

GENETICS AND MOLECULAR BIOLOGY

The Wnt/ β -catenin signaling pathway is involved in regulating feather growth of embryonic chicks

W. Y. Xie, M. J. Chen, S. G. Jiang, H. C. Yan, X. Q. Wang and C. Q. Gao¹

College of Animal Science, South China Agricultural University/Guangdong Provincial Key Laboratory of Animal Nutrition Control/Key Laboratory of Chicken Genetics, Breeding and Reproduction, Ministry of Agriculture, Guangzhou 510642, China

ABSTRACT Avian feathers have robust growth and regeneration capability and serve as a useful model for decoding hair morphogenesis and other developmental studies. However, the molecular signaling involved in regulating the development of feather follicles is unclear. The purpose of this study was to investigate the role of the Wnt/ β -catenin pathway in regulating feather morphogenesis in embryonic chicks through *in ovo* injection of different doses of Dickkopf-1 (DKK1, a specific inhibitor of the target of the Wnt/ β -catenin pathway). A total of 120 fertilized embryo eggs were randomly divided into 4 treatments, including a noninjection group (control group) and groups injected with 100 μ L of phosphate-buffered saline (PBS)/egg (PBS control group), 100 μ L of PBS/egg containing 600-ng DKK1/egg (600-ng DKK1 group), and 100- μ L PBS/egg containing 1,200-ng DKK1/egg (1,200-ng DKK1 group). Feathers and skin tissues were sampled on embryonic (E) day 15 and the day of hatching to examine the feather mass, diameter and density of

feather follicles, and the protein expression of the Wnt/ β -catenin pathway. The results showed that, compared with CON and PBS treatment, the injection of DKK1 into the yolk sac of chick embryos had no significant effect on the hatching rate and embryo weight ($P > 0.05$), while it significantly decreased the relative mass of feathers in the whole body ($P < 0.05$). The high dose of DKK1 (1,200-ng DKK1/egg) decreased the relative mass of feathers on the back, chest, belly, neck, wings, head, and legs, which was more obvious than that in the 600-ng DKK1 group, which presented a dose-dependent effect. In addition, DKK1 injection significantly downregulated the protein expression levels of β -catenin, transcription factor 4, Cyclin D1, and c-Myc ($P < 0.05$). The immunofluorescence result of β -catenin was consistent with the Western blotting assay results. Altogether, these observations suggested that the Wnt/ β -catenin signaling pathway is involved in regulating feather follicle development and feather growth during the embryonic development of chicks.

Key words: feather growth, *in ovo* injection, DKK1, Wnt/ β -catenin, chicks

2020 Poultry Science 99:2315–2323

<https://doi.org/10.1016/j.psj.2020.01.002>

INTRODUCTION

Feathers play a crucial role in thermoregulation, energy metabolism, and self-protection of birds. Feather growth and development are important characteristics of poultry in commercial environments. However, nearly 15% of chickens have different degrees of feather development problems in large-scale poultry production. Such problems have seriously affected energy efficiency,

the appearance of living bodies, and the quality of carcasses.

Avian feathers consist of terminally differentiated keratinocytes with complex structures (Lin, et al., 2013; Feo, et al., 2016; Li, et al., 2017). Feather development begins in the feather follicle, which is a complex miniorgan composed of epidermal and mesenchymal (dermal) components, that controls feather growth, replacement, and morphological structure (Yue, et al., 2012; Moller and Nielsen, 2017) and undergoes the growth, regression, and resting phases (Bai, et al., 2015; Chen, et al., 2015). Throughout the feather (hair) cycle, the growth of feather (hair) follicles is regulated by the interaction between adjacent dermal papilla and stem cells (Hardman, et al., 2015; Wang, et al., 2017), and many factors are involved in the process (Krause and Foitzik, 2006).

Previous studies have shown that several signals regulate the growth and development of hair follicles, such as

© 2020 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received July 25, 2019.

Accepted January 20, 2020.

The authors declare no competing or financial interests.

¹Corresponding author: cqgao@scau.edu.cn

Wnt/ β -catenin (Lei, et al., 2017), bone morphogenetic protein (Zhang, et al., 2010), fibroblast growth factor (Cheng, et al., 2018), and other factors, such as transforming growth factor- β signaling (Chen, et al., 2016). Moreover, our previous study found that the Wnt/ β -catenin signaling pathway-related proteins were upregulated during feather morphogenesis in embryonic chicks. However, whether the Wnt/ β -catenin signaling pathway directly regulates feather follicle morphogenesis is unclear.

Dickkopf-related protein 1 (DKK1), which interacts with the Wnt receptor lipoprotein receptor-related protein 5/6 (LRP5/6), is a secreted antagonist of the canonical Wnt signaling pathway (Li, et al., 2010; Ahn, et al., 2011). In the presence of DKK1, β -catenin is phosphorylated and cannot bind to intranuclear lymphoid enhancing factor (LEF)/transcription factor 4 (TCF4) transcription factors (Zorn, 2001; Kawano and Kypta, 2003). This situation inhibits the downstream regulation of the cell cycle and related target genes, blocking the Wnt signaling pathway (Clevers, 2006).

To better understand the feather development processes and the contribution of the Wnt/ β -catenin signaling pathway to these processes, our present study aimed to investigate the molecular mechanism by which the Wnt/ β -catenin signaling pathway regulates feather follicle morphogenesis and feather growth by *in ovo* injection of different doses of DKK1.

MATERIAL AND METHODS

Experimental Animals

Fertilized eggs were purchased from a commercial chicken farm (Qingyuan Chickens Company, Guangdong, China). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of South China Agricultural University (Guangzhou, China), and all experimental methods and management procedures were approved by the Animal Care Committee of South China Agricultural University (Guangzhou, China).

Experimental Design and Treatments

A total of 160 fertilized eggs were incubated in an incubator (Keyu, Shandong, China) with a relative humidity and temperature of 60% and 37.8°C, respectively. A regular overturn of the eggs was continued for 180 s every 2 h until embryonic day 18, and the eggs were transferred to the same hatchery for the last 3 D of incubation. On day 6 of incubation, eggs were candled, and those that were infertile or contained early dead embryos were removed. Then, a total of 120 fertilized eggs (46.3 ± 0.3 g) were divided into 4 treatment groups of 30 eggs per group. The feather buds began to form on embryonic day (E) 9, and a previous study has demonstrated that *in ovo* injection at E9 has no effect on the shelling rate (Sun, et al., 2018). Therefore, all *in ovo* injection solutions were

freshly prepared at 9 D of incubation, and the solutions were injected into the egg yolk sac.

