Methionine improves feather follicle development in chick embryos by activating Wnt/β-catenin signaling

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ABSTRACT This study was conducted to explore the regulatory role of methionine (**Met**) in feather follicle and feather development during the embryonic period of chicks. A total of 280 fertile eggs (40 eggs/group) were injected with 0, 5, 10, 20 mg of L-Met or DL-Met/per egg on embryonic day 9 (E9), and whole-body feather and skin tissues were collected on E15 and the day of hatching (**DOH**). The whole-body feather weight was determined to describe the feather growth, and the skin samples were subjected to hematoxylin and eosin staining and Western blotting for the evaluation of feather follicle development and the expressions of Wingless/Int (**Wnt**)/β-catenin signaling pathway proteins, respectively. The results showed that L- or DL-Met did not affect the embryo weight ($P > 0.05$), but increased the absolute and relative whole-body feather weights. Specifically, 5 and 10 mg of L-Met and 5, 10, and 20 mg of DL-Met significantly increased the absolute feather weight at E15 ($P < 0.05$), and 10 mg of L-Met and 5 and 10 mg of DL-Met significantly increased the absolute and relative feather weight on the DOH ($P < 0.05$). Moreover, a main effect analysis suggested that changes in the embryo and feather weights were related to the Met levels ($P < 0.05$) but not the Met source ($P > 0.05$). The levels of L- and DL-Met were quadratically correlated with the absolute and relative feather weights of chicks on the DOH ($P < 0.05$). Correspondingly, all doses of L- and DL-Met significantly increased the diameter and density of feather follicles on the DOH ($P < 0.05$), as well as the activity of Wnt/β-catenin on E15 and the DOH ($P < 0.05$). In conclusion, injection of either L- or DL-Met can improve feather follicle development by activating Wnt/β-catenin signaling, and thereby promoting feather growth; furthermore, no difference in feather growth was found between L- and DL-Met treatments. Our findings might provide a nutritional intervention for regulating feather growth in poultry production.

Key words: feather, feather follicle, methionine, Wnt/β-catenin signaling

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INTRODUCTION

In the poultry industry, the nutritional requirements for feather growth are often ignored due to a lack of direct economic benefits. However, feathers play a critical role in conserving heat to help reduce the maintenance energy requirements and improve the poultry production performance (Leeson and Walsh, 2004; Clark et al., 2011). In addition, feathers contribute to preventing skin abrasions and infections and thus improving the uniformity of carcasses. Poor feathers will reduce both the slaughter quality of poultry and the net profit (Lopez-Coello, 2003; Zeng et al., 2015). In general, all follicles that regulate feather growth begin to appear during embryo development (Wu et al., 2008b; Xu et al., 2007), and their periodic growth is controlled by feather follicle stem cells located in the collar bulge (Xu et al., 2011). The nutrients needed for the maintenance or growth of stem cells are provided by the dermal papilla at the bottom of the collar bulge (Yu et al., 2004).

The development of feather follicles is regulated by multiple signals, among which canonical Wingless/Int (**Wnt**) signaling is indispensable. As an evolutionarily conserved pathway, Wnt/β-catenin signaling is widely active in feather follicles. Upon activation, β-catenin accumulates in the cytoplasm and enters the nucleus, where it displaces Groucho from transcription factor (**TCF**)/lymphoid enhancing factor transcription factors, and the resultant TCF/lymphoid enhancing factor-β-catenin complexes act as bipartite transcriptional activators of specific target genes such as cyclin D1 and c-Myc

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(Logan and Nusse, 2004). Wnt/β-catenin signaling modulates epithelial–mesenchymal cell actions, including the regulation of feather follicle morphogenesis and cycles (Yue et al., 2006). Additionally, β-catenin is prominently expressed at the early embryo stage and significantly declines during embryonic development (Wu et al., 2008a). In mammals, activation of the Wnt/β-catenin signaling pathway has also been shown to regenerate hair follicles for the treatment of hair loss (Mayumi et al., 2007).

Keratin is the main protein in poultry feathers (Harrap and Woods, 1964), which are mainly composed of cysteine (Ward and Lundgren, 1954; Wheeler and Latshaw, 1981). Methionine (**Met**) can be converted by transsulfuration into cystine, which is involved in the synthesis of feather keratin and is important for feather growth (Champe and Maurice, 1984; Zeng et al., 2015). The main components of common poultry diets are wheat, corn, and soybeans, which are usually poor in Met. Therefore, Met is considered as the first-limiting amino acid in poultry diets (Vieira et al., 2004; Albrecht et al., 2017; Xue et al., 2017), as well as the main nutrient factor affecting both feather coverage and length (Zeng et al., 2015). In general, DL-Met is commonly added to feed, but most studies indicate that DL-Met must be converted into biologically active L-Met to be effectively utilized at the cellular level (Dilger and Baker, 2007). Undeniably, it can be assumed that there is no substantial difference between L-and DL-Met (Katz and Baker, 1975; Milgen et al., 2013). Current studies on the effects of L- or DL-Met on the development of feather follicles are limited to descriptions of characteristics and molecular mechanisms have rarely been reported. Albrecht et al. (2019) revealed that L-Met deletion disrupts Wnt/β-catenin signaling in HeLa cells, and this disruption can be rescued by S-adenosylmethionine (**SAM**, a direct metabolite of Met) supplementation during Met depletion. However, it is still unknown whether Met from different sources promotes feather follicle and feather development by activating Wnt/β-catenin.

