Characterization of the novel duplicated *PRLR* gene at the late-feathering *K* locus in Lohmann chickens

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Abstract

A partial duplication of the prolactin (PRL) receptor gene (designated as *dPRLR*) has been identified at the late-feathering (LF) *K* locus on chromosome Z of some chicken strains recently, implying that *dPRLR* is probably a candidate gene associated with LF development in chickens. However, little is known about the structure, functionality, and spatiotemporal expression of the *dPRLR* gene in chickens. In this study, using 3'-RACE and RT-PCR, the full-length cDNA of the *dPRLR* obtained from the kidneys of male Lohmann layer chickens carrying a *K* allele was cloned. The cloned *dPRLR* is predicted to encode a membrane-spanning receptor of 683 amino acids, which is nearly identical to the original *PRLR*, except for its lack of a 149-amino acid C-terminal tail. Using a 5× STAT5–Luciferase reporter system and western blot analysis, we demonstrated that *dPRLR* expressed in HepG2 cells could be potentially activated by chicken PRL and functionally coupled to the intracellular STAT5 signaling pathway, suggesting that *dPRLR* may function as a novel receptor for PRL. RT-PCR assays revealed that similar to the original *PRLR* gene, *dPRLR* mRNA is widely expressed in all embryonic and adult tissues examined including the skin of male Lohmann chickens with a *K* allele. These findings, together with the expression of *PRL* mRNA detected in the skin of embryos at embryonic day 20 and 1-week-old chicks, suggest that skin-expressed *dPRLR* and *PRLR*, together with plasma and skin-derived PRL, may be involved in the control of the LF development of chicks at hatching. Moreover, the wide tissue expression of *dPRLR* implies that *dPRLR* may regulate other physiological processes of chickens carrying the *K* allele.

Key Words

- ▶ chickens
- ▶ duplicated prolactin receptor
- ▶ skin
- ▶ feather development

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Introduction

Prolactin (PRL) is a polypeptide hormone and belongs to the growth hormone (GH)/PRL family, which also includes somatotactin, placental lactogens, and the newly identified PRL-like (PRL-L) protein (Goffin *et al.* 1996, Bole-Feysot *et al.* 1998, Zhu *et al.* 2004,

Ben-Jonathan *et al.* 2008, Wang *et al.* 2010b). PRL is primarily synthesized in and released from the anterior pituitary and participates in many physiological processes including reproduction, osmoregulation, immunomodulation, behavior, and growth and development in

vertebrates (Freeman *et al.* 2000, Ben-Jonathan *et al.* 2008, Nguyen *et al.* 2008). It is clear that the diverse actions of PRL are mediated by the PRL receptor (PRLR), which contains a large extracellular domain, a transmembrane domain (TMD), and an intracellular domain (Bole-Feysot *et al.* 1998). Upon the binding of PRL, the PRLR can initiate multiple intracellular signaling cascades, including the activation of the JAK2–STAT5 signaling pathway, and thus control the subsequent expression of target genes (Bole-Feysot *et al.* 1998, Brooks 2012).

Specifically, in chickens, PRL has been reported to regulate a number of physiological processes, such as the initiation and maintenance of incubation behavior, immunomodulation, and regulation of gonadal development and functions (Sharp *et al.* 1979, 1988, Lea *et al.* 1981, Doneen & Smith 1982, Talbot *et al.* 1991). As in mammals, the broad spectrum of PRL actions in chickens has also been reported to be mediated by a PRLR, which is encoded by a *PRLR* gene on chromosome Z and widely expressed in a variety of tissues (Tanaka *et al.* 1992, Ohkubo *et al.* 1998b, Bu *et al.* 2013).

Strikingly, in addition to a *PRLR* gene localized on chromosome Z, a partially duplicated *PRLR* gene (designated as *dPRLR* gene herein) has also been identified recently (Elferink *et al.* 2008). The *dPRLR* gene was found within the sex-linked late-feathering (LF) *K* locus on chromosome Z, within which a partial duplication of sperm flagellar 2 gene (named *dSPEF2*) was identified simultaneously (Elferink *et al.* 2008). It has been reported that this dominant sex-linked *K* allele is associated with the LF phenotype of newly hatched chicks with the retarded emergence of flight feathers, while the recessive *k+* allele determines the early-feathering (EF) phenotype of chicks (Serebrovsky 1922, Elferink *et al.* 2008). The identification of the *dPRLR* gene within the *K* locus (but not within the *k+* locus) suggests that *dPRLR* is probably a candidate gene associated with LF development in chicks (Elferink *et al.* 2008, Wang *et al.* 2010a, Luo *et al.* 2012). However, some fundamental issues regarding *dPRLR* gene remain to be addressed: i) whether the *dPRLR* gene is expressed in various tissues of chickens carrying the *K* allele; ii) whether the *dPRLR* gene can encode a functional receptor for PRL; and iii) how the *dPRLR* gene is involved in the control of the phenotypic traits of chickens, such as the LF development of chicks.

Pursuing our recent studies and focusing on the molecular characterization of chicken *PRLR* gene (designated as the original *PRLR* gene herein) including its intricate gene structure, tissue expression, promoter usage, and interaction with its two ligands (PRL and PRL-L;

Wang *et al.* 2010b, Bu *et al.* 2013), the present study aims to further characterize this duplicated *PRLR* gene (*dPRLR*) within the *K* locus in a commercial chicken strain – Lohmann layer. Our results showed that *dPRLR* is likely to encode a novel functional receptor for PRL and is widely expressed in all chicken tissues examined including the skin with a spatiotemporal expression pattern similar to that of the original *PRLR* gene. The results of the present study establish an important molecular basis for the elucidation of the roles of the *dPRLR* gene in chickens further, including its potential action on feather development. Moreover, an extra copy of the *PRLR* gene (*dPRLR*), found to be ubiquitously expressed in all the tissues of chickens carrying the *K* allele examined in this study, also suggests that chickens with different genotypes (with or without the *dPRLR* gene) might be an ideal and unique model to decipher the diverse roles of PRLR and its ligand(s) in vertebrates, such as its roles in skin biology (Elferink *et al.* 2008, Foitzik *et al.* 2009).

Materials and methods

Chemicals and hormones

All the chemicals were obtained from Sigma–Aldrich, and restriction enzymes were obtained from Takara (Dalian, China), unless stated otherwise. Antibodies against β -actin (catalog no. 4967), STAT5 (catalog no. 9358), and phosphorylated STAT5 (Tyr⁶⁹⁴) (catalog no. 4322) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Total RNA extraction

Chickens (or embryos) (Lohmann Sandy strain) were purchased from a local commercial company and killed. As an initial step toward uncovering the roles of the *dPRLR* gene within the *K* locus in chicken strains, only male chickens with the genotype *K/k+* (LF phenotype) and female chickens with the genotype *k+/w* (EF phenotype) were used in this study (Fig. 1). The genotype of male chickens or embryos (*K/k+*) was first examined by PCR amplification of the 78 bp breakpoint junction fragment from genomic DNA extracted from the liver or blood as reported previously (Elferink *et al.* 2008, Wang *et al.* 2010a). Then, it was further confirmed by evaluating the copy number of the *PRLR* gene in chickens using quantitative real-time PCR assays (GH receptor (*GHR*) gene close to *PRLR* gene (Fig. 1) was used as an internal control to evaluate the copy number of *PRLR* gene in each

Figure 1

(A) A 6-day-old female chick with the EF phenotype (genotype $k+/w$); (B) a 6-day-old male chick with the LF phenotype (genotype $K/K+$). Arrows in (A) and (B) indicate the different developmental status of flight feathers in the female (EF, ♀) and male (LF, ♂) chicks. (C) Organization of the $k+$ allele and K allele in chicken sex chromosome Z. The $k+$ allele contains *PRLR* and *SPEF2* genes only, while the K allele contains the original *PRLR* and *SPEF2* genes and the partially duplicated *PRLR* (*dPRLR*) and *SPEF2*

(*dSPEF2*) genes arranged in an opposite direction (Elferink *et al.* 2008). In the K allele, unlike the original *PRLR* gene in the region adjacent to it, the *dPRLR* gene has an incomplete last exon (named exon 16' in this study), which is linked to intron 4 (in the antisense direction) of *dSPEF2* gene. Arrows represent the putative transcription start sites of each gene. *GHR* gene is located on chromosome Z close to the *PRLR* gene.

chicken) (Fig. 1; Supplementary Figure 1, see section on supplementary data given at the end of this article). Tissues from embryos (at embryonic day 12 (E12), E16, and E20), 1-week-old chicks, and 2-month-old and adult chickens, including the whole brain, heart, small intestine, kidneys, liver, lungs, muscle, ovaries, testis, pituitary, spleen, pancreas, and skin (around the feathers), were collected, frozen in liquid nitrogen, and stored at -80°C prior to use. Total RNA was extracted from the tissues using RNazol (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions and dissolved in diethyl pyrocarbonate-treated H_2O . All the experiments were carried out according to the guidelines of the Animal Ethics Committee of Sichuan University.