The 4 treatment groups consisted of (1) a noninjected group (negative control, CON group), (2) injection with 100- μ L phosphate-buffered saline (PBS)/egg (positive control, PBS group), (3) injection with 100 μ L of PBS/egg containing 600-ng DKK1/egg (600-ng DKK1 group), and (4) injection with 100 μ L of PBS/egg containing 1,200-ng DKK1/egg (1,200 ng DKK1 group).

Preparation of DKK1 Solutions and *in ovo* Injection Procedure

DKK1 (#5439-DK-CF, R&D Systems, Minneapolis, MN) was dissolved in PBS, and the injected solution was filtered using a 0.22- μ m syringe filter.

The *in ovo* injection procedure was performed according to the study by Sun et al. (2018) with modifications. Under the candle, the embryo, which appeared as a dark floating silhouette with the head as a dark spot, was located. At 2/3 of the way from the large end to the small end of each egg with a yolk sac, the target site of injection on the shell was disinfected with an alcohol swab (Supplementary Figure 1). A small hole was drilled on the injection site. Using a needle (0.5 mm \times 38 mm), 100 μ L of injection solutions was injected into the yolks (depth, 2 cm) to provide a dose of 0, 600, or 1,200 ng DKK1/egg. The pinhole site on the shell was immediately sealed with paraffin wax. After that, eggs were returned to the incubator. All eggs were held outside the incubator for less than 30 min while injected. The eggs from the noninjected positive control group remained outside the incubator for the same length of time.

Sampling and Measurement

Feathers and skin tissue samples were obtained on E15 and the day of hatching (DOH), and all hatched chicks were counted and then weighed to obtain the chick hatch weight on the DOH. The numbers of failed pips, healthy chicks, and weak chicks were also recorded. Hatchability was calculated as a percentage of hatched birds out of the total number of injected eggs for each treatment. Part of the skin tissue was frozen in liquid nitrogen and stored at -80°C for Western blotting, and some was stored in 10% buffered formalin for further histological processing.

When feathers were collected, those from the back, chest, belly, neck, leg, head, and wing were separated. The back was defined as above the clavicle and above the wing; the chest was the area below the neck and above the belly; the belly referred to the part below the xiphoid and the ischia bone; the leg was from the ankle to the hip; the neck was based on the leading edge of the trapezius; the wing was from the shoulder joint to the edge of the wing; and finally, the head was all organs above the neck (cervical spine). Then, the feathers were placed in an oven, dried at 65°C for 24 h, and weighed with an electronic balance (Precisa,

Dietikon, Switzerland; precision 0.0001 g). The whole-body relative feather mass (or each zone's relative feather mass, %) was calculated as follows: (The absolute feather mass/the body weight of chicks on E15/DOH) \times 100.

Histological Processing

Skin samples were obtained from chick embryos after the embryos were preserved in 10% neutral buffered formalin for approximately 24 h. Skin specimens of approximately 1.5 cm² were excised from the back, neck, chest, leg, and belly and stored in 10% neutral buffered formalin for tissue sectioning and hematoxylin and eosin (H&E) staining.

The tissue was placed into the labeled embedding box, dehydrated, cleared in xylene (transparent), and impregnated with wax. The tissue was subjected to the following series of solutions: 75% ethanol for 2 h, 85% ethanol for 2 h, 95% ethanol for 2 h, 100% ethanol 3 times for 1 h each, 50% ethanol + 50% xylene for 1.5 h, and xylene for 1.5 h. It was dipped in wax for 2 h in a paraffin-embedding processor (Jinhua Kedi Instrumental Equipment Co., Ltd., Zhejiang, China), and it was finally embedded. Serial longitudinal and transverse sections of skin were cut at the desired thickness of 5 μ m with a Thermo-HM 3401 (Thermo Scientific). Then, the sections of skin samples were transferred to dry overnight on a dryer (HI CAPACITY, Thermo Scientific) and stored at room temperature until ready for use.

H&E Staining

After the sections were mounted on slides, a modification of the H&E staining protocol described by Heidelberg (2012) was applied. A drop of Permount was placed over the tissue on each slide, and a coverslip was added. Then, the slides were viewed using a Nikon microscope (Ti-2u, Tokyo, Japan).

Immunofluorescence Analysis and Quantification

Skin tissues were fixed in 4% paraformaldehyde, washed with PBS containing 100 mmol/L glycine, and the paraffin sections deparaffinized. The sections of skin samples were incubated with β -catenin (1:500 in antibody diluent; #6302, Abcam, Cambridge, MA) overnight at 4°C. After washing with PBS 6 times for 5 min each time, goat antirabbit IgG (E030120) fluorescein isothiocyanate-conjugated antibody (1:400 dilution, San Francisco, CA) was added for 30 min. The nuclei were stained with 4', 6-diamidino-2-phenylindole (Sigma-Aldrich) for 5 min at room temperature. Fluorescence signals were observed with a fluorescence microscope (NIS-Elements, Nikon, Japan). Statistical analysis of the fluorescence signal intensity was achieved using the Image J software (version 1.8.0 112; National

Institute of Health, Bethesda, MD) according to the method reported by Zhou et al. (2019).