Therefore, the aim of this study was to determine the development of feather follicles in response to the supply of different levels and sources of Met. Furthermore, the changes in the Wnt/β-catenin signaling pathway during the embryonic period and after in ovo injection were investigated.

MATERIALS AND METHODS

Ethical Statement

All experimental procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of South China Agricultural University (Guangzhou, China), and experiments were approved by the Animal Ethics Committee of South China Agricultural University.

Preparation of Met Solutions and In Ovo Injection Procedure

L-Met purchased from Sigma-Aldrich (purity > 98%, St. Louis, MO) and DL-Met purchased from Evonik Nutrition & Care GmbH (purity > 99%, Essen, Germany) were dissolved in PBS and filtered using a 0.22 μm syringe filter.

The target site for injection into the shell was found by illumination (approximately at two-thirds from the blunt end of the embryo egg), and a small hole was drilled by a 5 mL disposable sterile syringe needle (BB000388, Xinjin Shifeng Medical Equipment Co., Ltd., Chengdu, China) at the target site after disinfection with a 75% alcohol-soaked cotton ball. Subsequently, 0.5 mL of solution was injected into the yolk with a 1 mL disposable sterile syringe (BB000242 Xinjin Shifeng Medical Equipment Co., Ltd.), because the yolk is the main source of nutrients for poultry embryos (Uni and Ferket, 2004; Oliveira et al., 2008). The hole on the shell was immediately sealed with paraffin, and the eggs were returned to the incubator. All the eggs were maintained outside the incubator for less than 30 min during the injection.

Experimental Design

The fertilized eggs of yellow-feathered broiler chicks (Qingyuan partridge chick), which were purchased from Qingyuan Chicken Company (Qingyuan, China), were first stored at 21°C for 24 h and subsequently incubated at 37.9°C with a relative humidity of 50% until hatching. After the illumination-based sorting of unfertilized and dead eggs on embryonic day 6 (E6), a total of 280 eggs were randomly allocated to 7 treatments with 40 eggs per treatment. The 7 treatments consisted of a control group treated with PBS (0 mg/egg Met), plus a 2 × 3 factorial arrangement based on 2 sources of Met (L-Met and DL-Met) and Met doses of 5, 10, and 20 mg/egg. The eggs were then injected with 0.5 mL of solution at E9. This time point was selected based on the results from preliminary studies (Supplementary Figure 1). From each treatment, 20 eggs at E15 and 20 chicks on the day of hatching (**DOH**) were sacrificed by CO_2 inhalation followed by cervical dislocation to ensure death, and samples were then collected.

Sampling and Measurement

The weight of the embryos on E15 and the chicks on the DOH was determined, and the embryos and chicks were then euthanized for tissue sampling. The feathers throughout the whole body were collected, dried, and weighed (relative feather weight = absolute feather weight/embryo weight or body weight). The skin samples were either fixed in fresh 4% paraformaldehyde for

Table 1. Effects of different sources and doses of Met on embryo and feather growth in chicks.

			E15			DOH		
Group	Met source	Met level (mg/egg)	Embryo weight	Absolute feather weight	Relative feather weight[1]	Body weight	Absolute feather weight	Relative feather weight[2]
1	Control (PBS)	0	10.56	0.21^{b}	1.99	31.11	0.65^{c}	2.11^{c}
2	L-Met	5	11.87	0.28^{a}	2.36	31.29	$0.68^{b,c}$	$2.18^{b,c}$
3	L-Met	10	11.04	0.28^{a}	2.49	31.57	0.73^{a}	2.31^{a}
4	L-Met	20	10.41	$0.25^{a,b}$	2.56	31.23	0.67^{c}	$2.16^{b,c}$
5	DL-Met	5	11.31	0.27^{a}	2.36	31.19	$0.71^{a,b}$	2.29^{a}
6	DL-Met	10	11.18	0.28^{a}	2.53	32.51	0.72^{a}	$2.22^{a,b}$
7	DL-Met	20	10.95	0.26^{a}	2.39	31.46	$0.69^{b,c}$	$2.18^{b,c}$
Pooled SEM			0.12	0.01	0.06	0.16	0.01	0.02
Main effect of Met level								
0 (PBS)			10.56^{b}	0.21^{b}	1.99^{b}	31.11^{b}	0.65^{c}	2.11^{c}
5			11.59^{a}	0.27^{a}	2.36^{a}	$31.24^{a,b}$	0.70^{b}	$2.23^{a,b}$
10			11.11^{a}	0.28^{a}	2.51^{a}	32.04^{a}	0.73^{a}	2.27^{a}
20			10.68^{b}	0.26^{a}	2.48^{a}	$31.35^{a,b}$	0.68^{b}	$2.17^{b,c}$
Main effect of Met source								
L-Met			11.11	0.27	2.48	31.37^{b}	0.69	2.22
DL-Met			11.15	0.27	2.42	$31.72^{a,b}$	0.71	2.23
P-values								
Treatment effect			0.065	0.020	0.170	0.441	<0.001	<0.001
Met level			0.009	<0.001	0.022	0.047	<0.001	<0.001
Met source			0.867	0.986	0.687	0.352	0.325	0.693
Met source × Met level			0.418	0.944	0.824	0.446	0.002	0.011
Linear effect								
L-Met			0.234	0.215	0.040	0.839	0.164	0.23
DL-Met			0.610	0.141	0.216	0.474	0.346	0.60
Quadratic effect								
L-Met			0.128	0.011	0.225	0.471	<0.001	<0.001
DL-Met			0.126	0.027	0.140	0.129	0.002	0.005

