

**Crosstalk between Prolactin, Insulin-like Growth Factors, and Thyroid
Hormones in Feather Growth Regulation in Neonatal Chick Wings**

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Abstract

The elongation of primary feathers in neonatal chicks is delayed by the late-feathering *K* gene located on the Z chromosome. We recently found that the *K* gene slows feather growth by reducing the number of functional prolactin (PRL) receptor (PRLR) dimers. In this study, we investigated the molecular mechanisms by which PRL promotes feather elongation. RT-qPCR and immunohistochemistry analyses revealed that PRLRs are predominantly localized in the pulp rather than in the epidermal layer of the feather follicle. Treatment of primary cultured feather pulp cells with PRL increased the expression of mRNAs for insulin-like growth factors (IGFs; IGF-1 and IGF-2) and type 2 deiodinase (DIO2). Furthermore, treatments with IGF-1 and triiodothyronine (T3) reciprocally enhanced the expression of mRNAs for DIO2 and IGFs. Additionally, BrdU staining in neonatal chicks showed that T3 promoted cell proliferation in both the epidermal layer and pulp cells, while this effect was suppressed by an IGF-1 receptor (IGF1R) inhibitor. These findings suggest a novel model in which PRL upregulates IGFs and DIO2 in feather pulp cells, creating a positive feedback loop between IGFs and T3, ultimately leading to the promotion of cell proliferation in both the epidermal layer and the pulp cells by IGFs. This is the first report proposing crosstalk between PRL, thyroid hormone (TH), and IGFs in feather follicles.

Keywords : Prolactin; thyroid hormone; IGF; iodothyronine deiodinase; feather growth

1. Introduction

Feathers are highly complex integumentary structures that have primarily evolved for flight in birds. In addition to enabling flight, avian feathers play vital roles in thermoregulation, protection from external stimuli, and various signaling functions, including communication related to dominance, age group, and species identity (Prum and Brush, 2003; You et al., 2024). Feather development occurs in the feather follicle (Yu et al., 2004), which consists of dermal tissue known as pulp and overlying epidermal layers. The collar bulge located at the base of the epidermis contains epidermal stem cells (Yue et al., 2005). During feather formation, these epidermal stem cells generate proliferative transit-amplifying (TA) cells in the collar region. As these TA cells proliferate, they are gradually pushed towards the follicle tip, where they differentiate into feather cells. These cells are pigmented by melanocytes, synthesize and accumulate β -keratin, undergo apoptosis, and ultimately form the feather (Chang et al., 2004; Nakaoka, 2024). A conceptual diagram illustrating these components and processes is provided in Fig. 1.

In neonatal chicks, the elongation of primary feathers is governed by genetic variation, specifically early feathering (wild type) and late feathering. These traits are controlled by a feathering locus on the Z chromosome (Hamoen et al., 2001), with the late-feathering K allele being dominant over the early-feathering k^+ allele (Lurette et al., 1993). Crossbreeding early-feathering males (k^+/k^+) with late-feathering females ($K/-$) results in all male offspring exhibiting late feathering, while all female offspring show early feathering. This makes the K allele a widely utilized genetic marker in poultry breeding for autosexing purposes (Majó et al., 1995). In 2008, the structure of the K allele was elucidated. It was found that while the k^+ allele consists of adjacent prolactin (PRL)

receptor (*PRLR*) and sperm flagellar 2 (*SPEF2*) genes, the *K* allele contains a fusion gene in which partially duplicated *PRLR* (*dPRLR*) and *SPEF2* (*dSPEF2*) genes are linked in a tail-to-tail arrangement, in addition to the intact *PRLR* and *SPEF2* genes (Elferink et al., 2008; Takenouchi et al., 2018). Recently, we demonstrated that the gene products of *dPRLR* and *dSPEF2* influence the amount of functional PRLR dimers, thereby inducing the late-feathering phenomenon in chickens (Okamura et al., 2019). Furthermore, it has been reported that the late-feathering gene in turkeys encodes a PRLR that lacks the C-terminal region, which significantly delays the elongation of wing feathers (Derks et al., 2018). These findings strongly suggest that PRL promotes feather elongation. However, the exact mechanism by which PRL acts on the feather follicle to promote this elongation remains unclear.