Western Blotting Analysis

Protein samples from skin tissues were extracted using radio immunoprecipitation assay lysis buffer. Proteins were separated on 10% SDS-polyacrylamide gels by electrophoresis as described previously (Xie, et al., 2019). The membranes were blocked with 2.5% skim milk and incubated with primary antibodies (1:1000 dilution) at 4°C overnight. The antibodies used were as follows: anti- β -catenin (#6302) was obtained from Abcam (Cambridge, MA); anti-TCF4 (#13027), anti-Cyclin D1 (#753), and anti- β -actin (#47778) were obtained from Santa Cruz Biotechnology (Dallas, TX); and anti-c-Myc (#5605) was obtained from Cell Signaling Technology (Beverly, MA). Then, the membranes were incubated with antimouse IgG (E030110, San Francisco, CA) and antirabbit IgG (E030120, San Francisco, CA) secondary antibodies. Proteins were visualized using the electrochemiluminescence-plus chemiluminescence detection kit (Beyotime Institute of Biotechnology, Beyotime, Shanghai, China). Enhanced chemiluminescence signals were scanned using a FluorChem M apparatus (Protein

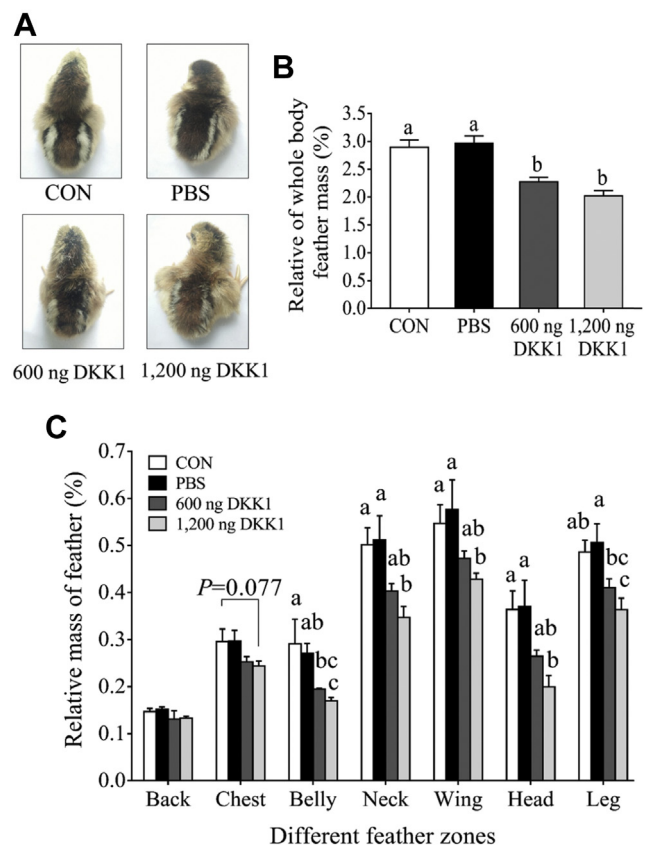


Figure 1. *In ovo* injection of DKK1 inhibits feather growth on the day of hatching (DOH) of broiler chicks. (A) Visual observation of broiler chicks. (B) The relative whole-body feather mass. (C) The relative feather mass in different feather zones. Values are presented as the means \pm standard error of the mean (SEM). Different letters in a column indicate a significant difference ($P < 0.05$, $n = 3$).