[a-c]No identical letters in the same column indicate significant differences between different treatment groups ($P < 0.05$).
Abbreviations: DOH, day of hatching; E15, embryonic day 15; Met, methionine.
Values are means ± SEMs with n = 6.
[1]Relative feather weight = absolute feather weight/embryo weight.
[2]Relative feather weight = absolute feather weight/body weight.

histological observation or snap frozen in liquid nitrogen for protein analysis.

Hematoxylin and Eosin Staining

The skin samples were fixed overnight with 4% paraformaldehyde, washed with PBS, dehydrated with different concentrations of alcohol (75, 85, 95, and 100%), and embedded in paraffin blocks. Sections with a thickness of 5 μm were deparaffinized, hydrated, and stained with hematoxylin and eosin. The density and diameter of the feather follicles were measured using Image-Pro Plus software (version 6.0; Media Cybernetics, Rockville, MD).

Immunofluorescence Analysis

The sections of skin samples were incubated with β-catenin (1:200 in antibody diluent; Abcam, Cambridge, MA) overnight at 4°C. Secondary staining was performed with Cy3-conjugated antibodies (1:200 in antibody diluent; Sangon Biotech, Shanghai, China), which were incubated with the skin samples at room temperature for 2 h. The nuclei were stained with 4′,6-diamidino-2-phenylindole (1:1,000 in PBS; Sigma-Aldrich) for 5 min at room temperature. Images of tissue sections were obtained under a fluorescence microscope (ECLIPSE-Ti, Nikon Corporation, Tokyo, Japan).

SAM Content in Skin Tissue

The contents of SAM in the skin tissues were determined using ELISA kits (Meimian Industrial Co. Ltd., Jiangsu, China) according to the manufacturer's recommended protocols.

Western Blot Analysis

Total protein from the skin tissues was extracted using radioimmunoprecipitation assay lysis buffer containing 0.1% phenylmethylsulfonyl fluoride, and the protein concentrations in the homogenates were determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL). We performed Western blotting as previously described (Zhou et al., 2019). Anti-β-catenin (#201328) antibody was obtained from Zen BioScience (Chengdu, Sichuan, China); anti-c-Myc (#5605) antibody was purchased from Cell Signaling Technology