PRL is known to induce molting in various bird species, including starlings (*Sturnus vulgaris*), song sparrows (*Melospiza melodia*), white-winged crossbills (*Loxia leucoptera*), and turkeys (*Meleagris gallopavo*) (Dawson, 2006; Dawson and Sharp, 1998; Deviche and Sharp, 2001; Lien and Siopes, 1993; Wingfield and Goldsmith, 1990). Similarly, thyroid hormones (TH) have been recognized for their role in inducing molting (Bass et al., 2007; Lien and Siopes, 1989). Recent studies have also reported that insulin-like growth factor 1 (IGF-1) induces molting in bearded reedlings (*Panurus biarmicus*) (Lendvai et al., 2021). Therefore, this study aims to explore the roles of IGFs and TH in the promotion of feather elongation by PRL. The findings presented here suggest a novel model of crosstalk between PRL, TH, and IGFs in feather follicles, where PRL upregulates IGFs and DIO2 in feather pulp cells, ultimately enhancing cell proliferation in both epidermal and pulp cells.

2. Materials and Methods

2.1. Animals

One-day-old Broiler (F1 generation of a cross between a female White Plymouth Rock and a male White Cornish) chicks were purchased from a commercial supplier (Fukuda Poultry Breeding Farm, Okayama, Japan). Due to the influence of the late feathering gene, females and males exhibit early and late feathering, respectively. All experiments were basically conducted using four chicks, with each experiment repeated three times. Depending on the purpose of the experiment, either females or males were used. The study was conducted at Okayama University, Japan, from 2018 and 2024. All animal procedures were conducted in accordance with the guidelines of the Experimental Animal Committee of Okayama University, and this study was specifically approved by the committee.

2.2. Total RNA preparation

One-day-old chicks were euthanized by carbon dioxide exposure followed by decapitation. The wings were cut off and sprayed with 70% ethanol. Using sterilized forceps, primary feather follicles were excised under a stereomicroscope in a 10 cm plastic dish containing sterile phosphate-buffered saline (PBS). The excised follicles were briefly immersed in 70% ethanol and then transferred to fresh sterile PBS. Using a sterile razor blade, the follicles were transversely cut 2-3 mm from the base, and the sample was prepared from the basal side of the cut. Follicles from a single individual were combined to create one sample. For the preparation of pulp and epidermal layers, follicle fragments from the basal side (2–3 mm from the base) of four individual birds were immersed in 20 ml of sterile PBS containing 500 U/ml of DISPASE II (Wako, Osaka, Japan) in a 10

cm dish and incubated overnight at 10°C. The pulp and epidermal layers were then carefully separated using forceps under a stereomicroscope. These tissues from each bird were pooled to create a single sample. Total RNA was extracted from these samples, as well as from cultured pulp cells and pituitaries, using TRI Reagent (COSMO BIO, Tokyo, Japan) according to the manufacturer's instructions.

2.3. Reverse-transcription PCR (RT-PCR) and real-time quantitative PCR (RT-qPCR)

Reverse transcription was performed using the Thermo Script RT-PCR system (Invitrogen, Carlsbad, CA, USA) or the ReverTra Ace qPCR RT Kit (TOYOBO, Shiga, Japan). Between 17 and 700 ng of total RNA from each sample was reverse-transcribed according to the manufacturer's instructions, and the resulting cDNA was used for reverse-transcription PCR (RT-PCR) and/or real-time quantitative PCR (RT-qPCR). RT-PCR was performed using Tks Gflex DNA polymerase (Takara Bio, Kusatsu, Japan) with a thermal cycler (GeneAtlas GA322; Astec, Fukuoka, Japan). The PCR conditions for Tks Gflex DNA polymerase were as follows: 94 °C for 1 minute, followed by cycling reactions including denaturation at 98 °C for 10 seconds, annealing at 60 °C for 15 seconds, and extension at 68 °C for 30 seconds. The sequences of all primers used in this study are listed in Table 1, and the primers and cycle numbers used for each cDNA amplification are described in the figure legends. A 5-μl aliquot of each PCR reaction was subjected to electrophoresis on a 2.0% agarose gel, stained with ethidium bromide, and visualized under ultraviolet illumination. RT-qPCR analysis was conducted using the Applied Biosystems 7300 Fast Real-Time PCR System or the LightCycler 96 System (Roche) with THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) or Taq Pro

Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), following the manufacturer's instructions. A melting curve analysis was performed from 60°C to 95°C to detect potential nonspecific products. The Ct values obtained from RT-qPCR were analyzed using the $\Delta\Delta C_t$ method. To minimize inter-plate variance, all comparative samples were measured on the same plate. Additionally, to account for within-plate variance, all samples were analyzed in triplicate simultaneously. Technical replicates were included, with each sample measured three to four times, to ensure the reliability of the results. Each experiment was conducted using three independently prepared sample groups, and statistical analyses were performed. Normalization was carried out using chicken actin B (*ACTB*) mRNA as an internal control. Statistical analysis and graph plotting were performed using GraphPad Prism 6 (GraphPad Software, CA, USA). An unpaired t-test (two-sample t-test) was used for the analysis. A *p* value of less than 0.05 was considered to be statistically significant.