Cloning of the full-length cDNA of *dPRLR* gene from the kidneys of LF male chickens

It has been reported that the last exon of *dPRLR* gene is incomplete and interrupted by a 78 bp breakpoint sequence

(Elferink *et al.* 2008), suggesting that the *dPRLR* gene may have a 3'-UTR that is different from that of the original *PRLR* gene. To determine the 3'-UTR sequence of *dPRLR*, gene-specific primers were designed to amplify the 3'-UTR of *dPRLR* from the kidneys of 1-week-old male Lohmann chicks (with the LF phenotype) using the SMART RACE cDNA Amplification Kit according to the manufacturer's instructions (Clontech). The amplified PCR products were cloned into a pTA2 vector (Toyobo, Osaka, Japan) and sequenced using the ABI3100 Genetic Analyzer (Beijing Genomics Institute, Shanghai, China). Then, according to the cloned unique 3'-UTR sequence of *dPRLR* and cDNA sequence of the original *PRLR* gene reported previously (Tanaka *et al.* 1992, Bu *et al.* 2013), new gene-specific primers were designed to amplify the full-length cDNA of *dPRLR* from chicken kidneys using high-fidelity *Taq* DNA polymerase (Toyobo; Table 1). The amplified PCR products were cloned into a pTA2 vector and sequenced. Finally, the full-length cDNA sequence containing an open reading frame (ORF) of the *dPRLR* gene was determined by sequencing at least three independent clones.

Table 1 Primers used^a in the study

Primers	Sense/antisense	Primer sequence (5'–3')	Size (bp)
Primers used for the rapid amplification of dPRLR 3'-ends (3'-RACE)			
<i>dPRLR-rF1</i>	Sense	GCAAACCTGACCACCATGGATCATGGA	
<i>dPRLR-rF2</i>	Sense	ACCGCCAGTGCCAGGGCCAAAGAT	
<i>dPRLR-rF3</i>	Sense	GTTGAGTGCTCTTGTTGCCATGGCT	
Primers used for the construction of dPRLR expression plasmids			
<i>dPRLR-eF1</i>	Sense	CGGGGTACCAAGAGGAAGTGGAAATCATGA	2109
<i>dPRLR-eR1</i>	Antisense	ACAAGTGTCAGACTAGGGCTAGCA	
Primers used for RT-PCR assays			
<i>β-Actin</i>	Sense	TGTGCTACGTCGCACTGGAT	401
	Antisense	GCTGATCCACATCTGCTGGA	
<i>dPRLR-f1</i>	Sense	CACTGGCTGATGTCAGATCT	1100
<i>dPRLR-r1</i>	Antisense	TCACCTGAGACCCAATGTTG	
<i>PRLR-f1</i>	Sense	CATGGAGTGAATGGAGCTCAG	1370
<i>PRLR-r1</i>	Antisense	TCACAGACTTATCAGCTAGG	
<i>cPRL</i>	Sense	TCACACAGAATCCCTACCATGA	472
	Antisense	GCTCCTCAATCTCTACAGCCT	
<i>cPRL-L</i>	Sense	TCCACCGAGCTGGAGAAGTACT	545
	Antisense	GTCACCTATCACATTGCTTCA	
Primers used for the detection of dPRLR type I and type II transcripts having distinct first exons ^b			
<i>dPRLR-F1</i>	Sense	CATGAATTGACATGGGTCTGA	2114–2760
<i>dPRLR-F2</i>	Sense	CAGTTCACCATGACTGAGAAT	2135–2478
<i>dPRLR-R1</i>	Antisense	ACAAGTGTCAGACTAGGGCTAGCA	
Primers used for quantitative real-time PCR assays ^c			
<i>cGHR</i>	Sense	CAGATACTGACAGGCTCCTGAGT	148
	Antisense	GAGATGGCATCACATGTGTCGCT	
<i>cPRLR</i>	Sense	CAGCCAGATCCTCCTGTGAATGT	126
	Antisense	CTCAGGGTAAGCCATCCCGATCT	
Primers used for the amplification of 78 bp breakpoint junction sequence ^d			
<i>dPRLR</i>	Sense	TGAAACCATCCCTGGAGAGATG	78
	Antisense	ACAAGTGTCAGACTAGGGCTAGCA	

^aAll primers were synthesized by Invitrogen.^bA common antisense (*dPRLR-R1*) primer was used to examine the expression of *dPRLR* type I and type II transcripts with distinct first exons (exon 1A' or exon 1G'). Multiple bands of different sizes (Fig. 7) were detected probably due to the alternative mRNA splicing of *dPRLR* gene in the 5'-UTR or coding region.^cPrimers used for evaluating the copy number of *PRLR* gene (*GHR* gene used as an internal control). The quantitative real-time PCR method was established in our previous study (Wang *et al.* 2007).^dPrimers were designed according to a previous report (Elferink *et al.* 2008).

RT-PCR

RT was carried out at 42 °C for 2 h in a total volume of 10 µl consisting of 2 µg of total RNA collected from different tissues, 1× single strand buffer, 0.5 mM of each deoxy-nucleotide triphosphate, 0.5 µg oligo-deoxythymide, and 100 U M-MLV reverse transcriptase (Promega). All negative control reactions were carried out under the same conditions without reverse transcriptase in the reaction mixture.

According to our previously established methods (Wang & Ge 2004, Wang *et al.* 2012a,b), RT-PCR assays were carried out to examine the mRNA expression of

dPRLR, *PRLR*, *PRL*, and *PRL-L* genes in chicken tissues; *β-actin* gene was used as an internal control for monitoring RNA loading and RT efficiency. PCR was carried out under the following conditions: 2-min denaturation at 95 °C, followed by 23 cycles (for *β-actin*: 30 s at 95 °C, 30 s at 56 °C, and 45 s at 72 °C) or 33 cycles (for *dPRLR* and *PRLR*: 30 s at 95 °C and 30 s at 60 °C and 90-s extension at 72 °C) or 35 cycles (for *cPRL* and *cPRL-L*: 30 s at 95 °C and 30 s at 58 °C and 45-s extension at 72 °C) of the reaction, ending with a 5-min extension step at 72 °C. Primers used for the RT-PCR assays are listed in Table 1. (Sense and antisense primers located on different exons were used for each

RT-PCR assay to ensure that the PCR product was amplified from the cDNA of the target gene.) The PCR products were visualized on a u.v.-transilluminator (Bio-Rad Laboratories, Inc.) after electrophoresis on 2% agarose gel containing ethidium bromide. The identity of the PCR products was verified by sequencing (Beijing Genomics Institute).

Functional characterization of dPRLR in cultured HepG2 cells

According to the cloned cDNA sequence of *dPRLR*, gene-specific primers flanking the start and stop codons were designed to amplify the ORF of *dPRLR* from adult chicken kidneys using high-fidelity *Taq* DNA polymerase (Toyobo; Table 1). The amplified PCR products were cloned into a pcDNA3.1(+) vector (Invitrogen) and sequenced.

