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Changes in prolactin receptor homodimer availability may cause late feathering in chickens

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ABSTRACT

Chicken early (EF) and late feathering (LF) are sex-linked phenotypes conferred by wild-type k^+ and dominant *K* alleles on chromosome Z, respectively. Besides prolactin (PRL) receptor (*PRLR*) and sperm flagellar 2 (*SPEF2*) genes, the *K* allele contains a fusion gene in which partially duplicated *PRLR* (*dPRLR*) and *SPEF2* (*dSPEF2*) genes are linked in a tail-to-tail manner. The causative *dPRLR* gene encodes a C-terminal truncated receptor. LF chickens have short or no primaries at hatching; however, their feather growth rate is higher than that of EF chickens. This study aimed to elucidate the molecular basis of the *K* allele's biphasic effect on feather development. By 3′RACE and RT-PCR analyses, we demonstrated that *dSPEF2* gene transcription occurred beyond all coding exons of the *dPRLR* gene on the opposite strand and that *dPRLR* mRNA was less abundant than *PRLR* mRNA. In addition, a 5′UTR splice variant (SPV) of PRL receptor mRNAs was increased in LF chickens. *In vitro* expression analysis of 5′UTR linked to the luciferase reporter gene revealed higher translation efficiency of SPV. RT-qPCR showed that the *dPRLR* mRNA level was higher in embryos; conversely, SPV was higher in hatched chickens, as was *dSPEF2* mRNA. These findings suggest that the *K* allele inhibits feather development at the fetal stage by expressing dPRLR to attenuate PRLR function and promotes feather growth after hatching by increasing PRLR through *dSPEF2* mRNA expression. Increased SPV may cause greater feather growth than that in EF chickens by increasing the availability of PRLR homodimers and enhancing PRL signaling.

1. Introduction

Feather growth is genetically controlled by the *K* locus on the Z chromosome in chickens (Hamoen et al., 2001). The sex-linked dominant *K* allele retards feather development and causes late feathering (LF), while the normal recessive k^+ allele is responsible for early feathering (EF) (Lirette et al., 1993). Hatched chicks have two rows of feathers on the outer blade of the wings: the upper row is the primary coverts and the lower row is the primaries. The *K* allele causes delayed emergence of primaries compared with the case for the k^+ allele. Among chicks born from the mating of an EF male and an LF female, males are all LF and females are all EF, so the *K* locus alleles are widely used for autosexing of day-old chicks in hatcheries (Leader and Siegel, 1957).

Studies on the molecular basis of the effects of the *K* allele have identified a fusion gene consisting of partially duplicated *PRLR* (*dPRLR*) and *SPEF2* (*dSPEF2*) genes between the original *PRLR* and *SPEF2* genes (Elferink et al., 2008; Takenouchi et al., 2018). The *PRLR* gene encodes a prolactin (PRL) receptor, which is 831 amino acids (aa) in length and transduces the PRL signal through receptor dimerization followed by the activation of related signaling proteins including Jak2 and Fyn. The *SPEF2* gene encodes sperm flagellar protein 2, which in mice plays a role in localization of the intraflagellar transport protein IFT20 to the manchette and is suggested to function as an adapter for dynein-mediated protein transport during spermatogenesis (Lehti et al., 2017). The *dPRLR* gene encodes a truncated receptor lacking the C-terminal 149 aa and the *dSPEF2* gene contains only 4 of 29 exons of the *SPEF2* gene; these two genes are linked in a tail-to-tail manner (Elferink et al.,

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2008).

Based on the results of microarray and quantitative reverse-transcription polymerase chain reaction (RT-qPCR), it has been suggested that the 1.78-fold increase in PRL receptor mRNAs (i.e., *PRLR* and *dPRLR* mRNAs; hereafter, PRLR and dPRLR are collectively referred to as the PRL receptor in this paper) results in the LF phenotype (Luo et al., 2012). Similarly, a PRL receptor with increased function has been proposed to cause the LF phenotype, based on expression and functional analyses demonstrating that *dPRLR* mRNA showed a spatio-temporal expression pattern similar to that of *PRLR* mRNA and that dPRLR was associated with Jak2/Stat5 signaling equivalent to that of PRLR (Bu et al., 2013a). In contrast, both *SPEF2* and *dSPEF2* genes but not PRL receptor genes have been suggested to be candidate genes for LF, based on RT-PCR and RT-qPCR findings (Zhao et al., 2016). Thus, there was controversy over the mechanism underlying the LF phenotype. Recently, however, the causal dominant mutation of the turkey LF phenotype was revealed to be a 5-bp deletion causing a frameshift resulting in the introduction of a stop codon causing deletion of the C-terminal 98 aa of the PRL receptor; it was strongly suggested that the lack of the C-terminal end of the PRL receptor is responsible for LF in both chicken and turkey (Derks et al., 2018), although the molecular mechanisms by which the PRL receptor mutations exert their effects remain unclear.