2.4. Immunostaining for PRLR

Tissue samples from female chicks were fixed in 4% paraformaldehyde at 4°C overnight, followed by dehydration through a graded ethanol series and embedding in paraffin. Sections (5 μ m thick) were cut using a microtome and mounted on glass slides. The sections were deparaffinized in xylene and rehydrated through graded ethanol solutions. Endogenous peroxidase activity was blocked by incubating the sections in 0.5% sodium metaperiodate for 15 minutes. The sections were then washed with PBS. Blocking was performed using 1% fetal bovine serum (FBS) for 1 hour at room temperature to reduce nonspecific binding. Due to the unavailability of a specific chicken PRLR antibody, we utilized a polyclonal antibody (bs-6445R; Bioss, USA) raised in

169 rabbits against a synthetic peptide corresponding to a partial sequence (251-350) of
170 human PRLR (Swiss Prot: P16471) as the primary antibody. Although the datasheet for
171 this antibody does not specify whether it recognizes chicken PRLR, the antigen region
172 corresponds to a conserved domain with 86.1% identity between chicken and human
173 PRLR. Based on this information, and considering that the antibody is polyclonal, we
174 proceeded with using it for this experiment. The antibody was diluted 1:400 in PBS
175 containing 1% FBS and 0.4% Triton X-100, and 50 μ l per slide was applied to the sections,
176 which were incubated overnight at 4°C in a moist chamber. After washing with PBS, the
177 biotinylated secondary antibody from the Vectastain ABC Kit (Vector Laboratories) was
178 diluted 1:300 in PBS containing 1% FBS and 0.4% Triton X-100. Then, 50 μ l was applied
179 per slide and incubated on the sections for 2 hours at room temperature. The sections were
180 washed again with PBS. Subsequently, 50 μ l per slide of the avidin-biotin complex (ABC)
181 reagent was applied according to the manufacturer's instructions (Vectastain ABC Kit,
182 Vector Laboratories) and incubated for 30 minutes. Immunoreactivity was visualized by
183 incubating the sections in a solution containing 50 ml of 0.05 M Tris buffer, 10 mg of
184 diaminobenzidine (DAB), and 10 μ l of H₂O₂. The reaction was monitored under a
185 microscope until the desired staining intensity was achieved, after which the reaction was
186 stopped by washing the sections in distilled water (D.W.). Finally, the sections were
187 dehydrated in ethanol, cleared in xylene, and mounted with coverslips. Since the chicken
188 PRLR was unavailable, a negative control was conducted by omitting the primary
189 antibody instead of using an absorbed antibody. This ensured that any observed staining
190 was specific to the primary antibody.

192 **2.5. Construction of the chicken PRL expression vector**

The cDNA encoding the open reading frame (ORF) of chicken PRL was amplified by RT-PCR from pituitary RNA of one-day-old chicks. PCR was performed using Tks Gflex DNA polymerase (Takara Bio, Shiga, Japan) with a forward primer containing an *Eco* R I site at the 5' end (5'-ACAGAATTTCTACCATGAGCAACAGA-3') and a reverse primer containing an *Xho* I site at the 5' end (5'-AGCCTCGAGGTACTTAGCAATTGCT-3'). The PCR product was digested with *Eco* RI and *Xho* I, and subsequently cloned into an *Eco* RI/*Xho* I-digested pCAGGS vector, generating a recombinant plasmid named pCAGGS-cPRL.

2.6. Transfection and protein production in HEK293T cells

HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) at 37 °C in a 5% CO₂ incubator. The cells were seeded into a 12-well plate at a density of 1.0×10^5 cells per well. Twenty-four hours after seeding, transfection was performed using Opti-MEM (Thermo Fisher Scientific, Tokyo, Japan) and PEI (Polysciences, Inc., Warrington, PA, USA). The transfection mixture was prepared by adding 1000 ng of the plasmid pCAGGS-cPRL (for the experimental group) or pCAGGS (for the control group), along with 8 µl of PEI, to Opti-MEM, adjusting the final volume to 100 µl per well, and incubated at room temperature for 15 minutes. After replacing the culture medium with DMEM containing 1% ITS-G Supplement (×100) (Wako Pure Chemical Industries, Osaka, Japan), 100 µl of the transfection mixture was added to each well, and the plate was gently shaken for 1 minute. The cells were then incubated at 37°C in 5% CO₂ for 24 hours, after which the conditioned media were

collected.