In this study, the expression plasmid encoding the original PRLR (831 amino acids, KC686695) of Lohmann chicks was also constructed and used as a positive control to test whether dPRLR is an additional functional receptor for PRL.

To test whether dPRLR is functional *in vitro*, human hepatocellular carcinoma (HepG2) cells expressing dPRLR were treated with recombinant chicken PRL (cPRL) prepared in our laboratory (Bu *et al.* 2013). Receptor activation was monitored using a 5× STAT5–Luciferase reporter system as reported previously (Cocolakis *et al.* 2008, Bu *et al.* 2013). In brief, the HepG2 cells were maintained in DMEM supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml of penicillin G, and 100 µg/ml of streptomycin (HyClone, Logan, UT, USA) in a 90 cm culture dish (Nunc, Rochester, NY, USA) and incubated at 37 °C with 5% CO₂. The HepG2 cells were plated in a six-well plate at a density of 3×10⁵ cells/well 24 h before transfection. A mixture containing 1000 ng of 5× STAT5–Luciferase reporter construct (an artificial promoter construct containing five STAT5-response elements fused to the luciferase gene), 100 ng of expression plasmid encoding pig STAT5A (Fang *et al.* 2012), 200 ng of expression plasmids encoding dPRLR (or full-length PRLR) or pcDNA3.1(+) vector, and 2 µl of jetPRIME was prepared in 100 µl of transfection buffer. Transfection was carried out according to the manufacturer's instructions (Polyplus-transfection SA, Illkirch, France) when the cells reached 70% confluency. After 24 h of culture, the cells were subcultured in a 96-well plate at a density of 2×10⁴ cells/well at 37 °C for 24 h. Then, the cells were treated with the gradient concentrations of recombinant cPRL (from 10^{−10} to 10^{−5} g/ml)

and incubated for an additional 18 h at 37 °C. After removal of the culture medium, 50 µl of 1× passive lysis buffer (Promega) were added to each well, and the luciferase activity of 15 µl of cellular lysates was measured using the Luciferase Assay Kit (Promega).

Western blot analysis

To investigate whether the activation of dPRLR can increase the levels of STAT5 tyrosine phosphorylation, HepG2 cells co-transfected with 500 ng of pig STAT5A and 500 ng of expression plasmid encoding dPRLR or PRLR (used as a positive control) or an empty pcDNA3.1 vector were cultured for 24 h in a six-well plate and treated with recombinant cPRL (300 ng/ml) for 30 min. Then, the whole-cell lysates were used to examine the phosphorylated STAT5 (Tyr⁶⁹⁴) using western blot analysis according to the manufacturer's instructions (Cell Signaling Technology). The levels of β-actin and total STAT5 (including STAT5A and STAT5B) proteins were also examined and used as internal controls in each experiment.

Statistical analysis

The luciferase activities in HepG2 cells treated with hormone were expressed as relative fold increase when compared with the control group treated with a hormone-free medium. The data were analyzed using one-way ANOVA followed by Dunnett's test using Graphpad Prism 4 (Graphpad Software, San Diego, CA, USA). To validate our results, all experiments were repeated at least two to four times.

Results

Cloning of the full-length cDNA of the *dPRLR* gene from the kidneys of LF chickens

A partial duplication of the *PRLR* gene (*dPRLR*) with an incomplete last exon has been identified within the *K* locus on chromosome Z recently (Elferink *et al.* 2008; Fig. 1); however, the cDNA sequence of *dPRLR* remains unclear. Therefore, in this study, using 3'-RACE PCR, we first amplified and cloned the 3'-UTR of *dPRLR* from the kidneys of male Lohmann chicks carrying a *K* allele. Sequence analysis revealed that the cloned 3'-UTR of *dPRLR* (448 bp) is different from that of the original *PRLR* gene (accession no.: JQ768800). Similar to the 3'-UTR of most eukaryotic genes, the 3'-UTR of *dPRLR* also contains a consensus polyadenylation site (AAUAAA) upstream of its

poly(A) tail (Supplementary Figure 2, see section on supplementary data given at the end of this article).

According to the 3'-UTR sequence of *dPRLR* and cDNA sequence of the original *PRLR* (Tanaka *et al.* 1992, Bu *et al.* 2013), new gene-specific primers were designed to amplify the full-length cDNA containing an ORF of *dPRLR* from the kidneys of male chicks with a *K* allele. The cloned *dPRLR* was 2519 bp in length, and its nucleotide sequence was nearly identical to that of *PRLR* except for its 3'-UTR (Supplementary Figure 2).

The cloned *dPRLR* is predicted to encode a novel receptor of 683 amino acids (accession no.: JQ768801). Similar to *PRLR*, *dPRLR* has a large extracellular domain containing two putative ligand-binding domains (LBD1 and LBD2), a single TMD, and a cytoplasmic tail of 221 amino acids (Brooks 2012). Sequence alignment further showed that *dPRLR* shares a nearly identical amino acid sequence identity with the original *PRLR* cloned from the kidneys of Lohmann chicks (accession no.: KC686695), with only four amino acid substitutions being observed between the two receptors. One substitution (Ser¹⁵⁹ → Asn¹⁵⁹) was observed at the extracellular LBD1, while the other three substitutions were found at the intracellular domain (Pro⁵⁷⁶ → Ser⁵⁷⁶, Ser⁶²⁸ → Asn⁶²⁸, and Glu⁶⁸³ → Ile⁶⁸³). In comparison with the original *PRLR*, *dPRLR* has a shorter intracellular domain and lacks a C-terminal tail of 149 amino acids. This means that there is a concurrent absence of the three tyrosine residues (Tyr⁷¹⁷, Tyr⁷⁵⁵, and Tyr⁸²⁰) on the C-terminal end, which are conserved in the *PRLR* of other vertebrate species, including humans, rats, mice, bullfrogs, lizards, and zebrafish (Fig. 2; Tanaka *et al.* 1992, Bu *et al.* 2013). However, a tyrosine residue at position 635 (Tyr⁶³⁵) and Box 1 and Box 2 motifs highly conserved in vertebrate *PRLR* are still retained within the short C-terminus of *dPRLR* (Fig. 2; Bole-Feysot *et al.* 1998, Brooks 2012).

dPRLR expressed in HepG2 cells is functionally coupled to the JAK–STAT signaling pathway

To examine whether *dPRLR* is functional *in vitro*, HepG2 cells expressing *dPRLR* were treated with recombinant cPRL, and then receptor activation was monitored using a 5× STAT5–Luciferase reporter system as reported previously (Cocolakis *et al.* 2008, Bu *et al.* 2013). As shown in Fig. 3A, cPRL could stimulate the luciferase activities of HepG2 cells in a dose-dependent manner via the activation of *dPRLR*, indicating that *dPRLR* is a novel functional receptor for PRL and potentially coupled to the intracellular JAK–STAT signaling pathway. Interestingly,

we also observed that the recombinant cPRL seemed to be 1.2- to 1.6-fold less potent in activating *dPRLR* than in activating the original chicken *PRLR* expressed in HepG2 cells in four independent experiments (Fig. 3A), although more assays would be required for confirmation over its statistical significance.

To further investigate whether the activation of *dPRLR* can increase the levels of STAT5 tyrosine phosphorylation, HepG2 cells co-transfected with *dPRLR* (or *PRLR*) were treated with cPRL for 30 min and phosphorylated STAT5 levels were examined in the whole-cell lysates using western blot analysis. As shown in Fig. 3B, similar to that of *PRLR*, the activation of *dPRLR* can increase the levels of STAT5 phosphorylation significantly. No significant change was observed in the negative control, i.e., HepG2 cells transfected with the empty pcDNA3.1(+) vector upon cPRL treatment (Fig. 3B). These findings clearly indicate that *dPRLR* expressed in HepG2 cells is functionally coupled to the intracellular STAT5 signaling pathway.