Interestingly, the LF phenotype differs between turkey and chicken. That is, LF turkeys generally show poor feathering even at a later age, but LF chickens show a higher growth rate of feathers after hatching than EF chickens (Derks et al., 2018; Zakrzewska and Savage, 1997). The present study was conducted to elucidate the molecular mechanism underlying this biphasic effect of the *K* allele on feather development in LF chickens. By cloning *dSPEF2* mRNA and quantifying the amount of relevant mRNAs, we now propose a new model that can explain the characteristics seen only in chickens. We also show for the first time that PRL signaling can be fine-tuned by regulating the transcriptional efficiency of PRLR mRNA by the alternative splicing of 5′UTR.

2. Materials and methods

One-day-old Broiler (F_1 generation of a cross between a female White Plymouth Rock and a male White Cornish) chicks and embryos were purchased from a commercial grower (Fukuda Poultry Breeding Farm, Okayama, Japan). The male (K/k^+) and female ($k^+/-$) chicks were housed in a room with free access to commercial food and water and were used in the experiments. Adult female White Leghorn chickens of the MK line ($K/-$) and MB line ($k^+/-$) were kindly provided by the Japanese Avian Bioresource Project Research Center (Hiroshima University, Hiroshima, Japan). All animal procedures were performed in accordance with the guidelines of the Experimental Animal Committee of Okayama University, and the committee specifically approved this study.

2.1. Molecular sexing of embryos

Five microliters of whole blood collected from each embryo was diluted with the same volume of distilled water. Diluted blood samples were denatured by adding 22.5 µl of 50 mM NaOH, vortexed for 15 min, incubated at 95 °C for 10 min, and then placed on an ice bath for 3 min. After neutralization by adding 2.5 µl of 1 M Tris-HCl and vortexing thoroughly for 1 min, the mixture was centrifuged at 12,000 rpm for 5 min at 4 °C, after which the supernatant was recovered. A 0.5-µl aliquot of the supernatant was subjected to polymerase chain reaction (PCR) (reaction volume of 25 µl) using Tks Gflex DNA polymerase (Takara Bio, Kusatsu, Japan), four primers P1–P4, and a thermal cycler (Life Eco; Nippon Genetics, Aichi, Japan). The sequences of all primers used in this study are listed in Table 1. The PCR conditions were as follows: 94 °C for 1 min, followed by 40 cycles of denaturation for 10 s at 98 °C, annealing for 15 s at 60 °C, and extension for 15 s at 68 °C. A 10-µl aliquot of each reaction was electrophoresed on a 2.0% agarose gel and stained with ethidium bromide. Cases were judged as males when there was one PCR amplification band and females when there were two (Itoh et al., 2001).

Table 1
Primers used in this study.

Primers	Gene	Sequence(5′ → 3′)
	For Sexing	
P1	*SPINDLIN* (FP)	AAGCATAGAAACAATGTGGGAC
P2	*SPINDLIN* (RP)	AACTCTGTCTGGAAGGACTT
P3	*NIPBL* (FP)	CTATGCCTACCACATTCCTATTTGC
P4	*NIPBL* (RP)	AGCTGGACTTCAGACCATCTTCT
	For 3′RACE	
P5	*SPEF2* (FP)	TCAGGATGACTTTAATCAATTTTCA
P6	*PRLR* (FP)	TTATCCTACCGCCAGTTCCAGG
P7	*dSPEF2* (FP)	ATCAGCCTCCTATGTTTGCCTAT
	For RT-PCR	
P8	*Luciferase* (FP)	ACTACCGGTAAAGCCACCATGGAAG
P9	*Luciferase* (RP)	GACCTCGAGATTACACGGCGATC
P10	*PRLR/dPRLR* (FP)	TCCGCTAGCAGACTGAATATTTTGCAGTCAGAG
P11	*PRLR/dPRLR* (RP)	CCCACCGGTGATTTCCACTTCCCCTTGGAAAAG
P12	*dSPEF2* (FP)	GGTTGGCCGTCATGTCCGAG
P13	*dSPEF2* (RP)	CAGTTGGTGTTTTCATTGATGTGA
P14	*PRLR/dPRLR* (FP)	TTTATCCTACCGCCAGTTCCAG
P15	*PRLR* (RP)	TGGAATGCAAATTCTCCATCTC
P16	*dPRLR* (RP)	GATCACCTGAGACCCAATGTTG
	For RT-PCR	
P17	*GAPDH* (FP)	GTGTTATCATCTCAGCTCCCTCAG
P18	*GAPDH* (RP)	GGTGCACGATGCATTGCTGACAA
P19	*PRLR* (FP)	GTTCACCATGACTGAGAATCTGC
P20	*PRLR* (FP)	CTCTGGATCTGCAGTCTAAGAG
P21	*PRLR* (RP)	TGGAATGCAAATTCTCCATCTC
P22	*dPRLR* (RP)	GATCACCTGAGACCCAATGTTG
P23	*PRLR/dPRLR* (FP)	TCCAATTGCTGCCTTTTTGAGGGT
P24	*PRLR/dPRLR* (RP)	AAGTAGCAGGAATTGGGACCTG
	For RT-qPCR	
P25	*PRLR/dPRLR* (FP)	GGCAAAAAGGAGAAGCCACA
P26	*PRLR/dPRLR* (RP)	CGTCAGATCACTTGATTATCAGACT
P27	*PRLR/dPRLR* (FP)	CCAGGCAAAAAGGAGAAGCC
P28	*PRLR/dPRLR* (RP)	AATTGGAAGTAGAGGCAGACTCTGTT
P29	*dSPEF2* (FP)	CTGATGAATGTACTTCTAACAGGGTCAT
P30	*dSPEF2* (RP)	GGAGTGGATGGAGCTTTTATCATG
P31	*PRLR/dPRLR* (FP)	TCAATCACAGTGTTCTCTCACTGA
P32	*PRLR* (RP)	TGACCTGTGCTGTGGTTTG
P33	*dPRLR* (RP)	AATTCTAACCTGAGACAAGTGTCAGACTA
P34	*Luciferase* (FP)	AGCGAAGGTTGTGGATCTGG
P35	*Luciferase* (RP)	GAGGCGAACTGTGTGTGAGA