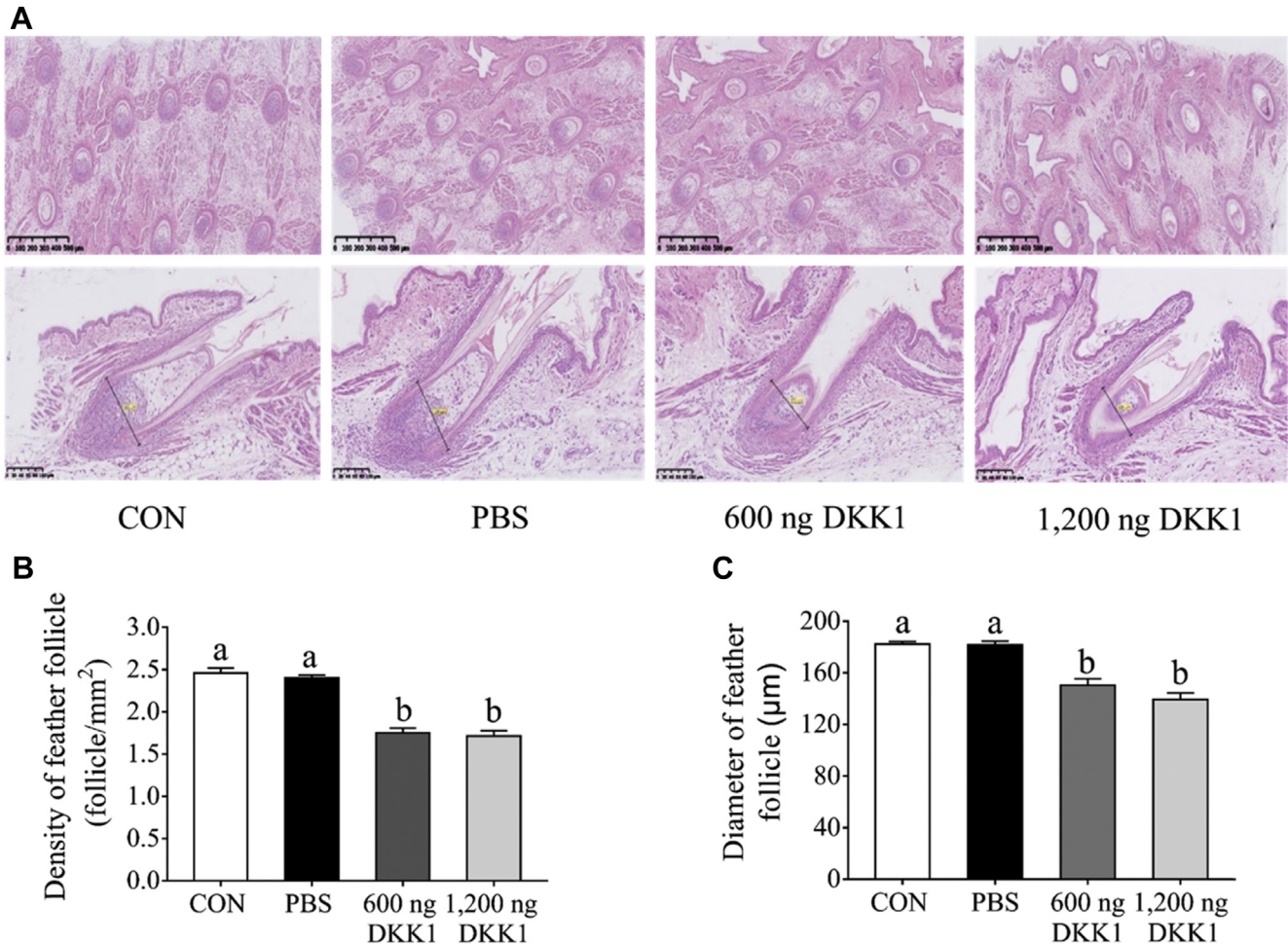


Figure 2. *In ovo* injection of DKK1 inhibits feather follicle development on the day of hatching (DOH) of broiler chicks. (A) The upper layer is a transverse section of the feather follicle. According to this method, the number of feather follicles was counted, and the result is presented in (B). Scale bars = 500 μ m; multiple: 50 \times ; H&E. The lower layer is the longitudinal section of feather follicles, and the diameter of feather follicles is indicated by black arrows, and the result is presented in (C). Scale bars = 100 μ m; multiple: 200 \times ; H&E. Values are presented as the means \pm standard error of the mean (SEM). Different letters in a column indicate a significant difference ($P < 0.05$, $n = 3$).

Simple, Inc., Santa Clara, CA), and the density of the bands was analyzed using ImageJ Analysis Software.

Observation and Quantitative Measurements

Using a Nikon biological microscope (Ti-2u, Tokyo, Japan), we first observed and photographed the distribution of feather follicles from each of the longitudinal skin sections to measure the diameter of feather follicles from 10 fields at a magnification of 200 \times at E15 and the DOH. In addition, the density of feather follicles (follicles/mm²) was counted from 10 fields of each transversal section at a magnification of 50 \times (or 100 \times) and an actual field area of 1.75 mm² (or 7 mm²). The diameter of a feather follicle was measured from the outermost layer of the follicle, where the maximum diameter was observed.

Statistical Analysis

Statistical tests were performed with SAS (version 9.2; SAS Institute Inc., Cary, NC), and the figures were made

using the GraphPad Prism software (version 7; GraphPad, La Jolla, CA). Statistical significance was determined by a *t* test or one-way ANOVA, with corrections for multiple comparisons, as appropriate. The values are presented as the means \pm standard error of the mean. $P < 0.05$ was considered statistically significant.

RESULTS

DKK1 Inhibits Feather Follicle Development and Feather Growth

The results of the embryo injection assay demonstrated that *in ovo* injection of DKK1 significantly decreased the relative mass of feathers of yellow-feathered broilers at E15 (Supplementary Figures 2A–2C) and the DOH (Figures 1A–1C) in comparison to that of the CON and PBS groups, while it had no significant effect on the hatching rate or hatching weight (Supplementary Table 1). Compared with the CON and PBS groups, DKK1 injection significantly decreased the relative mass of whole-body feathers (Supplementary Figure 2B, Figure 1B $P < 0.05$). In addition, in our present study, injection of

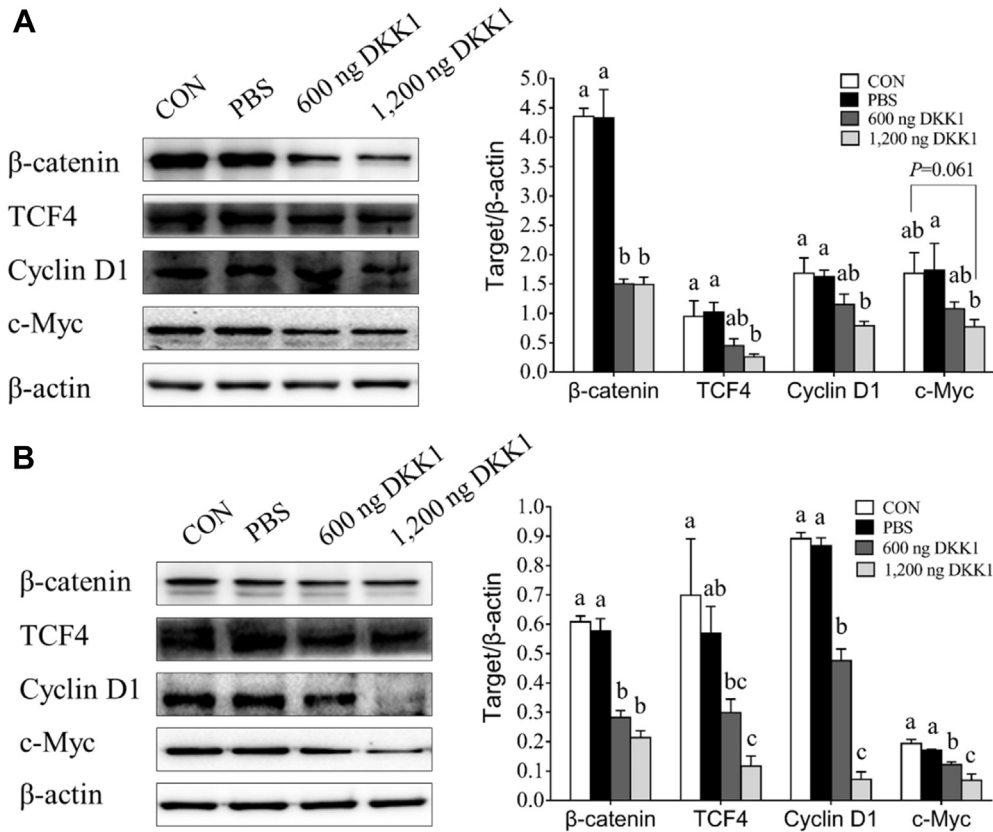


Figure 3. *In ovo* injection of DKK1 inhibits the Wnt/ β -catenin signaling pathway. Western blotting images of β -catenin, TCF4, c-Myc and Cyclin D1 in belly (A) and neck (B) skin feather follicle tissues. β -actin was used as a control. Statistical significance was determined by *t* test. The results are expressed as the means \pm SEM, and different letters in a column indicate a significant difference ($P < 0.05$, $n = 9$).

high doses of DKK1 (1,200 ng/egg) significantly decreased the relative mass of feathers on the back, chest, belly, neck, wing, and legs compared to that of the CON and PBS groups at E15 (Supplementary Figure 2C, $P < 0.05$), while injection of 600 ng or 1,200 ng DKK1/egg did not affect the relative mass of head feathers (Supplementary Figure 2C, $P > 0.05$).