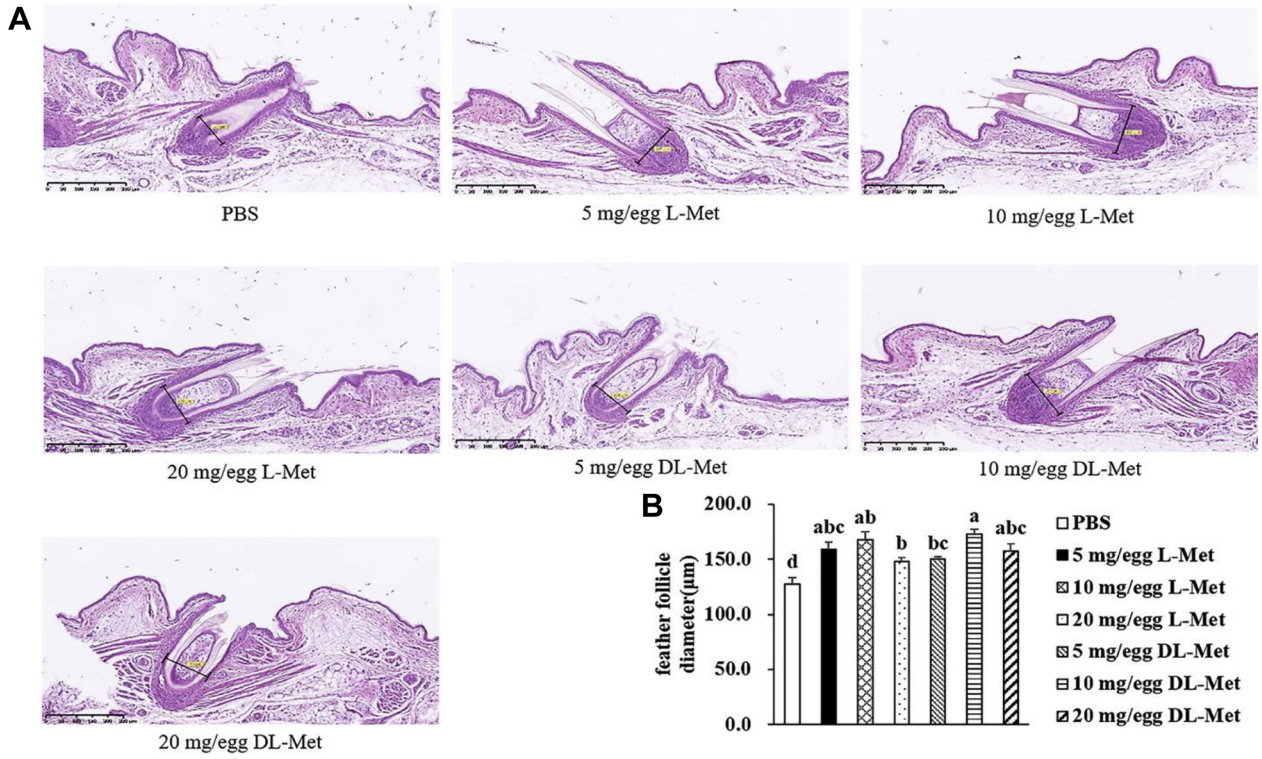

Figure 1. (A) Representative images of hematoxylin and eosin staining in the longitudinal sections of feather follicles (100×). (B) The feather follicle diameter was measured as indicated in the image. "a–d" indicate significant differences ($P < 0.05$). The values are means ± SEMs with n = 6. Abbreviation: Met, methionine.

(Beverly, MA); and anti-TCF4 (#13027), anti-cyclin D1 (#753), and anti-β-actin (#47778) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX). Additionally, anti-rabbit IgG (#7074) and anti-mouse IgG (#7056) secondary antibodies were purchased from Santa Cruz Biotechnology. The proteins were visualized using a BeyoECL Plus chemiluminescence detection kit (Beyotime Institute of Biotechnology, Shanghai, China) with a FluorChem M imaging system (ProteinSimple, San Jose, CA). The band density was analyzed using ImageJ software (version 1.8.0_112, National Institutes of Health, Bethesda, MD).

Statistical Analysis

The data were analyzed with SAS software (version 9.2; SAS Institute Inc., Cary, NC). With the exception of the control group, the means from the 6 Met-supplement groups were analyzed based on a 2 × 3 factorial arrangement (Met source × Met level) using the general linear model procedure in SAS software. The model included the main effects of the Met source and level as well as the Met source × Met level interaction. Primary and quadratic linear trend analyses of various indexes obtained with different levels and sources of Met were performed using the orthogonal polynomial comparison method. All data are presented as means ± SEMs. The differences among the different groups were analyzed by one-way ANOVA followed by Duncan's multiple range test. P-values < 0.05 were considered statistically significant.

RESULTS

Met Accelerates the Growth of Feathers

As shown in Supplementary Figure 1, the yolk membrane completely encloses the yolk at E9 (Supplementary Figure 1), which is conducive to in ovo injection. To determine the effect of Met on feather growth, we obtained the whole-body feather weights of the chicks in the middle (E15) and end of the experiment (DOH). The results are shown in Table 1. Neither the L-Met nor the DL-Met treatment significantly affected the embryo and chick weights compared with those obtained with the PBS treatment (E15, $P = 0.065$; DOH, $P = 0.441$). The injections of 5 and 10 mg of L-Met and 10 and 20 mg of DL-Met significantly increased the feather weight ($P < 0.05$). Additionally, 10 mg of L-Met significantly increased the absolute and relative feather weights ($P < 0.05$) on the DOH. The injections of 5 and 10 mg of DL-Met exerted the same effects on the absolute and relative feather weight ($P < 0.05$). Moreover, the main effect analysis showed that the feather weights were related to the Met level ($P < 0.05$) but not the Met source ($P > 0.05$). In addition, a regression analysis showed that the L-Met treatments exhibited linear and quadratic correlations with the relative and absolute feather weights