FP and RP denote forward primer and reverse primer, respectively.

2.2. Total RNA preparation and deoxyribonuclease I treatment

Total RNA was prepared from cultured cells (HEK293T), pooled feather follicles (three to five pieces), outer blade of wings (wing tips), and various peripheral tissues using the TriPure Isolation Reagent (Roche Diagnostics, IN, USA). After RNA integrity had been assessed by 1% agarose gel electrophoresis, and to remove co-extracted genomic DNA, the total RNA was treated with deoxyribonuclease I (amplification grade; Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions and used for the following analyses: rapid amplification of cDNA 3′ ends (3′RACE), reverse-transcription PCR (RT-PCR), and real time quantitative PCR (RT-qPCR).

2.3. 3′RACE

The 3′RACE analyses were performed using the GeneRacer kit (Invitrogen), in accordance with the manufacturer's instructions. Total RNA prepared from the primary flight feather follicles plucked from 8-day-old male Broiler chicks was reverse-transcribed using the

ThermoScript RT-PCR system (Invitrogen) and GeneRacer Oligo-dT. The PCR was carried out using platinum Taq (Invitrogen) and gene-specific forward primers. The GeneRacer 3′ primer and the GeneRacer 3′ nested primer were used in the first 3′RACE-PCR and the second (nested) 3′RACE-PCR, respectively, as the reverse primers. The gene-specific forward primer used for *dSPEF2* cDNA amplification was P5, and those for *dPRLR* cDNA amplification were P6 and P7. These primers were designed based on the reported chicken *SPEF2* transcript SPEF2-201 (ENSGALT00000039152.3 in EnsEMBL) and *dPRLR-dSPEF2* fusion gene (KU553470.1 in GenBank). After PCR, an aliquot of each reaction was electrophoresed on 1.0% or 2.0% agarose gel, stained with ethidium bromide, and photographed under UV illumination. The amplified cDNA fragments were extracted from the agarose gel, purified using NucleoSpin Gel and PCR Clean-up (Takara Bio), subcloned using a TOPO TA Cloning Kit for Sequencing (Thermo Fisher Scientific, Tokyo, Japan), and sequenced. Sequencing was carried out using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an automatic sequencer (ABI 3500/3500 × L Genetic Analyzer; Applied Biosystems). The obtained sequences were mapped to the chicken genome combined with the reported *dPRLR-dSPEF2* fusion gene (KU553470.1 in GenBank) and chicken *PRLR* gene (ENSGALG00000003446) extracted from the EnsEMBL chicken genome database (http://www.ensembl.org/Gallus_gallus/Info/Index).

2.4. RT-PCR

Reverse transcription was performed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), the SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity DNA Polymerase (Invitrogen), or the PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio), depending on the experiment. Here, 0.5–3 μg of each total RNA was reverse-transcribed, in accordance with the manufacturer's instructions. Subsequent PCR was carried out using Platinum Taq DNA polymerase or Tks Gflex DNA polymerase. The PCR cycle conditions were in accordance with the instructions for each enzyme. The primers used and the numbers of cycling reactions for each cDNA amplification are described in the figure legends. An aliquot of each reaction was electrophoresed on 0.7%–2.0% agarose gel, stained with ethidium bromide, and photographed under ultraviolet illumination. The amplified cDNA fragments were recovered from the gel and subjected to sequencing directly or after being subcloned.