2.7. Western blot analysis

After determining the protein concentration of the conditioned media, samples were diluted with RIPA buffer (Nacalai tesque, Kyoto, Japan) to achieve a final protein concentration of 30 ng in 40 μ l. Then, 10 μ l of 5 $\times$ SDS-loading buffer (125 mM Tris-HCl, pH 6.8; 4% SDS; 0.01% bromophenol blue; 20% glycerol; 10% β -mercaptoethanol) was added to each sample. The samples were heated at 95 $^{\circ}$ C for 5 minutes and subjected to electrophoresis on an 18% polyacrylamide gel at a constant current of 80 mA for 50 minutes. After electrophoresis, the proteins were transferred to a PVDF membrane (Fluoro TransR W; Pall Corporation, Port Washington, NY, USA) using a current of 310 mA for 1 hour. The membrane was blocked with 5% skim milk in TBS-T (0.1% Tween 20, 0.15 M sodium chloride, 0.05 M Tris, pH 7.6) for 1 hour at room temperature. Following the blocking step, the membrane was washed with TBS-T and then incubated with rabbit anti-mouse PRL antibody (AFP-131078; National Hormone & Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA, USA) diluted 1:10,000 in Can Get Signal[®] solution 1 (TOYOBO, Osaka, Japan) at 4 $^{\circ}$ C for 16 hours. The membrane was washed three times with TBS-T for 5 minutes each, followed by incubation with an anti-rabbit IgG-HRP secondary antibody (GE Healthcare, Little Chalfont, UK) diluted 1:10,000 in Can Get Signal[®] solution 2 (TOYOBO) for 2 hours at room temperature. After a final wash with TBS-T, the signal was detected using ECL Prime (GE Healthcare, Little Chalfont, UK) and visualized with a LAS-4000 mini imager (FUJIFILM, Tokyo, Japan).

2.8. Isolation and culture of feather pulp cells and treatment with PRL, T3, and IGF-1

Feather pulp cells were prepared from primary feather follicles of one-day-old female chicks. The procedure up to the treatment of follicle fragments with DISPASE II was conducted using the same method as described in Section 2.2. After DISPASE treatment, pulp tissue was separated from the follicles and placed in a 15 ml tube containing 10 ml of Trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 4 °C for 1 hour. Afterward, the tube was moved to a 37 °C water incubator and gently agitated for 1 hour. After 30 and 60 minutes in the water incubator, gentle pipetting was performed to create a cell suspension. Following centrifugation at 2,000 rpm for 5 minutes, the cells were resuspended in 10 ml of culture medium and seeded in a 10 cm dish. The culture medium consisted of D-MEM (High Glucose) with L-Glutamine, Phenol Red, and Sodium Pyruvate (Wako, Osaka, Japan), supplemented with 10% FBS, penicillin-streptomycin solution (Wako, Osaka, Japan), and amphotericin B suspension (Wako, Osaka, Japan). When the pulp cells reached semi-confluency, they were harvested, subcultured in 60 mm dishes, and treated for 24 hours with a final concentration of 10 nM 3, 3', 5-Triiodo-L-thyronine (T3; Sigma-Aldrich) or 50 ng/ml recombinant human IGF-1 (Amersham). For PRL treatment, the conditioned media of HEK293T cells transfected with pCAGGS-cPRL (for the experimental group) or pCAGGS (for the control group) (see section 2.6) were added to the dishes, and the cells were incubated for 48 hours.

2.9. *In vivo* treatment with triiodothyronine (T3), IGF1R inhibitor, and BrdU

One-day-old male chicks received intraperitoneal injections of treatment

solutions using syringes fitted with 26G needles. In each experiment, 4 chicks were used in both the experimental and control groups, with the respective treatment solutions or vehicle administered accordingly. The vehicle for T3 treatment consisted of 7.5 μ l DMSO (Nacalai tesque, Kyoto, Japan) and 92.5 μ l sesame oil, while for the simultaneous administration of T3 and the IGF1R inhibitor, the vehicle was 125 μ l DMSO. Each experiment was repeated three times to ensure reliability. For T3 treatment, each chick was administered a solution containing 0.75 mg T3 (Sigma-Aldrich), along with the previously mentioned vehicle. In the case of simultaneous administration of T3 and the IGF1R inhibitor, each chick was given a solution containing 0.75 mg T3, 3.75 mg IGF1R inhibitor (Linsitinib; Funakoshi, Tokyo, Japan), and 125 μ l DMSO. For BrdU administration, each chick received a solution containing 5 mg BrdU (Nacalai tesque, Kyoto, Japan), 50 μ l DMSO, and 450 μ l PBS. The experimental schedule involved administering BrdU 23 hours after the injection of either T3 or the combination of T3 and IGF1R inhibitor, with feather follicles sampled 1 hour after the BrdU administration.