Tissue expression of *dPRLR* mRNA in male embryos and chickens carrying a *K* allele

As the first step toward unraveling the roles of *dPRLR* gene in chickens, we used only male embryos and chickens (LF) carrying a *K* allele (genotype *K/k*+) to examine and compare the tissue expression patterns of *dPRLR* and *PRLR* (the original *PRLR*) genes in this study. As shown in Fig. 4, both *dPRLR* and *PRLR* genes were detected to be co-expressed in all tissues examined, the brain, small intestine, liver, lungs, muscle, and testis of embryos (from E12 to E20) and 1-week-old chicks using RT-PCR. Similarly, both receptor genes were also found to be co-expressed in all the 12 tissues examined (the brain, heart, small intestine, kidneys, liver, lungs, muscle, testis, pituitary, spleen, pancreas, and skin) of LF male chickens at the 2-month and adult stages (Fig. 5). Interestingly, we also observed that the tissue expression patterns of the two genes (*dPRLR* and *PRLR*) appeared to be more or less similar. For instance, the relatively strong PCR signals of the two *PRLR* genes were consistently observed in the whole brain, small intestine, kidneys, testis, and pituitary of male chickens at the 2-month and adult stages, even though our PCR method was not quantitative (Fig. 5).

In sharp contrast, only *PRLR* gene was detected to be expressed in all tissues examined in EF female chickens (or embryos) with the genotype *k*+/w (without *dPRLR* gene) (Figs 4 and 5).

Figure 3

(A) Activation of chicken dPRLR and PRLR expressed in HepG2 cells by recombinant cPRL treatment (10^{-10} to 10^{-5} g/ml, 18 h). A 5× STAT5–Luciferase reporter system was used to monitor receptor activation by co-transfection of a 5× STAT5–Luciferase reporter construct, pig STAT5A, and receptor expression plasmid in cultured HepG2 cells; co-transfection of an empty pcDNA3.1 (+) vector, pig STAT5A, and a 5× STAT5–Luciferase reporter construct in HepG2 cells was used as an internal control. Each data point represents mean $\pm$ s.e.m. of three replicates. (B) cPRL enhances STAT5 tyrosine phosphorylation of HepG2 cells expressing either dPRLR or chicken PRLR. HepG2 cells transiently expressing chicken PRLR or dPRLR (empty pcDNA3.1(+) vector used as a negative control) were treated with recombinant chicken prolactin (cPRL, 300 ng/ml) for 30 min and total cell lysates were analyzed by western blot assays using specific antibodies against phosphorylated STAT5 (pSTAT5), STAT5 (total STAT5), and β -actin (loading control). Phosphorylated and total STAT5 levels were determined using densitometric analysis, and STAT5 phosphorylation was expressed as relative fold increase in phosphorylated/total STAT5 levels vs respective control (CTL). Each data point represents mean $\pm$ s.e.m. of three replicates. * $P < 0.01$ vs respective control. Representative western blots (pSTAT5, upper panel; total STAT5, middle panel; and β -actin, lower panel) are shown at the bottom of each graph.

Expression of dPRLR, cPRLR, cPRL, and cPRL-L mRNAs in chicken skin tissue

Since dPRLR is probably a candidate gene associated with LF phenotype (Elferink *et al.* 2008, Wang *et al.* 2010a, Luo *et al.* 2012), using RT-PCR, we also examined the

expression of its mRNA in the skin of male chickens (with the genotype *K/k+*) at different developmental stages. As shown in Figs 5 and 6, both dPRLR and PRLR genes were detected to be co-expressed not only in the skin of male embryos from E12 to E20, but also in the skin tissue of 1-week-old, 2-month-old, and adult male chickens.

By contrast, only the PRLR gene was detected to be expressed in the skin tissue of females with the genotype *k+/w* at various stages, i.e., from E12 to the adult stage (Figs 5 and 6).

To determine whether the two PRLR ligands, PRL and PRL-L, could be produced locally (Wang *et al.* 2010b, Bu *et al.* 2013), the mRNA expression of cPRL and cPRL-L genes was also examined in the skin tissue of both sexes at different developmental stages, i.e., from E12 to the post-hatching stage (1 week), using RT-PCR. As shown in Fig. 6, an apparent PCR signal of PRL was consistently observed in the skin tissue of both male (genotype *K/k+*) and female (genotype *k+/w*) embryos at E20 and only a weak PCR signal was detected in the skin tissue of 1-week-old male and female chicks, whereas no PCR signal was detected at other stages. Unlike that of cPRL, only an extremely faint PCR signal of cPRL-L was observed in the skin tissue of both sexes at all the stages examined.

Expression of dPRLR type I and type II transcripts with distinct first exons in LF chicken tissues

In our recent study, we reported that chicken PRLR transcripts encoding PRLR (831 amino acids) should be subdivided into two types, type I and type II transcripts, according to the first exon (exon 1G or exon 1A) used in their 5'-UTRs (Bu *et al.* 2013). PRLR type I transcripts use exon 1G as their first exon, the expression of which is driven by *P1* promoter, whereas PRLR type II transcripts use exon 1A as their first exon and their expression is controlled by *P2* promoter (Fig. 7A; Bu *et al.* 2013). These findings led us to speculate that dPRLR gene could also produce two types of transcripts in chicken tissues similar to those produced by the original PRLR gene. To test this possibility, using a sense primer located on exon 1A (primer F1) or on exon 1G (primer F2) and an antisense primer (primer R1) specific to the unique 3'-UTR of dPRLR gene (Fig. 7A and Table 1), we carried out an RT-PCR to examine their existence in the small intestine and kidneys of male LF chicks. As shown in Fig. 7B and C, the PCR bands of expected sizes could be amplified from the two tissues examined, and sequence analyses further confirmed the existence of dPRLR type I and type II transcripts with distinct first exons in their 5'-UTRs. This finding not only indicates that, similar to the PRLR gene, the dPRLR gene could also generate type I and type II

Figure 4

RT-PCR detection of the expression of *dPRLR* and *PRLR* mRNAs in different tissues of developing embryos from E12 to E20 and 7-day-old (D7) chicks: (A) whole brain (Br); (B) small intestine (In); (C) liver (Li); (D) lung (Lu); (E) muscle (Mu); (F) gonads (Go; ovaries from female embryos or chicks or testis from male embryos or chicks). Both *dPRLR* gene and the original *PRLR* gene were detected to be co-expressed in all the tissues of male chicks (♂, LF) and embryos with the genotype *K/k*+. By contrast, only the original *PRLR* gene was detected to be expressed in all the tissues of female chicks

(♀, early-feathering) and embryos with the genotype *k*+/*w*. No PCR signal was detected in all the negative controls (–). Since *dPRLR* and *PRLR* mRNAs differ in their 3'-UTRs, a sense primer (r1) located on the coding region and an antisense primer (r1) specific either to *dPRLR* 3'-UTR or to *PRLR* 3'-UTR were designed for RT-PCR detection of the expression of *dPRLR* and *PRLR* in chicken tissues (Figs 4, 5 and 6). The location of the primers used for *PRLR* (*PRLR*-f1/r1) and *dPRLR* (*dPRLR*-f1/r1) is shown in Fig. 7A.

transcripts in chicken tissues, which use the duplicated exon 1G and exon 1A (named exon 1G' and exon 1A' in this study) as their first exons respectively, but also implies that the expression of *dPRLR* type I and type II transcripts is controlled by two functional promoters, designed as *P1'* and *P2'* promoters in this study, which were also duplicated from the original *PRLR* gene (Fig. 7A; Bu *et al.* 2013).

Discussion

This study provides clear evidence that *dPRLR* at the sex-linked LF *K* locus of chickens is widely expressed in all tissues examined, including the skin, with a spatiotemporal expression pattern more or less similar to that of the original

copy of the *PRLR* gene localized in the region adjacent to it. Furthermore, we demonstrated that the *dPRLR* gene is likely to encode a novel receptor for PRL. To our knowledge, this study represents the first to report the structure, functionality, and spatiotemporal expression of the *dPRLR* gene in LF chickens and sets an initial, nonetheless important, step toward uncovering the roles of the *dPRLR* gene in chickens carrying the *K* allele.

