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/)(Davies *et al.,* 2011) and the interactive maps of *FRZB* and other related proteins were constructed using the STRING database (Szklarczyk *et al.,* 2011). The MEGA6.0 software was used for homology alignment and phylogenetic tree (Kim and Yoon, 2014). The secondary structure of the proteins was predicted using the Hopfield neural network (http://npsa125 pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) (MacPhail *et al.,* 1989) while Netphone was used to predict the phosphorylation sites of *FRZB* (http://www.cbs.dtu.dk/services/NetPhos/)(Payne *et al.,* 1991).

Cell Culture and transfection

The cells were cultured in a medium containing 10% foetal bovine serum (FBS) and placed in an incubator at a constant temperature of 37°C CO_2. The DP cells were transferred to a 24-well plate and transfected with Lipofectamine™ 2000 (Invitrogen, CA) when the cells reached a confluency of 80% according to the instructions.

Apoptosis and proliferation assays

The cells were collected after first digesting with ethylenediaminetetraacetic acid free-free trypsin and then washed with pre-cooled phosphate buffered saline 2-3 times before finally adding the Annexin V-FITC reagent and PI dye for determining the level of apoptosis and protection from light. Fluorescence-activated cell sorting was performed using a BD FACSAria SORP flow cytometer (Becton Dickinson). The transfected cells were seeded into 96-well plates after 12 h and the cell proliferation was analysed using the Cell Counting Kit-8 (Vazyme) at 0, 24, 48 and 72 h, respectively. The OD value was detected at 450 nm using an Infinite M200 Pro (Tecan).

TOP/FOP flash Wnt reporting system

The TOP/FOP Flash assay is a luciferase reporter gene system that specifically evaluates the β-catenin and TCF signalling activity. The TOP-FLASH plasmid was co-transfected with pcDNA3.1FRZB siRNA-FRZB into DP cells, respectively, using Lipofectamine 2000 (Invitrogen, CA). The FOP FLASH (TOP-FLASH mutant) reporter plasmid served as the control group and after 48 h, the cells were lysed for assessing the luciferase activity assay (Zhao *et al.,* 2019).

Analysis of the experimental results

Preliminary statistics of the data were performed using Excel and the statistical analysis was performed using the SPSS 13.0 (IBM Corporation, Armonk, USA) (Richardson, 2005). ANOVA and *t*-test were used to analyse the significant differences ($P<0.05$ for consequential, $P<0.01$ for highly substantial). The data was represented graphically using GraphPad Prism 6 software (Lazareno, 1994).

RESULTS

Cloning and bioinformatics analysis of Angora Rabbit FRZB

The 957 bp long CDS of the rabbit *FRZB* gene, encoding 318 amino acids was successfully cloned by PCR. The molecular weight and isoelectric point of the *FRZB* protein were predicted by the ProtParam software to be 35.6 kDa and 8.48, respectively. The amino acid sequence of *FRZB* possesses 35 negatively charged residues (Asp+Glu) and 41 positively charged residues (Arg+Lys). The molecular formula is $C_{1563}H_{2490}N_{444}O_{460}S_{24}$. The average hydrophilicity value of -0.316 indicated the *FRZB* protein to be an unstable hydrophilic protein having an instability coefficient of 50.61. SignalP4.1 predicted *FRZB* is a secreted protein containing a signal peptide (Figure 1A). TMHMM indicated *FRZB* to include a transmembrane domain (Figure 1B). Also, *FRZB* was found to interact with many proteins, such as *WIF1, SFRP1, DKK1, WNT3A, MATN3 and LRP5* (Figure 1C). Phylogenetic tree analysis using MEGA6.0 construction showed *FRZB* to have the highest homology between *Homo sapiens* and *Rattus norvegicus* (Figure 1D). The protein secondary structure is shown in Figure 1E. Netphone predicted 16 serines, six threonines and two tyrosines of the *FRZB* protein to have putative phosphorylation sites (Figure 1F).