2.10. BrdU immunostaining and analysis

The immunostaining procedure closely followed the method described in section 2.4, with some modifications to enhance antigen reactivity. Specifically, the samples were incubated in 2N HCl at room temperature for 30 minutes, followed by neutralization in 0.1 M sodium borate at room temperature for 10 minutes. After this, they were treated with 0.1% trypsin at 37°C for 30 minutes before blocking. The primary antibody used was a Mouse Anti-BrdU monoclonal antibody (50 μ g/ml), diluted 1:100, and the secondary antibody was ImmPRESS Horse Anti-Mouse IgG. To quantify BrdU-positive nuclei, ImageJ software was used. Images were converted to 8-bit, and the

threshold was adjusted to detect stained regions. The watershed algorithm and Analyze Particles function, both available by default in ImageJ, were applied to separate overlapping nuclei and automatically count the BrdU-positive nuclei, respectively. In the epidermal layer, the total number of BrdU-positive nuclei was counted up to 500 μm from the base for graphing. For the pulp, the number of BrdU-positive nuclei was counted up to 700 μm from the base and expressed as the number of positive nuclei per 10,000 μm^2 for graphing. This automated counting method eliminates potential observer bias, ensuring reliable data quantification.

3. Results

3.1. Identification of PRL target cells in the feather follicle

To investigate the mechanism by which PRL promotes feather elongation, we first aimed to identify the target cells of PRL within the feather follicle. Primary feather follicles from one-day-old female chicks were isolated and enzymatically treated with dispase to separate the epidermal layers from the pulp. RT-qPCR analysis revealed predominant *PRLR* mRNA expression in the pulp (Fig. 2A). Furthermore, immunostaining of the feather follicle using an anti-human PRLR antibody demonstrated positive signals in the pulp, particularly localized in the collar mesenchyme, a specific region of the pulp surrounded by the collar (Fig. 2B). These results suggest that the collar mesenchyme is a key target of PRL action in the feather follicle.

3.2. Investigation of local mediators in PRL signaling

Next, we examined the potential role of IGFs and the active form of TH,

311 triiodothyronine (T3), as local mediators in PRL signaling. Since a decrease in PRL
312 signaling has been reported in late-feathering chicks (Okamura et al., 2019), it is
313 conceivable that the expression of factors mediating PRL action is also reduced in these
314 individuals. RT-qPCR analysis showed that the expression levels of *IGF1*, *IGF2*, and
315 *DIO2* were significantly lower in the primary feathers of late-feathering male chicks
316 compared to early-feathering females (Fig. 3A). Consistently, RT-qPCR confirmed that
317 *IGF1*, *IGF2*, and *DIO2* mRNA were predominantly expressed in the pulp, with
318 significantly lower levels detected in the epidermal layers (Fig. 3B).

319 Additionally, RT-PCR and RT-qPCR revealed that *IGF1R*, the common
320 receptor for IGF-1 and IGF-2, along with TH receptors *THRA* and *THRB*, were
321 expressed in both the epidermal layers and the pulp (Fig. 4A and Fig. 4B). To directly
322 assess the impact of PRL on the expression of *IGF1*, *IGF2*, and *DIO2*, PRL-containing
323 conditioned media were applied to primary cultured pulp cells isolated from the primary
324 feathers of early-feathering female chicks (Fig. 5A and Fig. 5B). RT-qPCR analysis
325 demonstrated that PRL significantly increased the expression of *IGF1*, *IGF2*, and *DIO2*
326 in these cells (Fig. 5C), indicating their role as local mediators of PRL's action in the
327 feather follicle.

329 3.3. Effects of TH and IGFs on feather pulp cells

330 To further explore the interaction between TH and IGFs, we investigated the
331 effects of T3 and IGF-1 on feather pulp cells *in vitro*. RT-qPCR analysis revealed that
332 treatment with T3 significantly upregulated the expression of *IGF1* and *IGF2* in primary
333 cultured feather pulp cells (Fig. 6A). Similarly, IGF-1 treatment markedly increased *DIO2*
334 expression in the same cells (Fig. 6B). These findings indicate a mutual promotion of

expression between TH and IGFs within the feather follicle, suggesting the existence of a positive feedback loop that may contribute to feather elongation.

