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 potently 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

Introduction
Prolactin (PRL) is a polypeptide hormone and belongs to the growth hormone (GH)/PRL family, which also includes somatolactin, placental lactogens, and the newly identified PRL-like (PRL-L) protein (Goffin et al. 1996, Bole-Feyso 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-Feytso 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-Feytso 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
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 H2O. 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; Table 1). 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.
RT-PCR 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 deoxynucleotide triphosphate, 0.5 µg oligo-deoxythymidine, 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

### Table 1 Primers used in the study

<table>
<thead>
<tr>
<th>Primers used for the rapid amplification of dPRLR 3'-ends (3'-RACE)</th>
<th>Sense/antisense</th>
<th>Primer sequence (5’-3’)</th>
<th>Size (bp)</th>
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<tr>
<td>dPRLR-rF1</td>
<td>Sense</td>
<td>GCAAACCTGACCATGGATCATGGAT</td>
<td>2109</td>
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<tr>
<td>dPRLR-rF2</td>
<td>Sense</td>
<td>ACCGCCAGTCCAGGGCCAAAGAT</td>
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<tr>
<td>dPRLR-rF3</td>
<td>Sense</td>
<td>GTTGAGTCTCTTGTTGCAAGGCTTGGCTT</td>
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<th>Primers used for the construction of dPRLR expression plasmids</th>
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<td>CCGGTTACACAGGAGAAATGGAATACATGA</td>
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<td>dPRLR-eR1</td>
<td>Antisense</td>
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<th>Primers used for RT-PCR assays</th>
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<th>Primer sequence (5’-3’)</th>
<th>Size (bp)</th>
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<td>β-Actin</td>
<td>Sense</td>
<td>TGTGCTACGTGCACCTGGAT</td>
<td>2114–2760</td>
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<tr>
<td>dPRLR-f1</td>
<td>Sense</td>
<td>CATGAATTGACATGGGTCGA</td>
<td>2135–2478</td>
</tr>
<tr>
<td>PRLR-f1</td>
<td>Sense</td>
<td>CATGGAGTGAATGGAGCTCAG</td>
<td>148</td>
</tr>
<tr>
<td>cPRL</td>
<td>Sense</td>
<td>CAGGCAGATCTCGTTGAGAATG</td>
<td>126</td>
</tr>
<tr>
<td>cPRL-L</td>
<td>Sense</td>
<td>TCGAGGAACTGCTGAGAATG</td>
<td>78</td>
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<th>Primers used for the detection of dPRLR type I and type II transcripts having distinct first exons</th>
<th>Sense/antisense</th>
<th>Primer sequence (5’-3’)</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>dPRLR-F1</td>
<td>Sense</td>
<td>CAGATACGTGACAGGTCGTGAG</td>
<td>78</td>
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<tr>
<td>dPRLR-F2</td>
<td>Sense</td>
<td>GAGATGGCATCACATGTGTCGCT</td>
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<tr>
<td>dPRLR-R1</td>
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<th>Primers used for quantitative real-time PCR assays</th>
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<th>Size (bp)</th>
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<td>cGHR</td>
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<td>cPRL</td>
<td>Sense</td>
<td>GAGATGGCATCACATGTGTCGCT</td>
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</tr>
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<td>cPRL-L</td>
<td>Sense</td>
<td>ACAAGTGTCAGACTAGGGCTAGCA</td>
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<th>Size (bp)</th>
</tr>
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<tr>
<td>dPRLR</td>
<td>Sense</td>
<td>TGAACATCCCTGTGGAGAGATG</td>
<td>78</td>
</tr>
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</table>

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 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 3×10^5 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% confluence. After 24 h of culture, the cells were subcultured in a 96-well plate at a density of 2×10^4 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 (Ellertink 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 \textit{dpLrL} and cDNA sequence of the original \textit{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 \textit{dpLrL} from the kidneys of male chicks with a K allele. The cloned \textit{dpLrL} was 2519 bp in length, and its nucleotide sequence was nearly identical to that of \textit{Prlr} except for its 3′-UTR (Supplementary Figure 2).

The cloned \textit{dpLrL} is predicted to encode a novel receptor of 683 amino acids (accession no.: JQ768801). Similar to \textit{Prlr}, \textit{dpLrL} 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 \textit{dpLrL} shares a nearly identical amino acid sequence identity with the original \textit{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 (Ser\textsuperscript{159} → Asn\textsuperscript{159}) was observed at the extracellular LBD1, while the other three substitutions were found at the intracellular domain (Pro\textsuperscript{576} → Ser\textsuperscript{576}, Ser\textsuperscript{628} → Asn\textsuperscript{628}, and Glu\textsuperscript{683} → Ile\textsuperscript{683}). In comparison with the original \textit{Prlr}, \textit{dpLrL} 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 (Tyr\textsuperscript{717}, Tyr\textsuperscript{755}, and Tyr\textsuperscript{820}) on the C-terminal end, which are conserved in the \textit{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 (Tyr\textsuperscript{635}) and Box 1 and Box 2 motifs highly conserved in vertebrate \textit{Prlr} are still retained within the short C-terminus of \textit{dpLrL} (Fig. 2; Bole-Feyssot et al. 1998, Brooks 2012).