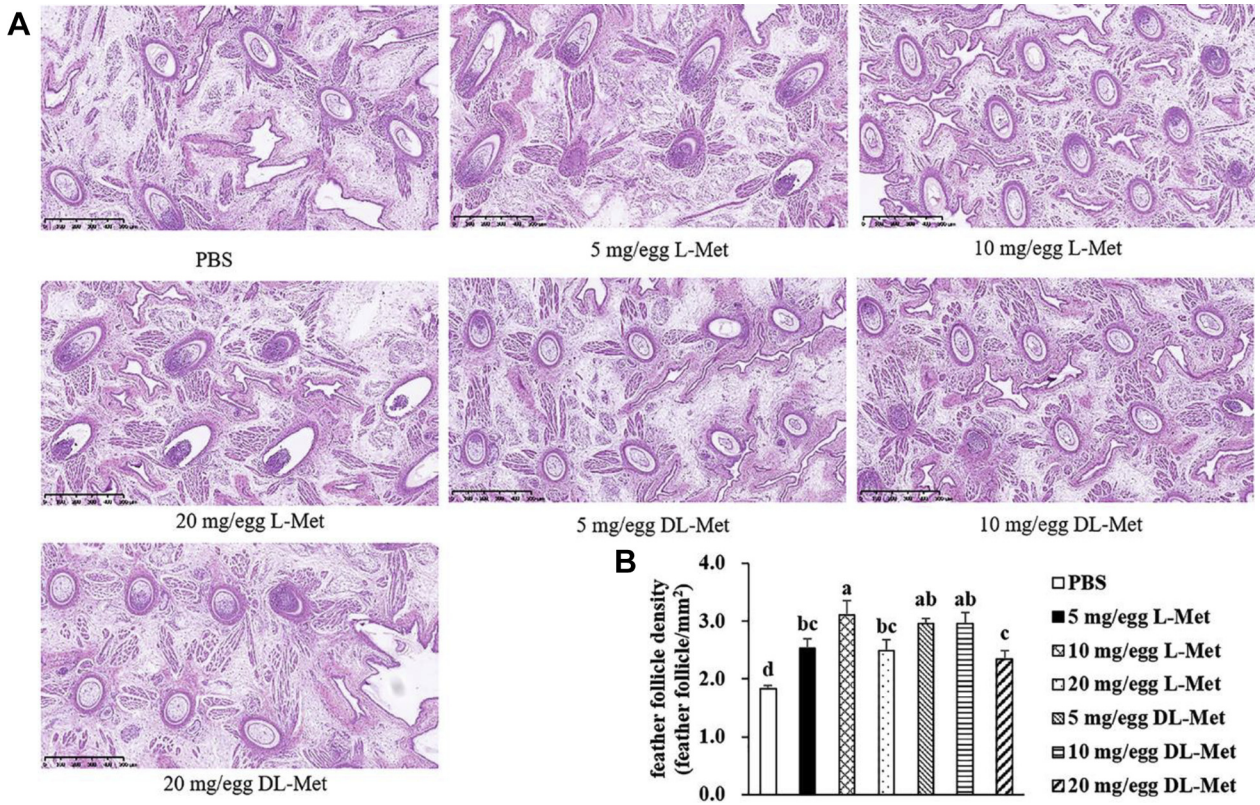

Figure 2. (A) Representative images of hematoxylin and eosin staining in the transverse sections of feather follicles (50×; n = 6). (B) The feather follicle density was measured as indicated in the image. "a–d" indicate significant differences ($P < 0.05$). The values are means ± SEMs with n = 6. Abbreviation: Met, methionine.

on E15 ($P < 0.05$), respectively, whereas the L- and DL-Met treatments were quadratically correlated with the absolute and relative feather weights on the DOH ($P < 0.05$). These results indicated that the effects of the L- and DL-Met treatments on increasing the feather weight did not exhibit a significant difference.

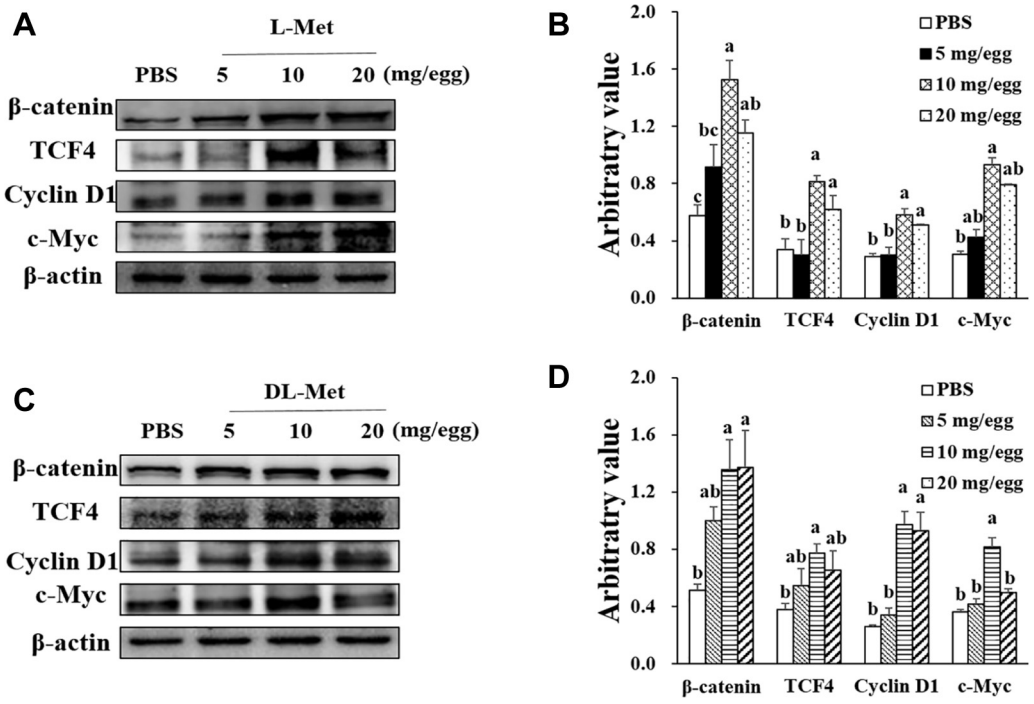

Figure 3. The expression of Wnt/β-catenin signaling pathway proteins in the skin of chicks injected with L-Met (A, B) and DL-Met (C, D) at E15, was measured by Western blotting. "a–c" indicate significant differences ($P < 0.05$), respectively. The values are means ± SEMs with n = 3. Abbreviation: Met, methionine.