3.4. Effects of TH and IGFs on the feather follicle

In vivo experiments supported the findings from cultured cells, showing that late-feathering male chicks exhibited significantly lower *DIO2* expression in feather follicles compared to early-feathering females (Fig. 3A). Administration of T3 to late-feathering males significantly increased the number of BrdU-positive nuclei in both epidermal and pulp cells, indicating enhanced cell proliferation (Fig. 7A, Fig. 7B). Additionally, RT-qPCR confirmed that T3 treatment upregulated the expression of *IGF1* and *IGF2* in feather follicles (Fig. 7C), consistent with the *in vitro* results. Furthermore, inhibition of IGF1R reduced cell proliferation in both epidermal and pulp cells (Fig. 8), demonstrating that IGFs mediate the proliferative effects of T3 and highlighting their importance in PRL-induced feather elongation.

4. Discussion

In this study, we identified the collar mesenchyme of the feather follicle as a key target of PRL, where PRLR is expressed. Our findings suggest that PRL indirectly promotes epidermal cell proliferation by stimulating the production of local mediators, such as IGFs and T3, in the pulp. Recent studies in mice have shown that PRL significantly upregulates *IGF2* expression in the choroid plexus during lactation, identifying IGF-2 as a crucial downstream mediator of PRL signaling (Phillipps et al., 2019). Similarly, research on mammary epithelial cells (MECs) demonstrated that IGF-2 is essential for PRL-induced cell proliferation via cyclin D1 activation (Briskin et al.,

2002). Furthermore, PRL enhances IGF2 mRNA levels during sexual maturity and early pregnancy, with IGF-2 acting as a key mediator in the PRL signaling pathway that promotes mammary epithelial cell proliferation and alveolar development (Hovey et al., 2003). Additionally, IGF-2 and PRL have been shown to cooperate in regulating the differentiation and growth of brown adipocytes. In PRL receptor knockout mice, reduced IGF-2 expression in brown adipose tissue leads to diminished adipocyte maturation and impaired thermoregulation (Viengchareun et al., 2008). Moreover, the activity of DIO2 is upregulated in rat brown adipose tissue by IGF-1 (Martinez-deMena and Obregón, 2005; Mills et al., 1987). In humans, IGF-1 and TH are also known to promote keratinocyte proliferation in hair follicles and maintain the anagen phase of hair growth (Ahn et al., 2012; Castela et al., 2017; Philpott et al., 1994; van Beek et al., 2008). Taken together, these findings suggest that IGF-2, along with IGF-1, may play a significant role in PRL-mediated signaling within feather follicles, indicating that similar mechanisms may regulate feather elongation as they do hair growth.

Molting is an energetically demanding process, with feathers comprising over 20% of a bird's dry body weight. Molt timing is tightly regulated to avoid conflict with critical life events, such as breeding or migration (Kiat et al., 2019; Lind and Jakobsson, 2001). Seasonal and reproductive status-related fluctuations in PRL and T4 levels have been reported (Dawson et al., 2009; Gumułka et al., 2021; Péczely et al., 2011), suggesting a central role for these hormones in controlling molt timing. Our model proposes that PRL and T4 induce a rapid increase in T3 and IGF activity within the feather follicle, facilitating feather elongation and ensuring efficient feather replacement during optimal periods.

Previous studies identified the collar as the site of epidermal call proliferation

in feather follicles (Yue et al., 2005). Our findings suggest that IGFs act in this region to promote the proliferation of transit-amplifying (TA) cells, which drive feather elongation. However, Wu et al. (2021) reported TA cell localization in the peripheral pulp of White Leghorn contour feathers, which we did not observe. This discrepancy may stem from differences in feather type (primary feathers vs. contour feathers) or the developmental stage of the birds (newly hatched vs. adult chickens). Dermal stem cells supply TA cells during feather growth, rapidly expanding the pulp to support feather morphogenesis and pigmentation (Li et al., 2017). IGFs produced in the pulp likely promote epidermal cell proliferation via autocrine and paracrine signaling.

Our findings highlight the importance of local mediators in PRL signaling. Specifically, IGFs and T3 act as key mediators in driving rapid cell proliferation necessary for feather elongation. This interplay between PRL, IGFs, and TH may explain the coordinated onset of molting and feather elongation during periods of heightened PRL secretion (Dawson, 2006; Lien and Siopes, 1989; Wingfield and Goldsmith, 1990).