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

To examine whether \textit{dpLrL} is functional \textit{in vitro}, HepG2 cells expressing \textit{dpLrL} 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, \textit{cPrl} could stimulate the luciferase activities of HepG2 cells in a dose-dependent manner via the activation of \textit{dpLrL}, indicating that \textit{dpLrL} is a novel functional receptor for \textit{Prlr} and potentially coupled to the intracellular JAK–STAT signaling pathway. Interestingly, we also observed that the recombinant \textit{cPrl} seemed to be 1.2- to 1.6-fold less potent in activating \textit{dpLrL} than in activating the original chicken \textit{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 \textit{dpLrL} can increase the levels of STAT5 tyrosine phosphorylation, HepG2 cells co-transfected with \textit{dpLrL} (or \textit{Prlr}) were treated with \textit{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 \textit{Prlr}, the activation of \textit{dpLrL} 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 \textit{cPrl} treatment (Fig. 3B). These findings clearly indicate that \textit{dpLrL} expressed in HepG2 cells is functionally coupled to the intracellular STAT5 signaling pathway.

Tissue expression of \textit{dpLrL} mRNA in male embryos and chickens carrying a K allele

As the first step toward unraveling the roles of \textit{dpLrL} gene in chickens, we used only male embryos and chickens (LF) carrying a \textit{K} allele (genotype \textit{K/k+}) to examine and compare the tissue expression patterns of \textit{dpLrL} and \textit{Prlr} (the original \textit{Prlr}) genes in this study. As shown in Fig. 4, both \textit{dpLrL} and \textit{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 (\textit{dpLrL} and \textit{Prlr}) appeared to be more or less similar. For instance, the relatively strong PCR signals of the two \textit{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 \textit{Prlr} gene was detected to be expressed in all tissues examined in EF female chickens (or embryos) with the genotype \textit{k+/w} (without \textit{dpLrL} gene) (Figs 4 and 5).
Amino acid sequence alignment of the duplicated PRLR (dPRLR: JQ768801, 683 amino acids) with chicken PRLR derived from the original PRLR gene (cPRLR: KC686695, 831 amino acids, cloned from the kidneys of Lohmann chickens) and PRLR from other vertebrate species including leopard geckos (lgPRLR: BAD24103), rats (rPRLR: NP_001029283), mice (mPRLR: NP_035299), humans (hPRLR: NP_000940), bullfrogs (rePRLR: BAD14941), and zebrafish (zfPRLR: NP_001122149). Arrowheads indicate the four amino acid substitutions (in bold) observed between dPRLR and the original PRLR. Signal peptide (SP) is underlined; LBD1 is shaded; LBD2 is shaded and underlined; TMD, Box 1 motif, and Box 2 motif are shaded. The tyrosine residue at position 635 (Tyr635 of cPRLR) conserved in chickens and other species is boxed and shaded. The other three conserved tyrosine residues (Tyr717, Tyr755, and Tyr820 of cPRLR) absent in dPRLR are boxed. Asterisk (*) indicates an extra tyrosine residue at position 600 (Tyr600, in bold) only observed in chicken dPRLR and PRLR. The conserved cysteine residues and WSXWS motif(s) at the extracellular LBD(s) critical for receptor folding, trafficking, and activation are also boxed (Brooks 2012).
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
transcripts in chicken tissues, which use the duplicated exon 1G and exon 1A (named exon 1G₀ and exon 1A₀ 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 P1₀ and P2₀ 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 PRLR gene (Fig. 7A; Bu et al. 2013).

**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

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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.

A 2-Month-old

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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 chicks (♂, 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.

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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 (−).
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 (Tyr<sup>717</sup>, Tyr<sup>755</sup>, and Tyr<sup>920</sup>) conserved in the PRLR of vertebrate species (Bole-Feyssot et al. 1998, Brooks 2012). In rats, these conserved tyrosine residues (Tyr<sup>479</sup>, Tyr<sup>515</sup>, and Tyr<sup>580</sup> in rat PRLR), particularly the last (Tyr<sup>580</sup>), 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/+−) 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 dSPEEF2 (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...
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 examined tissues 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 hypotalamic–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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