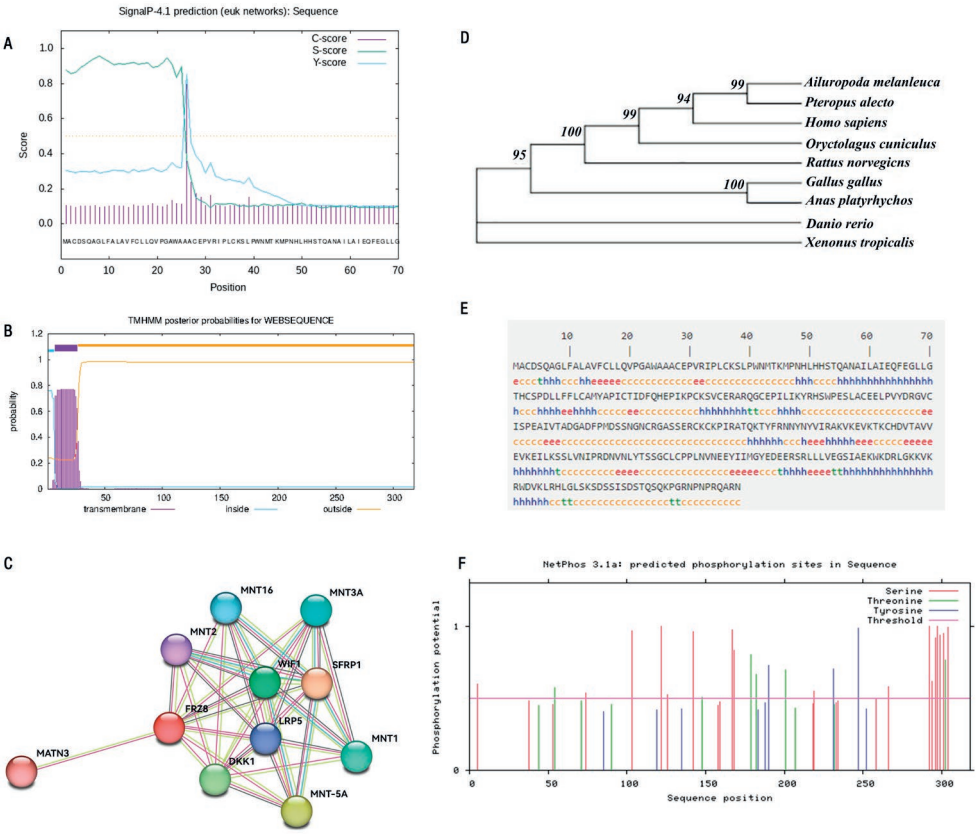

Figure 1: Analysis of the *FRZB* sequence. (A) Predicting the signal peptide of the *FRZB* protein; (B) Prediction of the transmembrane region of the *FRZB* protein; (C) Prediction of the *FRZB* phosphorylation site; (D) Phylogenetic tree analysis of the *FRZB* gene; (E) Secondary level of the *FRZB* protein structure prediction, his α-helix, e is extended chain, c is random coil; (F) Prediction of the *FRZB* phosphorylation site.

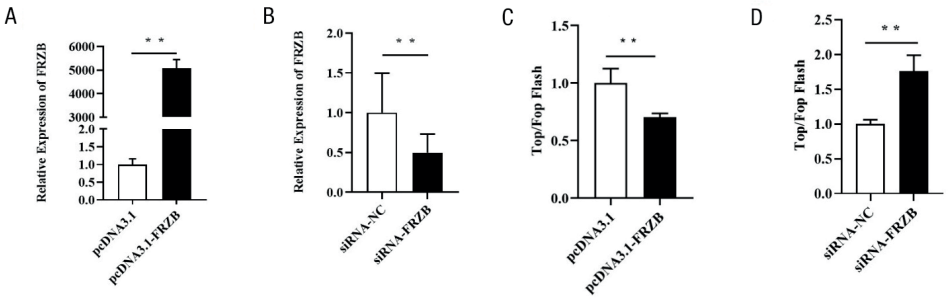

Figure 2: *FRZB* regulates the Wnt pathway activity. (A) Analysis of the effect of *FRZB* overexpression. (B) *FRZB* overexpression affects the regulation of Wnt pathway activity. (C) Analysis of the inhibitory effect of *FRZB*. (D) *FRZB* inhibition affects the regulation of the Wnt pathway activity. Inhibition **P*<0.05, ***P*<0.01.

Detecting the influence of FRZB on the Wnt signalling pathway

After constructing the pcDNA3.1-*FRZB* overexpression vector and designing and synthesising the siRNA of *FRZB*, its expression level was significantly increased or decreased after overexpression (*P*<0.05) (Figures 2A and 2C). The luciferase activity was analysed using the TOP/FOP luciferase reporter system to elucidate the regulation of Wnt signalling pathway activity by *FRZB*. The results showed that after *FRZB* overexpression, pcDNA3.1-*FRZB* was significantly lower than pcDNA3.1, which indicated that *FRZB* inhibits the transcriptional activity of β-catenin/TCF. However, when si-*FRZB* was transfected into the DP cells, it was found to be considerably higher than si-*FRZB* (*P*<0.05), indicating that it could promote the transcriptional activity of β-catenin/TCF. (Figures 2B and 2D).