Gene structure of the *dPRLR* gene at the sex-linked LF *K* locus

In this study, we first cloned the cDNA sequence containing an ORF of *dPRLR* from the kidneys of male

Figure 5

RT-PCR detection of the expression of *dPRLR* and *PRLR* mRNAs in different tissues (brain (Br), heart (He), small intestine (In), kidney (Ki), liver (Li), lung (Lu), muscle (Mu), ovary (Ov), testis (Te), pituitary (Pi), spleen (Sp), pancreas (Pa), and skin (Sk)) of 2-month-old (A) and adult (B) chickens. Both *dPRLR*

and *PRLR* genes were detected to be co-expressed in all the tissues of male chickens (♂, LF, genotype *K/k+*) carrying a *K* allele. By contrast, only the original *PRLR* gene was detected to be expressed in all the tissues of female chickens (♀, EF, genotype *k+/w*) without the *K* allele.

chicks with a *K* allele. Sequence analysis showed that the coding region sequence of *dPRLR* was nearly identical to that of the *PRLR* gene, except for several nucleotide differences, resulting in only four amino acid substitutions being observed between *dPRLR* and *PRLR*, indicating that the coding region of *dPRLR* gene has an exon-intron organization identical to that of the original *PRLR* gene (Fig. 7A; Tanaka *et al.* 1992, Elferink *et al.* 2008, Bu *et al.* 2013). Moreover, *dPRLR* has a spatiotemporal expression pattern more or less similar to that of the *PRLR* gene, suggesting that the promoter regions driving the expression of *dPRLR* have also been duplicated from the *PRLR* gene (Fig. 7A; Bu *et al.* 2013).

The only obvious difference observed between *dPRLR* and *PRLR* lies in their 3'-UTRs. It has been reported that the last exon of the *dPRLR* gene is incomplete and interrupted by a 78 bp breakpoint junction fragment, which links to intron 4 of the partially duplicated *SPEF2* gene (*dSPEF2*) (Fig. 1; Elferink *et al.* 2008). Consistent with this finding, the unique 3'-UTR of the *dPRLR* gene also contains a 78 bp breakpoint junction fragment and a 409 bp sequence antisense to the partial sequence of intron 4 of *dSPEF2* gene (Supplementary Figure 2). The identification of this unique 3'-UTR of *dPRLR* also provides us with an important and valuable molecular marker to detect the existence and mRNA expression of the *dPRLR* gene in chicken strains with the LF phenotype.

Figure 6

RT-PCR detection of the expression of *dPRLR*, *PRLR*, *cPRL-L*, and *cPRL* mRNAs in the skin tissue of developing embryos (from E12 to E20) and 7-day-old (D7) chicks. Both *dPRLR* gene and the original *PRLR* gene were detected to be co-expressed in the skin of male chicks (♂, LF) and embryos with the

genotype *K/k+*. By contrast, only the original *PRLR* gene was detected to be expressed in the skin of female chicks (♀, EF) and embryos with the genotype *k+/w*. The number in brackets indicates the number of PCR cycles used. No PCR signal was detected in all the negative controls (—).

Figure 7

(A) Schematic diagram showing the putative promoters and exon organization of *dPRLR* and *PRLR* genes in LF chickens. The original *PRLR* gene consists of at least 25 exons, including ten non-coding exons (exon 1A to exon 1J) upstream of the translation start site (ATG) and 15 exons (exons 2–16) within the coding region (Bu *et al.* 2013). The two promoters near exon 1G (promoter 1, *P1*) and exon 1A (promoter 2, *P2*) are responsible for the expression of *PRLR* type I and type II transcripts respectively in chickens (for detailed information on *cPRLR*, refer to Bu *et al.* (2013)). *dPRLR* gene has been proposed to be duplicated from the original *PRLR* gene and thus may have a structure identical to that of *PRLR* gene (including two promoters (*P1'* and *P2'*) and 25 exons (exon 1A' to exon 16')), except the 3'-region of the last exon (exon 16') (shaded) of *dPRLR* is distinct from that of *PRLR* gene. Similar to those of *PRLR* gene, the *P1'* promoter (near exon 1G') and *P2'* promoter (near exon 1A') of the *dPRLR* gene are probably responsible for the expression of *dPRLR* type I and type II transcripts respectively in LF chicken

tissues. Arrowheads indicate the locations of translation start codon (ATG) and stop codon (TAA) of *PRLR* and *dPRLR*. (B) RT-PCR detection of the expression of *dPRLR* type I transcripts with exon 1G' in the kidneys and small intestine of three LF male chicks (M1, M2, and M3). The location of primers F2 and R1 used is shown in Fig. 7A. (C) RT-PCR detection of the expression of *dPRLR* type II transcripts with an alternative first exon (exon 1A') in the kidneys and small intestine of three LF male chicks (M1, M2, and M3). The location of primers F1 and R1 used is shown in Fig. 7A. Multiple PCR bands can be observed in (B) and (C), suggesting that the complex alternative mRNA splicing also occurs within the 5'-UTR or coding region of the *dPRLR* gene, as has been observed in the original *PRLR* gene (Bu *et al.* 2013). No PCR band was detected in all the negative controls (–). Note: the location of the primers used for RT-PCR detection of the expression of *PRLR* (*PRLR*-f1/r1) and *dPRLR* (*dPRLR*-f1/r1) mRNAs (Figs 4, 5, and 6) in different chicken tissues is also shown in Fig. 7A.

The *dPRLR* gene may encode a novel functional PRLR

In this study, we found that *dPRLR* is likely to encode a receptor of 683 amino acids. Compared with the original PRLR, *dPRLR* lacks a stretch of 149 amino acid residues including three tyrosine residues (Tyr⁷¹⁷, Tyr⁷⁵⁵, and Tyr⁸²⁰) conserved in the PRLR of vertebrate species (Bole-Feysot *et al.* 1998, Brooks 2012). In rats, these conserved tyrosine residues (Tyr⁴⁷⁹, Tyr⁵¹⁵, and Tyr⁵⁸⁰ in rat PRLR), particularly the last (Tyr⁵⁸⁰), of PRLR have been

reported to be responsible for JAK2-mediated receptor tyrosine phosphorylation (Lebrun *et al.* 1995, Pezet *et al.* 1997, Bole-Feysot *et al.* 1998), which have been proposed to be the docking sites for STAT5 signaling molecules, thereby allowing them to be phosphorylated by JAK2 kinase (Pezet *et al.* 1997). However, our functional study on *dPRLR* clearly indicated that the absence of these conserved tyrosine residues could affect neither receptor-mediated STAT5 tyrosine phosphorylation nor PRL-induced gene

promoter activity, suggesting that dPRLR is a novel functional receptor for PRL. Our finding is partially consistent with the findings in rats and rabbits, in which the removal of the C-terminal portion or mutation of these tyrosine residues of the long-form PRLR does not inhibit PRL-stimulated STAT5 tyrosine phosphorylation (Goupille *et al.* 1997, Pezet *et al.* 1997). Since dPRLR still retains a tyrosine residue at position 635 (Tyr⁶³⁵) conserved in all the vertebrates and an additional tyrosine residue at position 600 (Tyr⁶⁰⁰) at its intracellular domain, it is possible that dPRLR may be phosphorylated at these specific site(s) and thus be capable of transmitting signals.

Wide expression of dPRLR gene in various tissues: implications for its potential diverse actions in LF chickens

It has been reported that PRLR is widely expressed in various tissues of chickens (Tanaka *et al.* 1992, Ohkubo *et al.* 1998a,b). In our recent study, we further confirmed this finding and pointed out that the transcription of PRLR gene is most probably controlled by multiple promoters, including a tissue-specific promoter (P1 promoter) and a generic promoter (P2 promoter) located upstream of the translation start site (Fig. 7A; Bu *et al.* 2013). In this study, we also showed that similar to the PRLR gene, the dPRLR gene is widely expressed in all chicken tissues examined and its expression is also probably controlled by multiple promoters, including P1' and P2' promoters, which were duplicated from the original PRLR gene (Fig. 7).