To further explore this interaction, we treated primary cultured feather pulp cells with T3 or IGF-1. T3 significantly increased *IGF1* and *IGF2* expression, while IGF-1 enhanced *DIO2* expression. These results suggest that IGFs and T3 promote each other's expression, creating a positive feedback loop that drives feather elongation. This mechanism may explain the coincidence of molt initiation with peak PRL concentration in birds (Dawson, 2006; Lien and Siopes, 1989; Wingfield and Goldsmith, 1990).

Based on these findings, we propose a model in which PRL promotes feather elongation by stimulating IGF and T3 production in the collar mesenchyme, promoting the proliferation of both epidermal and pulp cells (Fig. 9). This model provides new insights into the hormonal regulation of feather morphogenesis and lays the foundation

for future research into PRL, IGFs, and TH interaction in avian species. Additionally, these findings may offer insights into the broader mechanisms of molting and feather regeneration.

We have previously shown that estrogen regulates local T3 levels in chicken saddle feathers, contributing to the pronounced sexual dimorphism observed in these feathers (You et al., 2024). Taken together, our results suggest that feather morphogenesis is governed by a complex interplay between systemic endocrine hormones and local follicular signaling. Studies investigating the structure of these hormonal regulatory networks may advance our understanding of how feather type and growth are precisely regulated.

While this study focused on feather elongation, the mechanism controlling the cessation of feather growth once the feather reaches its predetermined length remain unclear. Despite the positive feedback between IGFs and T3, the factors that terminate elongation are largely unknown. Future research will need to address how this feedback loop is regulated to ensure that feather elongation stops at the appropriate stage, providing deeper insights into the intricate control of feather development.

5. Conclusion

This study proposes a model for the role of PRL in feather elongation, suggesting that PRL signaling interacts with molting-inducing factors, such as IGFs and TH, to promote feather growth. Our findings demonstrate that IGFs and TH mutually enhance each other's expression within the feather follicle, creating a positive feedback loop that stimulates the proliferation of both epidermal and feather pulp cells, ultimately driving feather elongation.

While we have elucidated the mechanisms that promote feather elongation, the process by which this growth is terminated to ensure that feathers reach a specific length remains an open question. Understanding this termination process is critical for a more complete picture of feather morphogenesis. Further investigations into the regulation of IGFs and T3 will be key to uncovering how feather growth is halted at the appropriate stage. These findings lay the groundwork for future studies aimed at unraveling the complex interactions between PRL, IGFs, and TH in avian species. Moreover, they may provide valuable insights into broader mechanisms governing molting and feather regeneration.

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Figure Legends

Fig. 1.

Structure of the chicken contour feather follicle and feather formation. Schematic diagrams of longitudinal and transverse sections of the developing feather follicle are shown. Cells proliferating in the collar at the base of the follicle are pushed toward the tip and then form the rachidial ridge and barbule ridge. These cells then undergo melanin pigmentation by melanocytes, produce and accumulate keratin, and eventually undergo keratinization to form the feather.

Fig. 2.

Distribution of PRLR-expressing cells in primary feather follicles of one-day-old female chicks. (A) Expression of *PRLR* mRNA in the epidermal layers (Epi) and pulp (Pulp). Representative RT-qPCR results are presented as mean $\pm$ SEM from triplicate determinations. **** $P < 0.0001$ indicates significant differences between Epi and Pulp. PCR primers for *PRLR* and *ACTB* were P1 and P2, and P3 and P4, respectively. (B) Distribution of immunoreactive (ir)-PRLR. Representative micrographs of immunostaining are shown with (left) and without (right) the anti-PRLR antibody. Scale bar=100 μ m. Dp: dermal papilla, Cm: collar mesenchyme, Epi: epidermal layer.

Fig. 3.

Expression of *IGF1*, *IGF2*, and *DIO2* mRNAs in primary feather follicles of one-day-old chicks. (A) Comparison of expression between late-feathering males (Late) and early-feathering females (Early). (B) Comparison of expression between the epidermal layers (Epi) and pulp (Pulp) in females. RT-qPCR results are shown as mean $\pm$ SEM from triplicate determinations. *** $P < 0.001$, **** $P < 0.0001$ indicate significant differences between the groups. PCR primers for *IGF1*, *IGF2* and *DIO2* were P5 and P6, P7 and P8, and P9 and P10, respectively.