FRZB regulates genes related to the Wnt signalling pathway

The mRNA expression levels of the genes related to the hair follicle cycle growth were detected after overexpressing and interfering with *FRZB* in the DP cells. The overexpression of *FRZB* was found to result in an increase in the expression levels of genes such as *SFRP2, BMP4 and WNT2* (*P*<0.01) (Figure 3A). In contrast, inhibition of FRZB was found to increase the expression of genes such as *LEF1, CCND1, DKK1* and *TCF7* (*P*<0.01) (Figure 3B). These results indicate that the *FRZB* gene is essential for hair follicle development by regulating the Wnt signalling pathway.

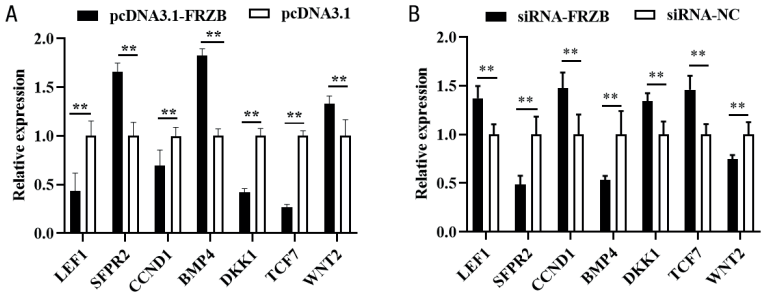

Figure 3: Effects of *FRZB* overexpression and inhibition on the *WNT* pathway-related gene expression. (A) *FRZB* overexpression down-regulated the expression of the genes *SFRP2, BMP4 and WNT2*. (B) *FRZB* was found to up-regulate the expression of the genes *LEF1, CCND1, DKK1 and TCF7*.

FRZB inhibits the proliferation and apoptosis of the DP cells

Cell proliferation was detected at 0 h, 24 h, 48 h and 72 h after *FRZB* overexpression and interference was detected by CCK-8. pcDNA3.1-*FRZB* was found to significantly inhibits the proliferation of the DP cells at 72 h after cell treatment ($P<0.01$) (Figure 4A), while siRNA-*FRZB* was found to significantly promote the expansion of the DP cells at 72 h after cell treatment ($P<0.01$) (Figure 4B). To detect the effects of overexpressing and inhibiting *FRZB* on the apoptosis of the DP cells, the FITC/PI method was used. The results showed that *FRZB* promotes the apoptosis of the long-haired rabbit DP cells and inhibits the proliferation of the DP cells. (Figures 4C and 4D).

DISCUSSION

Hair follicles are the only organs in mammals undergoing cyclical growth cycles throughout their lifespan, from organ regeneration and growth phase to the degeneration after apoptosis, regulating critical aspects of cellular and embryonic development (Chanda *et al.,* 2000). Many signalling pathways interact to control the growth and circulation of the hair follicles (Krause *et al.,* 2006). Among these signalling pathways, the Wnt pathway is widely accepted as exerting a function in hair morphology and hair follicle cycling (Myung *et al.,* 2013). Studies have identified the Wnt