The overlapping mRNA expression of dPRLR and PRLR genes in all tissues examined of LF chickens and their similar transcriptional regulatory mechanisms suggest that both the receptors are expressed and may function simultaneously in all the chicken tissues. Moreover, the wide tissue expression of dPRLR also strongly suggests that dPRLR may be not only involved in feather development (Elferink *et al.* 2008), but also may participate in other physiological processes by mediating the diverse actions of PRL and thereby control other phenotypic traits of chickens carrying the K allele.

Potential association of the dPRLR gene with the LF development of chickens

The LF phenotype of chicks has long been reported to be controlled by the K locus on chromosome Z (Iraqi & Smith 1995). Chicks with the K allele (genotypes K/w, K/K, and K/k+) often display a LF phenotype, while chicks with the genotypes k+/k+ and k+/w exhibit an EF phenotype at hatching. Since its discovery in chickens in 1922, the

K locus has been introduced into some chicken breeds and widely used for gender identification of newly hatched chicks in the poultry industry (Serebrovsky 1922). For instance, in commercial crosses of egg layers, LF females (K/w) are mated with homozygous EF males (k+/k+) to generate offspring, among which all males (genotype K/k+) have the LF phenotype and all females (genotype k+/w) have the EF phenotype; thus, both sexes of chicks could be easily identified according to the developmental status of flight feathers (Fig. 1). Recently, the K allele on chromosome Z has been reported to arise from a tandem duplication of a large fragment of 176 kb, which contains two partially duplicated genes: dPRLR and dSPEF2 (Iraqi & Smith 1995, Elferink *et al.* 2008). In that study, the authors proposed that dPRLR is probably a candidate gene involved in the delay of feather growth (Elferink *et al.* 2008). However, whether dPRLR gene could be expressed in chicken tissues including the skin and how it is involved in feather development remain unclear.

In this study, both dPRLR and PRLR genes were detected to be co-expressed in the skin tissue of LF male chickens at different developmental stages, i.e., from E12 to the adult stage. This finding, together with the evidence showing that dPRLR is functional *in vitro*, led us to speculate that the co-expression of dPRLR and PRLR in chicken tissues may increase the receptor numbers in target cell membranes and consequently modify the responsiveness of skin (or feather follicles) to PRL, thereby regulating LF development.

Although both dPRLR and PRLR have been shown to be functional in terms of their capability to activate the JAK-STAT signaling pathway, it should be noted that the two receptors differ significantly in their intracellular domains (Fig. 2). Thus, the possibility that the specific signaling event(s) triggered (or interfered) by dPRLR may affect feather growth cannot be ruled out completely. Regardless of the hypothetical model that is the best to explain the *in vivo* situation, the co-expression of dPRLR and PRLR in skin tissue hints that both the receptors are probably involved in the LF development of chicks (or embryos). Future study on the mRNA and/or protein localization of PRLR and dPRLR in skin tissue, particularly their precise localization in feather follicles, will enrich our hypotheses.

Using the transplantation of chicken skin with distinct feathering speed, Danforth (1929) reported that the feathering speed of transplanted skin from newly hatched chicks is primarily determined by the transplanted skin itself, but not by the surrounding host skin or hormones from host chicks, suggesting that the sex-linked

gene(s) responsible for the slow-feathering act locally in the skin or feather follicles rather than through the intermediation of gonadal or other hormones (Danforth 1929). Clearly, sex-linked *dPRLR* and *PRLR* genes co-expressed in the skin of slow-feathering chicks (or embryos) exactly meet the criterion.

The co-expression of *PRLR* and *dPRLR* in the skin tissue of LF chicks (or embryos) also raises an interesting issue as to where its ligand(s) comes from. It has been reported that pituitary lactotrophs begin to differentiate at E17 and secrete PRL into the blood and thus plasma PRL levels increase significantly in embryos at hatching (Harvey *et al.* 1979, Woods & Porter 1998, Fu & Porter 2004), supporting that plasma is probably a major source of PRL. Interestingly, the mRNA expression of *PRL* and *PRL-L*, both of which have been demonstrated to be the functional ligands of cPRLR in our recent study (Bu *et al.* 2013), could be detected in the skin tissue of embryos or chicks. Particularly, an apparent PCR signal of PRL, instead of that of *PRL-L*, was consistently observed in the skin of embryos at E20 and 1-week-old chicks. Our observation is consistent with the findings in humans and mice, in which the mRNA or protein of PRL, a newly defined hair growth-inhibitory hormone, is most prominently found in the hair follicle (Craven *et al.* 2001, Foitzik *et al.* 2003, 2006, Langan *et al.* 2010, Ramot *et al.* 2010). Our findings suggest that as in mammals (Foitzik *et al.* 2009), skin tissue is an extra source of PRL in birds. Presumably, plasma PRL, perhaps together with PRL produced locally, may activate *dPRLR* and *PRLR* co-expressed in the skin (or feather follicles) and thus regulate feathering speed at the late embryonic and early post-hatching stages (Fig. 8).

The tandem duplication of a 176 kb fragment at the *K* locus causes *dPRLR* and *dSPEF2* genes to link together at their 3'-regions (Fig. 1), also pointing out the possibility that the mRNA transcripts of the two duplicated genes may contain antisense sequences of the other gene (*PRLR* or *SPEF2*) and may lead to RNA interference and possibly influence the translation of both the duplicated and original genes and thereby control feather development (Elferink *et al.* 2008). However, using 3'-RACE assays, we found that *dPRLR* cDNA cloned from the kidneys does not contain the antisense sequence of the *SPEF2* gene. Similarly, the cDNA sequence of *dSPEF2* obtained from LF chicken intestine contains no antisense sequence of the *PRLR* gene (data not shown). Moreover, Luo *et al.* (2012) reported that the expression levels of *PRLR* mRNA in the skin tissue of slow-feathering chicks are 1.78-fold higher, but not lower, than those in EF chicks. These findings cast doubt on whether RNA interference could occur and

Figure 8

Hypothetical models for the roles of PRLR or dPRLR in the mediation of PRL actions in chickens with different feathering phenotypes. (A) In EF chickens without the *K* allele, only *PRLR* is widely expressed in all tissues, and thus the diverse actions of PRL including its potential action on feather development are mediated by a single PRLR via interaction with its membrane-proximal LBD2 (Bu *et al.* 2013). (B) In LF chickens carrying the *K* allele, both *dPRLR* and *PRLR* are co-expressed in all tissues examined including the skin, and thus the diverse actions of PRL in various tissues may be mediated by two PRLRs: PRLR and dPRLR, both of which were shown to be functionally coupled to the intracellular JAK–STAT signaling pathway *in vitro*. In LF chicks, the co-expression of *PRLR* and *dPRLR* in skin tissue suggests that both the receptors might be involved in the regulation of feather development through the mediation of the actions of PRL either from plasma or produced from skin tissue. However, this hypothesis needs further verification.

effectively regulate the mRNA levels of *PRLR* gene in skin tissue. Clearly, future studies on the two duplicated genes (*dPRLR* and *dSPEF2*), particularly on their mRNA sequences, mRNA localization, and protein levels in feather follicles of newly hatched chicks (or embryos), will provide more clues to whether RNA interference could play a substantial role in LF development.