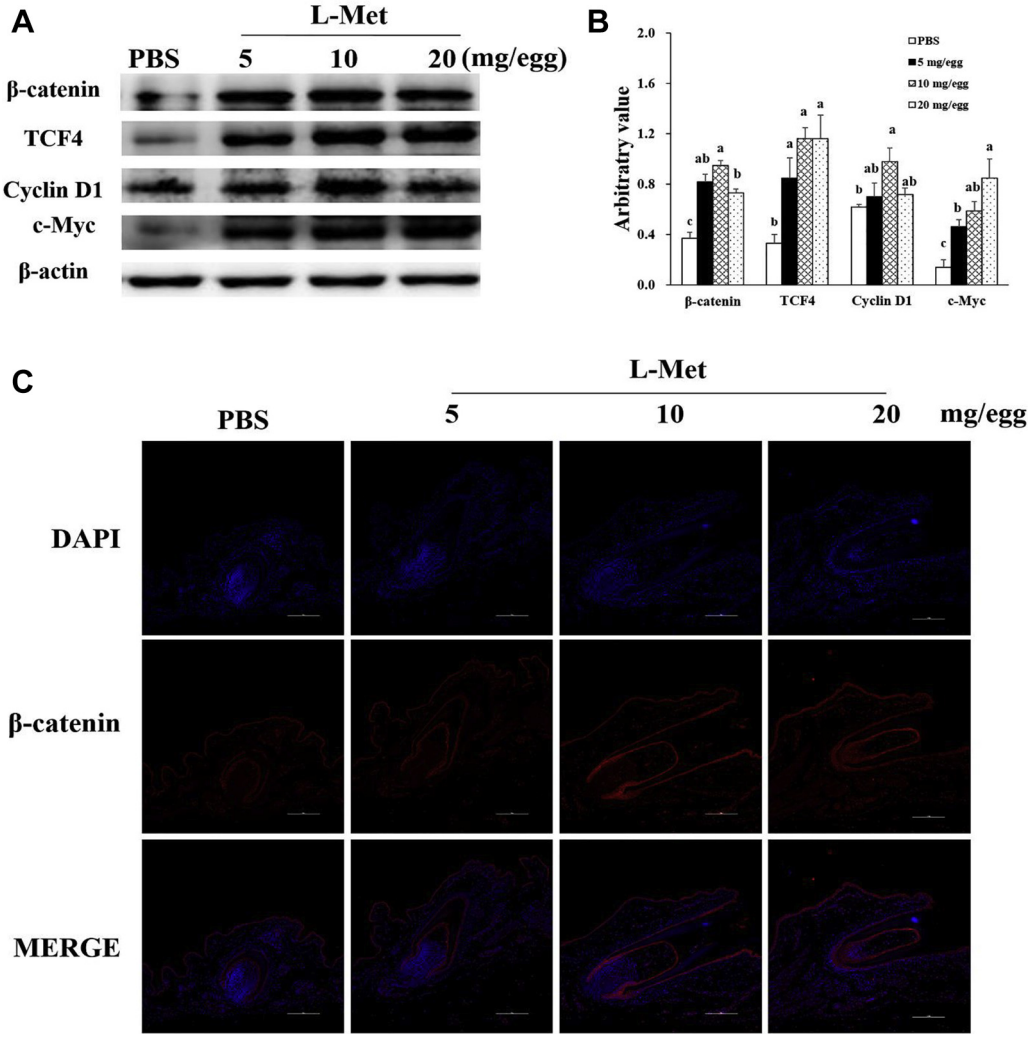

Figure 4. (A, B) The expression of Wnt/β-catenin signaling pathway proteins in the skin of chicks injected with L-methionine (L-Met) on the day of hatching was measured by Western blotting. "a–c" indicate significant differences ($P < 0.05$). The values are means ± SEMs with n = 3. (C) Representative immunofluorescence images following staining with the β-catenin antibody in the feather follicle (200×; n = 3).

Met Improves the Development of Feather Follicles

The development of feather follicles largely determines the growth of feathers (Yu et al., 2002). Thus, we performed histological measurements of the diameter and density of the feather follicles for the assessment of feather development. In general, L- and DL-Met, particularly the 10 mg dose, improved the development of feather follicles, and no significant difference was found between the L- and DL-Met treatments. Specifically, the results obtained from the longitudinal (Figures 1A–1B) and transverse (Figures 2A–2B) histological sections showed that supplementation with 5, 10, and 20 mg of L- or DL-Met significantly increased the diameter and density of feather follicles in the skin of chicks on the DOH compared with the results obtained with the PBS group ($P < 0.05$), and the 10 mg Met dose was identified as the optimal dose.