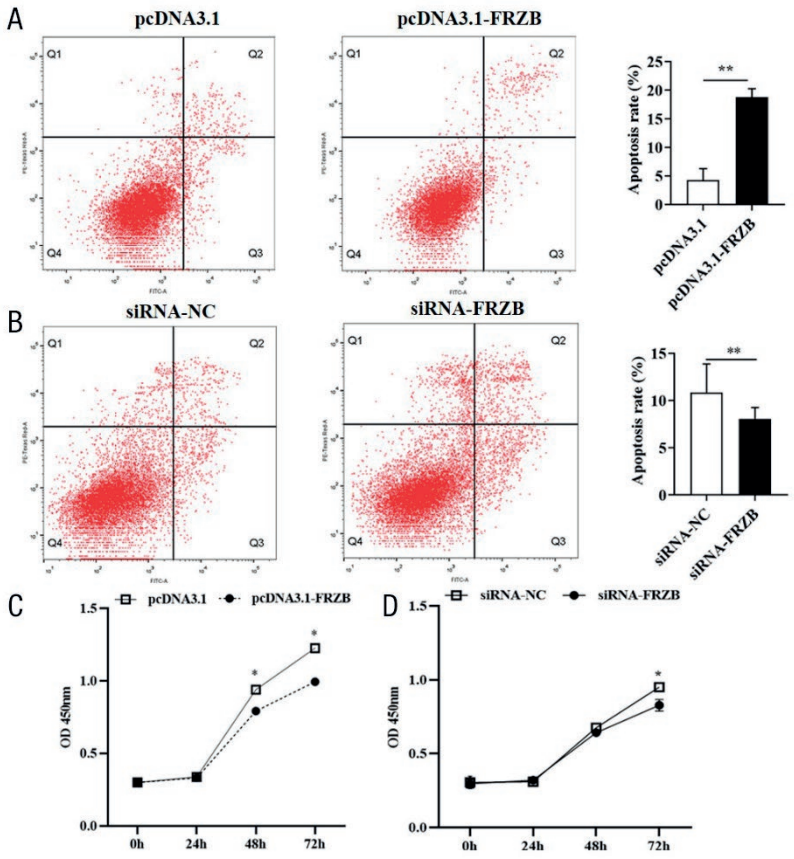

Figure 4: The effect of *FRZB* on the apoptosis and proliferation of the DP cells. (A) The development of overexpression of *FRZB* on the apoptosis of the DP cells. (B) Inhibition of apoptosis by *FRZB* in the DP cells. (C) and (D) the Cell Counting Kit-8 to detect cell proliferation.

signalling as regulating the β-catenin way of the mouse dermal papilla and play an essential role in promoting hair follicles for maintaining growth (Zhu *et al.,* 2020). This study found that the *FRZB* gene not only regulates hair follicle growth and development but also regulates skin morphogenesis. The rabbit *FRZB* sequence was cloned and the analysis indicated that the *FRZB* coding region contained 957 bp.

Bioinformatics analysis showed *FRZB* protein to be an unstable hydrophilic protein. According to the phylogenetic tree analysis, the *FRZB* gene of the rabbit has the highest homology with *Homo sapiens* and the *Rattus norvegicus* in evolution. *FRZB* is primarily an extracellularly secreted protein with a secondary structure primarily composed of random coils. According to bioinformatics analysis, *FRZB* interacts with many proteins such as *MATN3, SFRP1, WIF1, Wnt16 and DKK1*. Both *FRZB* and *DKK1* are inhibitors of the WNT/β-catenin pathway secretion (Hardy *et al.,* 2012). Studies have identified *DKK-1* as participating in the hair follicle development cycle by regulating the activity of hair follicle keratinocytes and the transition from the growth phase to the catagen phase (Kwack *et al.,* 2012). *SFRP2* regulates the growth of the hair follicles by inhibiting the activity and cell proliferation of the Wnt pathway (Kim and Yoon, 2014). *CCND1* is an essential regulator in the cell cycle which might inhibit apoptosis (Cai *et al.,* 2012). In addition, *LEF1* has been associated with hair follicle growth and its expression was found to increase during the anagen phase of hair follicle development, but attenuated during the catagen and telogen phases (Zhou *et al.,* 1995). The overexpression of *FRZB* was found to up-regulate the expression of the upstream genes *DKK1* and *SFRP2* in the WNT pathway. In contrast, the expression level of the genes was found to decrease. When FRZB was inhibited, there was an increase in the expression of *LEF1, CCND1* and *TCF7* downstream of the gene WNT pathway. The TOP/FOP luciferase reporter system detected that *FRZB* inhibits the transcriptional activity of β-catenin/TCF and regulates the downstream genes, which clarified the regulatory mechanism of *FRZB* on the Wnt pathway.

In our study, the overexpression of *FRZB* was found to inhibit the proliferation of gastric cancer cells, regulating the relationship between cell proliferation and differentiation (Qu *et al.,* 2008). *FRZB* overexpression can promote the apoptosis of DP cells and inhibit cell proliferation, suggesting that *FRZB* can inhibit the hair follicle cycle. Therefore, we believe that *FRZB* inhibits hair follicle growth, regulates the expression of the Wnt pathway-related genes and inhibits the activity of the Wnt pathway.

CONCLUSION

We successfully amplified the complete FRZB sequence for bioinformatics analysis. The *FRZB* gene was overexpressed to detect hair follicle development-related genes, suggesting that it plays a repressive role in hair follicle development. This study shows that *FRZB* regulates the growth cycle of rabbit hair follicles, which can provide a reference for further research on the molecular function of *FRZB*.

Acknowledgements: Our study was funded by the National Natural Science Foundation of China (Grant No. 32072724), the Modern Agricultural Industrial System Special Funding (CARS-43-A-1) and the Zhejiang Science and Technology Major Programme on Agricultural New Variety Breeding (2021C02068-7).

Authors contribution: Wang F.: writing-original draft, conceptualization and methodology. Zhang X.: validation and formal analysis. Dai Y.: validation and formal analysis. Zhao B.: investigation and resources. Wu X.: resources, project administration and funding acquisition. Chen Y.: writing – review & editing, project administration and funding acquisition.

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