At the time of hatching, we also found that the 1,200-ng DKK1 group had a significantly decreased relative mass of feathers on the belly, neck, wings, head, and legs compared with that of the CON and PBS groups (Figure 1C, $P < 0.05$), and there was a tendency toward a decreased relative mass of chest feathers (Figure 1C, $P = 0.077$). However, 600-ng/egg DKK1 injection did not decrease the relative mass of feathers (Figure 1C, $P > 0.05$). These results suggested that the effect of *in ovo* injection of DKK1 on the relative mass of feathers has a time-dose effect.

Furthermore, we performed H&E staining to calculate feather follicle density and diameter after *in ovo* injection of DKK1 (Supplementary Figure 3A; Figure 2A). The current results showed that the injection of 600-ng or 1,200-ng DKK1/egg significantly decreased the feather follicle density and feather follicle diameter of chicks compared with those of the CON and PBS groups at E15 (Supplementary Figures 3B, 3C, $P < 0.05$) and the DOH (Figures 2B, 2C, $P < 0.05$). The density of feather follicles significantly decreased from 2.46/mm² (or 8.68/mm²) in the CON group to 1.71/mm² (or

3.24/mm²) in the 1,200 ng DKK1/group on the DOH (or E15) (Figure 2B; Supplementary Figure 3B, $P < 0.05$), while the diameter of feather follicles significantly decreased from 182.29 μ m (or 130.67 μ m) to 139.29 μ m (or 98.00 μ m) on the DOH (or E15) (Figure 2C; Supplementary Figure 3C, $P < 0.05$). These findings suggested that *in ovo* injection of DKK1 significantly inhibits feather follicle development in chicks.

DKK1 Decreases the Protein Expression of the Wnt/ β -Catenin Signaling Pathway

Then, we investigated whether DKK1 blocks the activation of the Wnt/ β -catenin pathway in skin feather follicles of chicks. At E15, we found that the high dose of DKK1 (1,200 ng DKK1/egg) significantly downregulated the expression levels of β -catenin and its downstream target proteins TCF4, Cyclin D1, and c-Myc in the skin feather follicle tissues (Supplementary Figures 4A, 4B, $P < 0.05$). In addition, the results of Western blotting showed that the 600-ng and 1,200-ng DKK1/egg groups significantly downregulated the expression levels of β -catenin and its downstream targets TCF4, Cyclin D1, and c-Myc in the feather follicle tissues of belly and neck skin on the DOH (Figure 3A, 3B, $P < 0.05$), which were consistent with the results of feather mass. These findings demonstrated that DKK1 inhibited feather follicle formation and feather

growth by blocking the expression of β -catenin and its downstream targets, and it had a time-dose effect.

In line with the aforementioned findings, we validated the expression level of β -catenin on the DOH by immunofluorescence, as shown in Figure 4. Compared with the results in the CON and PBS groups, the β -catenin fluorescence signal decreased in the 600-ng and 1,200-ng DKK1 injection groups and was mainly expressed in the epidermal layer. In the CON and PBS groups, the expression level of β -catenin was higher, which was consistent with the Western blotting results, and was expressed in the follicle sheath, feather sheath, and epidermis. Generally, the expression level of nuclear and cytoplasmic β -catenin was downregulated in response to DKK1, which further suggested that DKK1 inhibited feather follicle development and feather growth by blocking Wnt-mediated activation of β -catenin signaling.

DISCUSSION

A previous study showed that overexpression of DKK1 inhibits the early development of hair follicles and hair growth (Andl, et al., 2002). In the present study, we found that *in ovo* injection of 600-ng or 1,200-ng DKK1 into the embryo significantly decreased the relative mass of feathers and the diameter and density of feather follicles in chicks compared with those of the CON and PBS control groups. These results are consistent with previous observations that overexpression of DKK1 in embryonic feathers inhibited feather follicles (Chang, et al., 2004). In addition, DKK1 injection into the hypodermis of mice during the anagen phase resulted in premature onset of catagen and led to decrease hair follicle length (Mi, et al., 2012). In addition, hair follicle stem cell treatment with DKK1 led to the inhibition of hair shaft elongation (Jeong, et al., 2017). We hypothesized that DKK1 inhibits feather follicle development and feather growth by inhibiting classical Wnt/ β -catenin signaling in skin feather follicle tissue, during which DKK1 binds to the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) receptor and facilitates its internalization, thereby obstructing the interaction between Wnt ligands and their receptors (Niehrs, 2006). Previous studies have demonstrated that DKK1 regulates hair follicle density, hair end diameter (Becker, et al., 2011), hair follicle diameter (Qiu, et al., 2017), and hair follicle periodic circulation by inhibiting canonical Wnt signaling (Mi, et al., 2012; Kim, et al., 2014). These results, in combination with our findings, revealed the significant role of β -catenin in feather follicle development and feather growth.

To validate our viewpoint, we determined the skin follicle tissue protein expression levels of the Wnt/ β -catenin signaling pathway by Western blotting and immunofluorescence. The results showed that the expression level of β -catenin was reduced after injection of DKK1 into embryo eggs compared with