Low-dose Met Has No Significant Effect on the SAM Content in Skin Tissue

Compared with the PBS group, the SAM levels on E15 were significantly increased by the 20 mg treatment ($P < 0.05$, Supplementary Figure 2A). On the DOH, no significant difference in the SAM levels was found among the 4 groups ($P > 0.05$, Supplementary Figure 2B).

Met Activates Wnt/β-Catenin Signaling

Periodic activation of Wnt/β-catenin signaling is responsible for the cyclic activity observed within feather follicles. The expression of β-catenin, TCF4, cyclin D1, and c-Myc on E15 in the groups injected with L-Met at doses of 10 and 20 mg ($P < 0.05$, Figures 3A–3B) and DL-Met at a dose of 10 mg ($P < 0.05$, Figures 3C–3D) was significantly upregulated compared with that in the PBS group. In addition, the injections of 5, 10, and

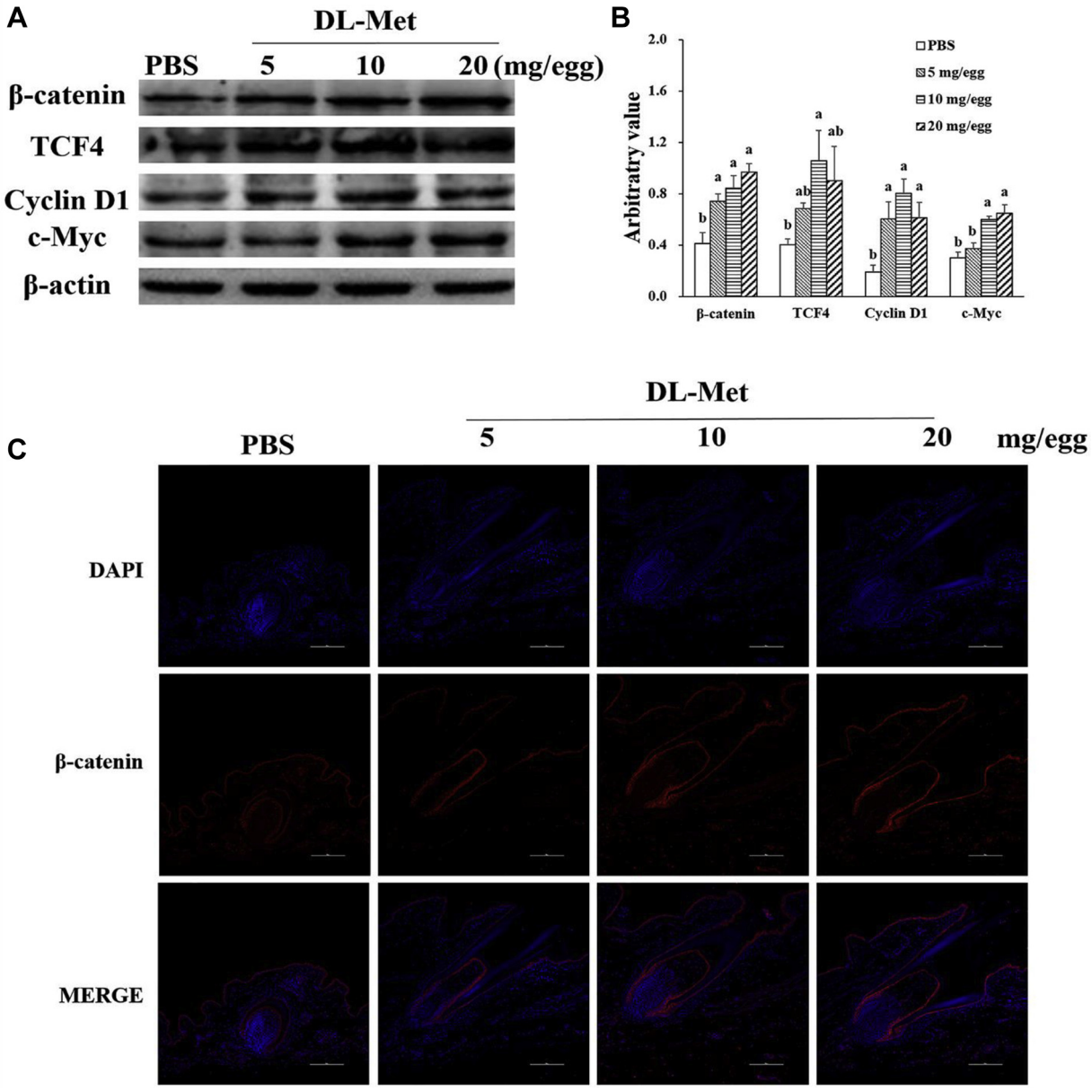

Figure 5. (A, B) The expression of Wnt/β-catenin signaling pathway proteins in the skin of chicks injected with DL-methionine (DL-Met) on the day of hatching was measured by Western blotting. "a, b" indicate significant differences ($P < 0.05$). The values are means ± SEMs with n = 3. (C) Representative immunofluorescence images following staining with the β-catenin antibody in the feather follicle (200×; n = 3).