It has been reported that in addition to being associated with LF development, the *K* allele is associated with other effects, such as reduction of egg production and increasing the mortality rate (Somes 1975, Harris *et al.* 1984, Dunnington *et al.* 1986, Smith & Fadly 1988, O'Sullivan *et al.* 1991). Some adverse effects are probably associated with the presence of the endogenous retrovirus 21 (*ev21*) gene; our preliminary study (G Bu, G Huang, H Fu, J Li, S Huang & Y Wang, unpublished observations) demonstrated that *ev21* is likely to be located in the first intron of the *dPRLR* (or *PRLR*) gene, at the *K* locus in LF chickens (Bacon *et al.* 1988, Smith & Fadly 1988, Boulliou *et al.* 1992), while some negative side effects can be attributed to the presence and expression of *dPRLR* gene according to the findings of previous studies and the present study (Elferink *et al.* 2008, Wang *et al.* 2010a). In view of the fact that *dPRLR* is widely expressed in all tissues examined and PRL plays critical roles in a number of physiological processes of chickens, such as the initiation and maintenance of incubation behavior, egg-laying, metabolism, and regulation of hypothalamic–pituitary–gonadal axis (Sharp *et al.* 1988, 1998, Talbot *et al.* 1991), it is tempting to speculate that similar to PRLR, *dPRLR* is capable of mediating the diverse actions of PRL *in vivo* and thus may also affect other phenotypic traits of chickens (Fig. 8). Clearly, more studies are required to fully uncover the roles of *dPRLR* in chickens, including i) extensive studies on mRNA and protein levels of *dPRLR* (and *PRLR*) gene in various tissues at different developmental stages in both sexes of chickens with distinct genotypes (*K/K*, *K/k*+, *k*+/+*k*+, *K/w*, and *k*+/+*w*); ii) intensive studies on the intracellular signaling event(s) triggered (or interfered) by *dPRLR*; and iii) extensive investigations on the coordinated actions of *dPRLR* and *PRLR* and their ligand(s) in the control of the physiological processes and phenotypic traits of chickens. Undoubtedly, these systematic studies will help us to re-evaluate whether it is necessary to introduce the *K* allele into some chicken strains from a whole new perspective.

In summary, *dPRLR* gene within the LF (*K*) locus of chickens was characterized in the present study. The results showed that similar to *PRLR*, *dPRLR* is likely to encode a novel PRLR and is widely expressed in various tissues, including the skin of male chickens carrying a *K* allele.

Moreover, *PRL* mRNA was detected to be expressed in the skin tissue of embryos at E20 and in 1-week-old chicks. Our findings not only suggest that skin-expressed *dPRLR* and *PRLR*, together with plasma and skin-derived PRL, may play a role in the control of LF development in chicks, but also imply that *dPRLR*, either alone or in combination with *PRLR*, is actively involved in many other physiological processes, as evidenced by its wide tissue expression (Fig. 8). Therefore, the quest for fully deciphering the roles, both advantageous and disadvantageous to the poultry industry, of the *dPRLR* gene within the sex-linked LF *K* locus in chickens is still ongoing.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-13-0068>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Bacon LD, Smith E, Crittenden LB & Havenstein GB 1988 Association of the slow feathering (*K*) and an endogenous viral (*ev21*) gene on the Z chromosome of chickens. *Poultry Science* **67** 191–197. (doi:10.3382/ps.0670191)
- Ben-Jonathan N, LaPensee CR & LaPensee EW 2008 What can we learn from rodents about prolactin in humans? *Endocrine Reviews* **29** 1–41. (doi:10.1210/er.2007-0017)
- Bole-Feyssot C, Goffin V, Edery M, Binart N & Kelly PA 1998 Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocrine Reviews* **19** 225–268. (doi:10.1210/er.19.3.225)
- Boulliou A, Le Pennec JP, Hubert G, Donal R & Smiley M 1992 The endogenous retroviral *ev21* locus in commercial chicken lines and its relationship with the slow-feathering phenotype (*K*). *Poultry Science* **71** 38–46. (doi:10.3382/ps.0710038)