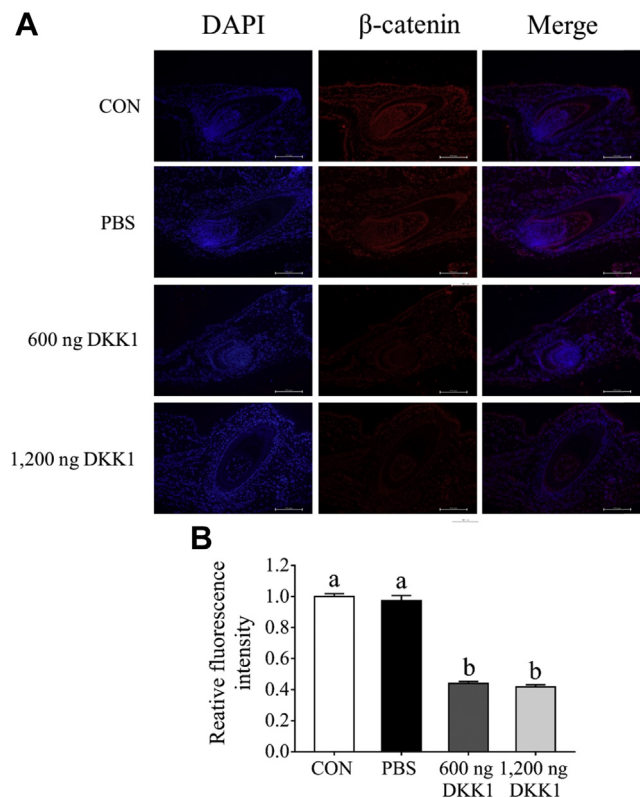


Figure 4. Immunofluorescence images of β -catenin in the neck skin tissue after *in ovo* injection of DKK1. (A) Longitudinal sections of skin of 5 μ m were fluorescently marked using an antibody for β -catenin, with DAPI as the nuclear counterstain. (B) Statistical analysis of the fluorescence signal intensity in feather follicle based on the images shown in (A). Scale bars = 100 μ m; multiple: 200 \times . The data are mean \pm SEM (n = 3).

expression in the CON and PBS control groups. These findings are in accordance with the results of Kim et al. (2014), who revealed that DKK1 downregulated the expression of β -catenin in hair follicles. Our present study also found that the expression levels of the downstream targets TCF4, Cyclin D1, and c-Myc were significantly downregulated by treatment with DKK1. These results indicated that the Wnt/ β -catenin signaling pathway is involved in regulating feather follicle development. Cyclin D1 plays a key role in the regulation of cell cycle progression, and the TCF4 binding site is located in its promoter region, indicating that the β -catenin/TCF4 pathway is involved in the regulation of Cyclin D1 expression (Lin, et al., 2000). To date, c-Myc expression has been reported only in human and mouse hair follicle cycles (Bull, et al., 2001; Barajon, et al., 2015) and plays a role in the proliferation or differentiation of hair follicle keratinocytes at different stages of mouse hair follicle morphogenesis (Wang, et al., 2012). Our findings are in agreement with previous studies, which reported that DKK1 blocked canonical Wnt-mediated activation of β -catenin signaling (Bafico, et al., 2001; Mi, et al., 2012; Lim, et al., 2014). Collectively, these results imply that DKK1 acts as a growth inhibitor of feather follicle development and feather growth by

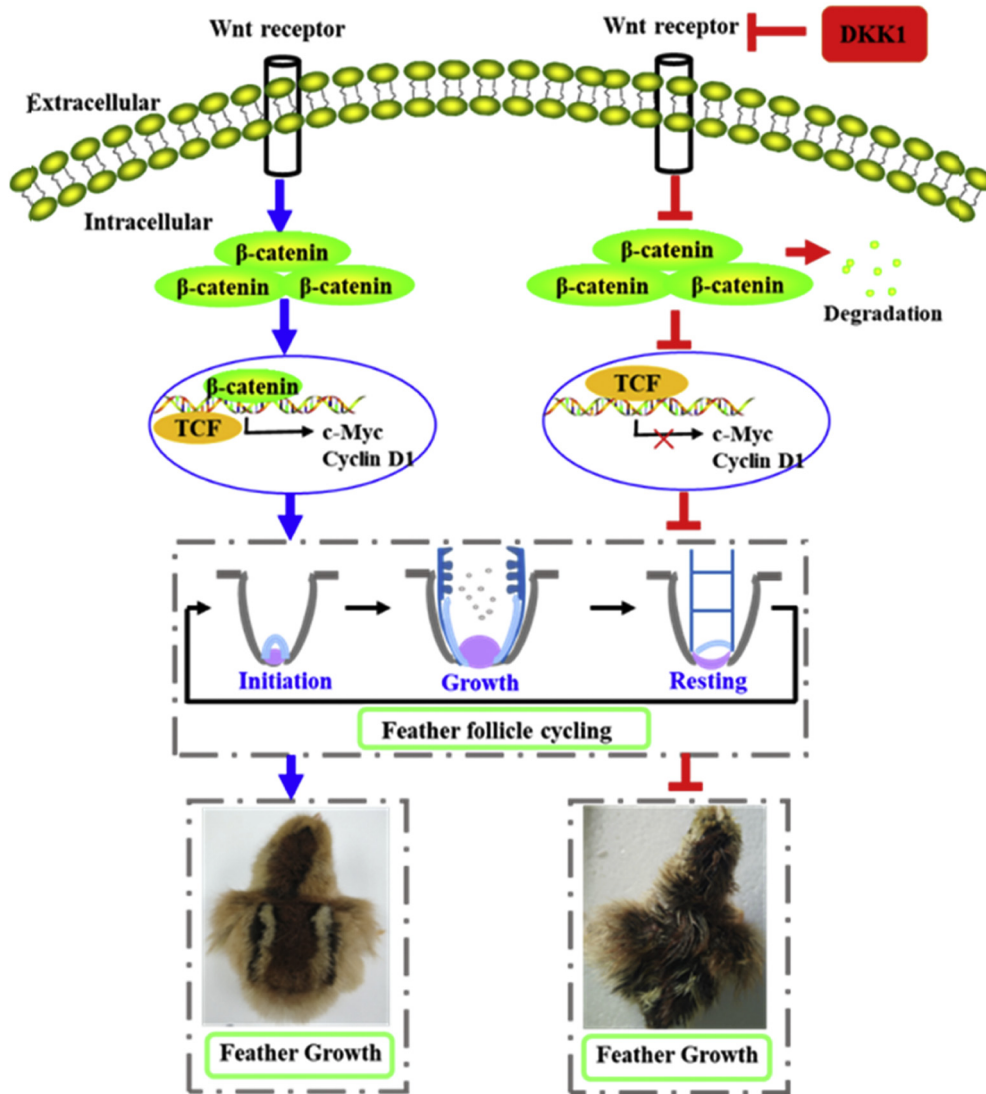


Figure 5. Schematic showing the effects of the Wnt/ β -catenin signaling pathway and DKK1 on feather follicle morphogenesis and feather growth in broiler chicks. “ \rightarrow ” represents promotion, “ \dashv ” represents suppression.

blocking the Wnt/ β -catenin signaling pathway (Figure 5).