20 mg of L-Met substantially increased the expression of β-catenin, TCF4, and c-Myc on the DOH and the 10 mg dose increased the expression of cyclin D1 ($P < 0.05$, Figures 4A–4B). The injections of 5, 10, and 20 mg of DL-Met significantly increased the expression of β-catenin and cyclin D1, the 10 and 20 mg doses of DL-Met increased the expression of c-Myc, and the 10 mg dose of DL-Met significantly increased the expression of TCF4 ($P < 0.05$, Figures 5A–5B). The immunofluorescence results for β-catenin were consistent with the Western blot results (Figures 4C and 5C). These data suggested that the injections of L and DL-Met, particularly the dose of 10 mg, can activate Wnt/β-catenin signaling.

DISCUSSION

This study indicates that Met plays a major role in embryonic physiology. The positive effects of the in ovo injection of Met on chick feather growth were similar to those observed in ducks, and dietary Met supplementation increased the feather weight compared with that obtained with the control group (Zhao et al., 2018). Moreover, a quadratic regression model of the feather weight indicated that the weight increased quadratically with the addition of Met during the embryonic period, which is in accordance with the results observed in a previous study of duck feathers conducted by Zeng et al.

(2015). In addition, our data also demonstrated that the levels of L- and DL-Met were quadratically correlated with the absolute and relative feather weights on the DOH. The results suggested that 10 mg of Met was the optimal dose for feather follicle development. Excessive Met might exhibit some toxicity on chick development (Robel, 1977; Farran and Thomas, 1992; Wylie et al., 2003).

DL-Met was synthesized by mixing racemic (50:50) D- and L-isomers. In vivo, D-Met is first oxidized to 2-keto-4-(methylthio) butanoic acid by D-amino acid oxidase (D-AAOX; EC 1.4.3.3), and 2-keto-4-(methylthio) butanoic acid is then converted into L-Met by transaminase (Zhang et al., 2018). Previous studies showed that supplementation with DL-Met exerted the same effect on the growth of chicks (Katz and Baker, 1975) and piglets (Milgen et al., 2013) as supplementation with L-Met. In our present study, we did not find any differences between the effects of L- and DL-Met supplementation on chick and feather weights. These results indicate that the biological effects of DL-Met are similar to those of L-Met.

The density and diameter of feather follicles almost fully determine the density and quality of feathers, respectively (Chen et al., 2012). Feather follicle stem cells control the growth cycle of feather follicles and feathers, and dermal papillae provide nutrients for feather follicles and feathers (Yue et al., 2005; Xu et al., 2007). As expected, the observed increase in feather weight was accompanied by increases in the feather follicle density and diameter. Previous studies conducted by Nazem et al. (2015) showed that 50 mg of DL-Met significantly increased the density and diameter of chicken embryo feather follicles compared with those obtained with 20 mg of DL-Met. However, our study found that a smaller dose of Met (10 mg) exerted the most significant effects, and no difference was detected between the 2 sources of Met.

Feather follicles are formed by the interaction of the dermis and epidermis during the embryonic phase (Yu et al., 2002). Several signaling molecules have been found to participate in the formation and development of follicles, and these include Wnt/β-catenin, Sonic Hedgehog, bone morphogenetic proteins, and Notch (Harris et al., 2010; Ouji et al., 2013; Ouspenskaia et al., 2016; Cheng et al., 2018). Albrecht et al. (2019) proved that Met deficiency causes a decrease in Wnt/β-catenin activity in vitro. Based on these results, we were not surprised that the Wnt/β-catenin signaling pathway was upregulated after L- or DL-Met injection. The activation of downstream targets in this pathway, such as c-Myc and cyclin D1, contributes to enlargement of the feather bud (Chiu, 2008). Similarly, our data showed that the size and number of feather follicles were significantly increased by L- and DL-Met supplementation, particularly at doses of 10 mg/egg. Albrecht et al. (2019) further discovered that the Met-mediated increase in Wnt/β-catenin activity was achieved by the degradation of GSK3β by the first metabolite, SAM, which results in the generation of methyl groups. However, we found that the SAM levels at E15 obtained after treatment with the 20 mg dose were significantly higher than those obtained with the other groups, and no significant difference was detected on the DOH. This outcome might be related to the long interval between detection and injection. Therefore, further studies on the molecular mechanism through which Met promotes Wnt/β-catenin signaling in feather follicle cells are needed to clarify the remaining questions.

Overall, our results demonstrate that Met, particularly at the 10 mg dose, promotes follicle and feather growth in developing chick embryos by activating the Wnt/β-catenin signaling pathway, and no significant difference was found between the L- and DL-Met treatments. Therefore, the injection of Met during the embryonic stage is an effective nutritional intervention for regulating feather growth in poultry production. Moreover, our findings might provide an available target and intervention strategy for human alopecia cure.

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Conflict of Interest Statement: The authors have declared no conflicts of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.psj.2020.05.047.

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