- Brooks CL 2012 Molecular mechanisms of prolactin and its receptor. *Endocrine Reviews* **33** 504–525. (doi:10.1210/er.2011-1040)
- Bu G, Ying Wang C, Cai G, Leung FC, Xu M, Wang H, Huang G, Li J & Wang Y 2013 Molecular characterization of prolactin receptor (cPRLR) gene in chickens: gene structure, tissue expression, promoter analysis, and its interaction with chicken prolactin (cPRL) and prolactin-like protein (cPRL-L). *Molecular and Cellular Endocrinology* **370** 149–162. (doi:10.1016/j.mce.2013.03.001)
- Cocolakis E, Dai M, Drevet L, Ho J, Haines E, Ali S & Lebrun JJ 2008 Smad signaling antagonizes STAT5-mediated gene transcription and mammary epithelial cell differentiation. *Journal of Biological Chemistry* **283** 1293–1307. (doi:10.1074/jbc.M707492200)
- Craven AJ, Ormandy CJ, Robertson FG, Wilkins RJ, Kelly PA, Nixon AJ & Pearson AJ 2001 Prolactin signaling influences the timing mechanism of the hair follicle: analysis of hair growth cycles in prolactin receptor knockout mice. *Endocrinology* **142** 2533–2539. (doi:10.1210/en.142.6.2533)
- Danforth CH 1929 Two factors influencing feathering in chickens. *Genetics* **14** 256–269.
- Doneen BA & Smith TE 1982 Ontogeny of endocrine control of osmoregulation in chick embryo. II. Actions of prolactin, arginine vasopressin, and aldosterone. *General and Comparative Endocrinology* **48** 310–318. (doi:10.1016/0016-6480(82)90142-3)
- Dunnington EA, Siegel PB & Gross WB 1986 Sex-linked feathering alleles (K, k+) in chickens of diverse genetic backgrounds. *Avian Pathology* **15** 139–148. (doi:10.1080/03079458608436273)
- Elferink MG, Vallee AA, Jungerius AP, Crooijmans RP & Groenen MA 2008 Partial duplication of the PRLR and SPEF2 genes at the late feathering locus in chicken. *BMC Genomics* **9** 391. (doi:10.1186/1471-2164-9-391)
- Fang Q, Li J, Lu X, Nie Q, Zhang X & Wang Y 2012 Pig signal transducer and activator of transcription 3, 5a and 5b genes: cDNA cloning, tissue expression, and identification of their transcript variants. *Journal of Sichuan University (Natural Science Edition)* **49** 879–886.
- Foitzik K, Krause K, Nixon AJ, Ford CA, Ohnemus U, Pearson AJ & Paus R 2003 Prolactin and its receptor are expressed in murine hair follicle epithelium, show hair cycle-dependent expression, and induce catagen. *American Journal of Pathology* **162** 1611–1621. (doi:10.1016/S0002-9440(10)64295-2)
- Foitzik K, Krause K, Conrad F, Nakamura M, Funk W & Paus R 2006 Human scalp hair follicles are both a target and a source of prolactin, which serves as an autocrine and/or paracrine promoter of apoptosis-driven hair follicle regression. *American Journal of Pathology* **168** 748–756. (doi:10.2353/ajpath.2006.050468)
- Foitzik K, Langan EA & Paus R 2009 Prolactin and the skin: a dermatological perspective on an ancient pleiotropic peptide hormone. *Journal of Investigative Dermatology* **129** 1071–1087. (doi:10.1038/jid.2008.348)
- Freeman ME, Kanyicska B, Lerant A & Nagy G 2000 Prolactin: structure, function, and regulation of secretion. *Physiological Reviews* **80** 1523–1631.
- Fu X & Porter TE 2004 Glucocorticoid induction of lactotrophs and prolactin gene expression in chicken embryonic pituitary cells: a delayed response relative to stimulated growth hormone production. *Endocrinology* **145** 1322–1330. (doi:10.1210/en.2003-1064)
- Goffin V, Shiverick KT, Kelly PA & Martial JA 1996 Sequence–function relationships within the expanding family of prolactin, growth hormone, placental lactogen, and related proteins in mammals. *Endocrine Reviews* **17** 385–410. (doi:10.1210/edrv-17-4-385)
- Goupille O, Daniel N, Bignon C, Jolivet G & Djiane J 1997 Prolactin signal transduction to milk protein genes: carboxy-terminal part of the prolactin receptor and its tyrosine phosphorylation are not obligatory for JAK2 and STAT5 activation. *Molecular and Cellular Endocrinology* **127** 155–169. (doi:10.1016/S0303-7207(97)04005-7)
- Harris DL, Garwood VA, Lowe PC, Hester PY, Crittenden LB & Fadly AM 1984 Influence of sex-linked feathering phenotypes of parents and progeny upon lymphoid leukosis virus infection status and egg production. *Poultry Science* **63** 401–413. (doi:10.3382/ps.0630401)
- Harvey S, Davison TF & Chadwick A 1979 Ontogeny of growth hormone and prolactin secretion in the domestic fowl (*Gallus domesticus*). *General and Comparative Endocrinology* **39** 270–273. (doi:10.1016/0016-6480(79)90121-7)
- Iraqi F & Smith EJ 1995 Organization of the sex-linked late-feathering haplotype in chickens. *Animal Genetics* **26** 141–146. (doi:10.1111/j.1365-2052.1995.tb03153.x)
- Langan EA, Ramot Y, Goffin V, Griffiths CE, Foitzik K & Paus R 2010 Mind the (gender) gap: does prolactin exert gender and/or site-specific effects on the human hair follicle? *Journal of Investigative Dermatology* **130** 886–891. (doi:10.1038/jid.2009.340)
- Lea RW, Dods AS, Sharp PJ & Chadwick A 1981 The possible role of prolactin in the regulation of nesting behaviour and the secretion of luteinizing hormone in broody bantams. *Journal of Endocrinology* **91** 89–97. (doi:10.1677/joe.0.0910089)
- Lebrun JJ, Ali S, Goffin V, Ullrich A & Kelly PA 1995 A single phosphotyrosine residue of the prolactin receptor is responsible for activation of gene transcription. *PNAS* **92** 4031–4035. (doi:10.1073/pnas.92.9.4031)
- Luo C, Shen X, Rao Y, Xu H, Tang J, Sun L, Nie Q & Zhang X 2012 Differences of Z chromosome and genomic expression between early- and late-feathering chickens. *Molecular Biology Reports* **39** 6283–6288. (doi:10.1007/s11033-012-1449-7)
- Nguyen N, Stellwag EJ & Zhu Y 2008 Prolactin-dependent modulation of organogenesis in the vertebrate: recent discoveries in zebrafish. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology* **148** 370–380. (doi:10.1016/j.cbpc.2008.05.010)
- Ohkubo T, Tanaka M, Nakashima K & Sharp PJ 1998a Relationship between prolactin receptor mRNA in the anterior pituitary gland and hypothalamus and reproductive state in male and female bantams (*Gallus domesticus*). *General and Comparative Endocrinology* **111** 167–176. (doi:10.1006/gcen.1998.7099)
- Ohkubo T, Tanaka M, Nakashima K, Talbot RT & Sharp PJ 1998b Prolactin receptor gene expression in the brain and peripheral tissues in broody and nonbroody breeds of domestic hen. *General and Comparative Endocrinology* **109** 60–68. (doi:10.1006/gcen.1997.7008)
- O'Sullivan NP, Dunnington EA, Smith EJ, Gross WB & Siegel PB 1991 Performance of early and late feathering broiler breeder females with different feeding regimens. *British Poultry Science* **32** 981–995. (doi:10.1080/00071669108417423)
- Pezet A, Ferrag F, Kelly PA & Edery M 1997 Tyrosine docking sites of the rat prolactin receptor required for association and activation of stat5. *Journal of Biological Chemistry* **272** 25043–25050. (doi:10.1074/jbc.272.40.25043)
- Ramot Y, Biro T, Tiede S, Toth BI, Langan EA, Sugawara K, Foitzik K, Ingber A, Goffin V, Langbein L *et al.* 2010 Prolactin – a novel neuroendocrine regulator of human keratin expression *in situ*. *FASEB Journal* **24** 1768–1779. (doi:10.1096/fj.09-146415)
- Serebrovsky AS 1922 Crossing-over involving three sex-linked genes in chickens. *American Naturalist* **56** 571–572. (doi:10.1086/279898)
- Sharp PJ, Scanes CG, Williams JB, Harvey S & Chadwick A 1979 Variations in concentrations of prolactin, luteinizing hormone, growth hormone and progesterone in the plasma of broody bantams (*Gallus domesticus*). *Journal of Endocrinology* **80** 51–57. (doi:10.1677/joe.0.0800051)
- Sharp PJ, Macnamee MC, Sterling RJ, Lea RW & Pedersen HC 1988 Relationships between prolactin, LH and broody behaviour in bantam hens. *Journal of Endocrinology* **118** 279–286. (doi:10.1677/joe.0.1180279)
- Sharp PJ, Dawson A & Lea RW 1998 Control of luteinizing hormone and prolactin secretion in birds. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology & Endocrinology* **119** 275–282. (doi:10.1016/S0742-8413(98)00016-4)
- Smith EJ & Fadly AM 1988 Influence of congenital transmission of endogenous virus-21 on the immune response to avian leukosis virus infection and the incidence of tumors in chickens. *Poultry Science* **67** 1674–1679. (doi:10.3382/ps.0671674)

- Somes RG Jr 1975 Pleiotrophic effects of the sex-linked delayed feathering gene, K-n, in the chicken. *Poultry Science* **54** 208–216. (doi:10.3382/ps.0540208)
- Talbot RT, Hanks MC, Sterling RJ, Sang HM & Sharp PJ 1991 Pituitary prolactin messenger ribonucleic acid levels in incubating and laying hens: effects of manipulating plasma levels of vasoactive intestinal polypeptide. *Endocrinology* **129** 496–502. (doi:10.1210/endo-129-1-496)
- Tanaka M, Maeda K, Okubo T & Nakashima K 1992 Double antenna structure of chicken prolactin receptor deduced from the cDNA sequence. *Biochemical and Biophysical Research Communications* **188** 490–496. (doi:10.1016/0006-291X(92)91082-2)
- Wang Y & Ge W 2004 Developmental profiles of activin β A, β B, and follistatin expression in the zebrafish ovary: evidence for their differential roles during sexual maturation and ovulatory cycle. *Biology of Reproduction* **71** 2056–2064. (doi:10.1095/biolreprod.104.032649)
- Wang CY, Wang Y, Kwok AH & Leung FC 2007 Identification of two novel chicken GHRH receptor splice variants: implications for the roles of aspartate 56 in the receptor activation and direct ligand receptor interaction. *Journal of Endocrinology* **195** 525–536. (doi:10.1677/JOE-07-0167)
- Wang X, Nahashon S, Feaster TK, Bohannon-Stewart A & Adefope N 2010a An initial map of chromosomal segmental copy number variations in the chicken. *BMC Genomics* **11** 351. (doi:10.1186/1471-2164-11-351)
- Wang Y, Li J, Yan Kwok AH, Ge W & Leung FC 2010b A novel prolactin-like protein (PRL-L) gene in chickens and zebrafish: cloning and characterization of its tissue expression. *General and Comparative Endocrinology* **166** 200–210. (doi:10.1016/j.ygcen.2009.10.007)
- Wang Y, Meng F, Zhong Y, Huang G & Li J 2012a Discovery of a novel glucagon-like peptide (GCGL) and its receptor (GCGLR) in chickens: evidence for the existence of GCGL and GCGLR genes in nonmammalian vertebrates. *Endocrinology* **153** 5247–5260. (doi:10.1210/en.2012-1586)
- Wang Y, Wang CY, Wu Y, Huang G, Li J & Leung FC 2012b Identification of the receptors for prolactin-releasing peptide (PrRP) and Carassius RFamide peptide (C-RFa) in chickens. *Endocrinology* **153** 1861–1874. (doi:10.1210/en.2011-1719)
- Woods KL & Porter TE 1998 Ontogeny of prolactin-secreting cells during chick embryonic development: effect of vasoactive intestinal peptide. *General and Comparative Endocrinology* **112** 240–246. (doi:10.1006/gcen.1998.7168)
- Zhu Y, Stiller JW, Shaner MP, Baldini A, Scemama JL & Capehart AA 2004 Cloning of somatolactin alpha and beta cDNAs in zebrafish and phylogenetic analysis of two distinct somatolactin subtypes in fish. *Journal of Endocrinology* **182** 509–518. (doi:10.1677/joe.0.1820509)

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