A previous study showed that DKK1 affects the development of teeth, the head, and eyes, which is regulated by the Wnt/ β -catenin signaling pathway (Wang, et al., 2014; Liu, et al., 2017). However, in the present study, although phenotypic changes were found in feather growth, we did not detect that the injection of DKK1 had a significant effect on the organ index or hatching embryo weight. The difference among the studies might be because the injection dose of DKK1 is low, and the low dose of DKK1 could not affect the development of other tissues.

Recently, nutrition scientists at home and abroad have carried out a large number of *in ovo* feeding studies. This is because the method of *in ovo* feeding technology has many advantages. Most studies have confirmed that *in ovo* feeding has significant effects on increasing chick glycogen storage, improving intestinal development, and promoting muscle and bone

growth (Zhang, et al., 2016; Kermanshahi, et al., 2017; Yu, et al., 2018). However, because the extent of penetration and decay of DKK1 in eggs are unclear, further studies using a gene delivery system, such as replication competent avian sarcoma (RCAS) virus infection (Chang, et al., 2004), are needed to further explore how (directly or indirectly) the Wnt/ β -catenin signaling pathway regulates the feather growth of chicks.

CONCLUSION

We can conclude that *in ovo* injection of DKK1 significantly inhibits feather follicle development and feather growth by blocking the Wnt/ β -catenin signaling pathway in chicks, indicating that the signaling pathway is involved in mediating the feather follicle development and feather growth of broiler chicks.

Funding: This work was supported by the Natural Science Foundation of Guangdong Province, China

(2018B030315001), the National Natural Science Foundation of China (31972585), the Pearl River Technology Science and Technology Nova Projects of Guangzhou, China (201710010110), Guangdong Provincial Promotion Project on Preservation and Utilization of Local Breed of Livestock and Poultry, and the Technical System of Poultry Industry of Guangdong Province, China (2019KJ128).

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.01.002>.