Fig. 4

Expression of *IGF1R* and TH receptors (*THRA*, *THRB*) in the epidermal layers (Epi) and pulp (Pulp) of primary feather follicles in one-day-old chicks. (A) Representative electrophoretic patterns of RT-PCR products for *IGF1R*, *THRA*, *THRB*, and *GAPDH*. Three independently prepared pooled samples, each derived from four individual, are shown for each target. The PCR primers and cycle numbers for *IGF1R*, *THRA*, *THRB*, and *GAPDH* were as follows: P11 and P12 for *IGF1R* (35 cycles), P13 and P14 for *THRA* (35 cycles), P15 and P16 for *THRB* (35 cycles), and P17 and P18 for *GAPDH* (23 cycles). NC represents the PCR product from the negative control, which does not contain template DNA. (B) Comparison of expression between the epidermal layers (Epi) and pulp (Pulp). Representative RT-qPCR results are shown as mean $\pm$ SEM from triplicate determinations. * $P < 0.05$ indicates significant differences between Epi and Pulp. PCR primers for *IGF1R*, *THRA*, and *THRB* were P11 and P12, P13 and P14, and P15 and P16, respectively.

Fig. 5

Effect of recombinant chicken PRL on cultured pulp cells from primary feather follicles in one-day-old female chicks. (A) Detection of recombinant chicken PRL in conditioned media by Western blotting using an anti-PRL antibody. The autoluminogram shown is a representative example of the Western blots. "PRL" and "Ctrl" indicate conditioned media from HEK293T cells transfected with either pCAGGS-cPRL or the empty vector pCAGGS, respectively. The molecular weight of recombinant chicken PRL is 22.8 kDa. (B) Phase-contrast micrograph of the primary cultured feather pulp cells used in this study. (C) Effect of conditioned media containing recombinant chicken PRL on *IGF1*, *IGF2*, and *DIO2* mRNA expression in cultured feather pulp cells. Representative RT-qPCR results are presented as mean $\pm$ SEM from triplicate determinations. "PRL" and "Ctrl" indicate conditioned media from HEK293T cells transfected with either pCAGGS-cPRL or the empty vector pCAGGS, respectively. * $P < 0.05$ indicates significant differences between PRL and Ctrl. PCR primers for *IGF1*, *IGF2*, and *DIO2* were P5 and P6, P7 and P8, and P9 and P10, respectively.

Fig. 6

Effect of T3 and IGF-1 on cultured pulp cells from primary feather follicles in one-day-old female chicks. (A) Effect of T3 on *IGF1* and *IGF2* mRNA expression. (B) Effect of IGF-1 on *DIO2* mRNA expression. Representative RT-qPCR results are presented as mean $\pm$ SEM from triplicate determinations. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences between the groups. PCR primers for *IGF1*, *IGF2*, and *DIO2* were P5 and P6, P7 and P8, and P9 and P10, respectively.

Fig. 7

Effect of T3 injection on feather follicles in one-day-old male chicks. (A) BrdU immunostaining of feather follicles from T3-treated (T3) and vehicle-treated (Ctrl) chicks. Representative micrographs are shown. Arrowheads indicate positive signals. Scale bar = 100 μ m. DP: dermal papilla, Epi: epidermal layer. (B) Comparison of the number of BrdU-positive nuclei in the epidermal layer and pulp. (C) RT-qPCR analysis of the effect of T3 injection on *IGF1* and *IGF2* mRNA expression. PCR primers used were P5 and P6 for *IGF1*, and P7 and P8 for *IGF2*. Representative results in (B) and (C) are presented as mean $\pm$ SEM from triplicate determinations. *P < 0.05 and **P < 0.01 indicate significant differences between T3-treated and Ctrl groups.

Fig. 8

Effect of simultaneous injection of T3 and IGF1R inhibitor on cell proliferation in feather follicles of one-day-old male chicks. (A) BrdU immunostaining of feather follicles from T3-treated (T3) and T3 plus IGF1R inhibitor (Inh)-treated (T3 + Inh) chicks. Representative micrographs are shown. Arrowheads indicate positive signals. Scale bar = 100 μ m. DP: dermal papilla, Epi: epidermal layer. (B) Comparison of the number of BrdU-positive nuclei in the epidermal layer and pulp. Representative results are presented as mean $\pm$ SEM from triplicate determinations. *P < 0.05 and ***P < 0.001 indicate significant differences between T3-treated and T3 plus IGF1R inhibitor (Inh)-treated groups.

Fig. 9

Proposed mechanism of PRL action in feather elongation. PRL binds to PRLR in the feather follicle, enhancing the expression of *IGFs* and *DIO2*. DIO2 converts

663 inactive T4 to active T3, increasing local T3 levels. This interaction promotes cell
664 proliferation, leading to feather growth (see text for a detailed discussion of the molecular
665 interactions).
666