REFERENCES

- Ahn, V. E., M. L. Chu, H. J. Choi, D. Tran, A. Abo, and W. I. Weis. 2011. Structural basis of wnt signaling inhibition by dickkopf binding to lrp5/6. *Dev. Cell* 21:862–873.
- Andl, T., S. T. Reddy, T. Gaddapara, and S. E. Millar. 2002. WNT signals are required for the initiation of hair follicle development. *Dev. Cell* 2:643–653.
- Bafico, A., G. Liu, A. Yaniv, A. Gazit, and S. A. Aaronson. 2001. Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat. Cell Biol.* 3:683–686.
- Bai, X., M. Lei, J. Shi, Y. Yu, W. Qiu, X. Lai, Y. Liu, T. Yang, L. Yang, and R. B. Widelitz. 2015. Roles of GasderminA3 in catagen-telogen transition during hair cycling. *J. Invest. Dermatol.* 135:2162–2172.
- Barajon, L., C. Rumio, E. Donetti, A. Imberti, M. Brivio, and P. Castano. 2015. Pattern of expression of c-Myc, Max and Bin1 in human anagen hair follicles. *Br. J. Dermatol.* 144:1193–1203.
- Becker, M. R., Y. S. Choi, S. E. Millar, and M. C. Udey. 2011. Wnt signaling influences the development of murine epidermal Langerhans cells. *J. Invest. Dermatol.* 131:1861–1868.
- Bull, J. J., S. Mullerover, S. V. Patel, C. M. Chronnell, I. A. McKay, and M. P. Philpott. 2001. Contrasting localization of c-Myc with other Myc superfamily transcription factors in the human hair follicle and during the hair growth cycle. *J. Invest. Dermatol.* 116:617–622.
- Chang, C. H., T. X. Jiang, C. M. Lin, L. W. Burrus, C. M. Chuong, and R. Widelitz. 2004. Distinct Wnt members regulate the hierarchical morphogenesis of skin regions (spinal tract) and individual feathers. *Mech. Dev.* 121:157–171.
- Chen, C. C., M. V. Plikus, P. C. Tang, R. B. Widelitz, and M. C. Cheng. 2016. The modulatable stem cell niche: tissue interactions during hair and feather follicle regeneration. *J. Mol. Biol.* 428:1423–1440.
- Chen, C. F., J. Foley, P. C. Tang, A. Li, T. X. Jiang, P. Wu, R. B. Widelitz, and M. C. Cheng. 2015. Development, regeneration, and evolution of feathers. *Annu. Rev. Anim. Biosci.* 3:169–195.
- Cheng, D., X. Yan, G. Qiu, J. Zhang, H. Wang, T. Feng, Y. Tian, H. Xu, M. Wang, and W. He. 2018. Contraction of basal filopodia controls periodic feather branching via Notch and FGF signaling. *Nat. Commun.* 9:1345–1355.
- Clevers, H. 2006. Wnt/ β -catenin signaling in development and disease. *Cell* 127:469–480.
- Feo, T. J., E. Simon, and R. O. Prum. 2016. Theory of the development of curved barbs and their effects on feather morphology. *J. Morphol.* 277:995–1013.
- Hardman, J. A., I. S. Haslam, N. Farjo, B. Farjo, and R. Paus. 2015. Thyroxine differentially modulates the peripheral clock: lessons from the human hair follicle. *PLoS One* 10:e0121878.
- Heidelberg, S. B. 2012. Hematoxylin and Eosin, 3ed. Springer Berlin Heidelberg, pp. 3984–4071.
- Jeong, H. S., H. K. Mi, M. K. Kim, J. C. Kim, and Y. K. Sung. 2017. Attenuation of Dickkopf 1-induced hair growth inhibition in cultured human hair follicles by tianeptine. *Ann. Dermatol.* 29:102–105.
- Kawano, Y., and R. Kypta. 2003. Secreted antagonists of the Wnt signalling pathway. *J. Cell Sci.* 116:2627–2634.
- Kermanshahi, H., A. Golian, N. K. Emami, A. Daneshmand, D. G. Tabari, and S. A. Ibrahim. 2017. Effects of in ovo injection of threonine on hatchability, intestinal morphology, and somatic attributes in Japanese quail (*Coturnix japonica*). *J. Appl. Anim. Res.* 45:437–441.
- Kim, B. K., H. Y. Lee, I. Kim, K. Choi, J. Park, and S. K. Yoon. 2014. Increased expression of Dkk1 by HR is associated with alteration of hair cycle in hairpoor mice. *J. Dermatol. Sci.* 74:81–87.
- Krause, K., and K. Foitzik. 2006. Biology of the hair follicle: the basics. *Semin. Cutan. Med. Surg.* 25:2–10.
- Lei, M., L. J. Schumacher, Y. C. Lai, W. T. Juan, C. Y. Yeh, P. Wu, T. X. Jiang, R. E. Baker, R. B. Widelitz, and L. Yang. 2017. Self-organization process in newborn skin organoid formation inspires strategy to restore hair regeneration of adult cells. *Proc. Natl. Acad. Sci. USA.* 114:E7101.
- Li, A., S. Figueroa, T. X. Jiang, P. Wu, R. Widelitz, Q. Nie, and C. M. Chuong. 2017. Diverse feather shape evolution enabled by coupling anisotropic signalling modules with self-organizing branching programme. *Nat. Commun.* 8: ncomms14139.
- Li, Y., W. Lu, T. D. King, C. C. Liu, G. N. Bijur, and G. Bu. 2010. Dkk1 stabilizes wnt co-receptor lrp6: implication for wnt ligand-induced lrp6 down-regulation. *PLoS One* 5:e11014.
- Lin, M. H., C. Leimeister, M. Gessler, and R. Kopan. 2000. Activation of the Notch pathway in the hair cortex leads to aberrant differentiation of the adjacent hair-shaft layers. *Development* 127:2421–2432.
- Lin, S. J., R. B. Widelitz, Z. Yue, A. Li, X. Wu, T. X. Jiang, P. Wu, and C. M. Chuong. 2013. Feather regeneration as a model for organogenesis. *Dev. Growth Differ.* 55:139–148.
- Liu, T., L. Zhang, Y. Wang, H. Zhang, L. Li, and X. Bao. 2017. Dickkopf-1 inhibits wnt3a-induced migration and epithelial-mesenchymal transition of human lens epithelial cells. *Exp. Eye Res.* 161:43–51.
- Moller, A. P., and J. T. Nielsen. 2017. The trade-off between rapid feather growth and impaired feather quality increases risk of predation. *J. Ornithol.* 159:1–7.
- Mi, H. K., M. K. Kim, J. C. Kim, and Y. K. Sung. 2012. Dickkopf 1 promotes regression of hair follicles. *J. Invest. Dermatol.* 132:1554–1560.
- Niehrs, C. 2006. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* 25:7469–7481.
- Qiu, W., M. Lei, L. Zhou, X. Bai, X. Lai, Y. Yu, T. Yang, and X. H. Lian. 2017. Hair follicle stem cell proliferation, akt and wnt signaling activation in tpa-induced hair regeneration. *Histochem. Cell Biol.* 14:749–758.
- Sun, X. M., X. D. Liao, L. Lu, L. Y. Zhang, Q. G. Ma, L. Xi, and X. G. Luo. 2018. Effect of in ovo zinc injection on the embryonic development, tissue zinc contents, antioxidation, and related gene expressions of broiler breeder eggs. *J. Integr. Agr.* 17:648–656.
- Wang, A. B., Y. V. Zhang, and T. Tumber. 2017. Gata6 promotes hair follicle progenitor cell renewal by genome maintenance during proliferation. *Embo J.* 36:61–78.
- Wang, B. M., J. Z. Zeng, and Y. Liu. 2014. Regulatory role of dkk1 in organ development and tumorigenesis. *Chin. J. Biochem. Mol. Biol.* 30:731–738.
- Wang, N., T. Yang, J. Li, M. Lei, J. Shi, W. Qiu, and X. Lian. 2012. The expression and role of c-Myc in mouse hair follicle morphogenesis and cycling. *Acta Histochem.* 114:199–206.
- Xie, W. Y., Z. Fu, N. X. Pan, H. C. Yan, X. Q. Wang, and C. Q. Gao. 2019. Leucine promotes the growth of squabs by increasing crop milk protein synthesis through the TOR signaling pathway in the domestic pigeon (*Columba livia*). *Poult. Sci.* 98:5514–5524.
- Yu, L. L., T. Gao, M. M. Zhao, P. A. Lv, L. Zhang, J. L. Li, Y. Jiang, F. Gao, and G. H. Zhou. 2018. Effects of in ovo feeding of l-arginine on breast muscle growth and protein deposition in post-hatch broilers. *Animal* 12:2256–2263.

- Yue, Z., T. X. Jiang, P. Wu, R. B. Widelitz, and M. C. Cheng. 2012. Sprouty/FGF signaling regulates the proximal-distal feather morphology and the size of dermal papillae. *Dev. Biol.* 372:45–54.
- Zhang, J., X. C. He, W. G. Tong, T. Johnson, L. M. Wiedemann, Y. Mishina, J. Q. Feng, and L. Li. 2010. Bone morphogenetic protein signaling inhibits hair follicle anagen Induction by restricting epithelial stem/progenitor cell activation and expansion. *Stem Cells* 24:2826–2839.
- Zhang, L., X. D. Zhu, X. F. Wang, J. L. Li, F. Gao, and G. H. Zhou. 2016. Individual and combined effects of in-ovo injection of creatine monohydrate and glucose on somatic characteristics, energy status, and post-hatch performance of broiler embryos and hatchlings. *Poult. Sci.* 95:2352–2359.
- Zhou, J. Y., Z. Wang, S. W. Zhang, H. L. Lin, C. Q. Gao, J. C. Zhao, C. B. Yang, and X. Q. Wang. 2019. Methionine and its hydroxyl analogues improve stem cell activity to eliminate deoxynivalenol-induced intestinal injury by reactivating Wnt/ β -catenin signaling. *J. Agric. Food Chem.* 67:11464–11473.
- Zorn, A. M. 2001. Wnt signalling: antagonistic Dickkopfs. *Curr. Biol.* 11:R592–R595.