2.5. Plasmid construction

By PCR cloning with the appropriate primers, the plasmid pEGFP-c1 (Takara Bio) was modified to construct a CAG enhancer/CMV promoter-driven luciferase reporter gene with the 5′ untranslated region (5′UTR) of *dPRLR* and *PRLR* mRNAs in front of its ORF. First, the pEGFP-c1 was digested with the restriction enzymes *Age*I (NEB, Ipswich, MA, USA) and *Xho*I (NEB) to remove ORF of the *egfp* gene, electrophoresed on 1% agarose gel, and purified using the NucleoSpin Gel and PCR clean-up. The firefly luciferase gene fragment was generated by PCR using the Tks Gflex DNA polymerase and the plasmid pGL3-basic (Promega, Madison, WI, USA) as a template. The forward primer P8 and the reverse primer P9 used contained *Age* I and *Xho* I recognition sites, respectively. The PCR-amplified luciferase gene fragment was purified and digested with *Age*I and *Xho*I, and then ligated with the *Age*I/*Xho*I double-digested pEGFP-c1 using the DNA ligation kit Mighty Mix (Takara Bio). Transformation in *E. coli* strain DH5α was performed in line with the standard protocol. The sequence of the resulting plasmid, named pLuc, was verified by sequencing. Second, the 5′UTRs of *dPRLR* and *PRLR* mRNAs were generated by RT-PCR using total RNA from feather follicles of adult female White Leghorn chickens of the MK line ($K/-$) and MB line ($k^+/-$). The total RNA was reverse-transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR, and subsequent PCR was carried out with Platinum Taq DNA Polymerase. The primers used were the forward primer P10 and the reverse primer P11, containing *Nhe*I and *Age*I recognition sites, respectively. After purification, each 5′UTR (Maj and SPV from MB and MK lines, respectively) was digested with *Nhe*I (NEB) and *Age*I, ligated with the *NheI/Age*I double-digested pLuc, and used for DH5α transformation. The sequences of the resulting plasmids, named pLuc-Maj5′UTR and pLuc-SPV5′UTR, were verified by sequencing. To obtain plasmid DNA for transfection experiments, purification of plasmids was performed using NucleoSpin Plasmid Easy Pure (Takara Bio).

2.6. Luciferase reporter assay

HEK293T cells were obtained from the Health Science Research Resources Bank (Osaka, Japan), maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air, and subcultured into 12-well cell culture plates with 1.0×10^5 cells/well 24 h before transfection. Transfection was performed with PEI (Polysciences, Warrington, PA, USA). In brief, for one well, 500 ng of the experimental plasmid (pLuc-Maj5′UTR or pLuc-SPV5′UTR), 2 ng of *Renilla* luciferase reporter vector (phRL-TK vector; Promega), and 4 μl of PEI were added to Opti-MEM to make a total volume of 100 μl, and the mixture was incubated for 15 min at room temperature. A 100-μl aliquot of this gene transfer mixture was added to each well and mixed for 1 min by shaking the plates. The *Renilla* luciferase reporter vector was used to check the transfection efficiency. Four hours after transfection, the culture medium was replaced with DMEM supplemented with 1% ITS-G Supplement (×100) (Wako Pure Chemical Industries, Osaka, Japan). The dual-luciferase assay was performed 24 h after transfection using the Dual-luciferase reporter assay system (Promega), in accordance with the manufacturer's instructions. Transfected cells were washed in phosphate-buffered saline, and were lysed with the reporter lysis buffer provided in the system. The lysates were assayed for luciferase activity using a T-20/20 Luminometer (Promega). The luciferase activity was normalized to the *Renilla* luciferase activity of each sample. Each assay was performed in quadruplicate, and each experiment was repeated at least three times. In addition, total RNA was prepared from transfected cells and used for real-time quantitative PCR (RT-qPCR) of luciferase mRNA.

2.7. RT-qPCR

A total of 0.5 μg of total RNA was reverse-transcribed in a 10-μl reaction mixture using PrimeScript RT Master Mix (Perfect Real Time), in accordance with the manufacturer's instructions. RT-qPCR was carried out using the SYBRPremix Ex Taq II (Tli RNaseH Plus) and the Applied Biosystems 7300 Fast Real-Time PCR System. The 10-μl PCR mixture consisted of 2 μl of RT reaction, 0.4 μl of 10 μM each of forward and reverse primers, 5 μl of SYBR®Premix Ex Taq II, 0.2 μl of ROX Reference Dye, and 2.4 μl of distilled water. The cycle parameters were as follows: initial denaturation for 30 s at 95 °C, followed by 40 cycles of denaturation for 5 s at 95 °C and annealing for 31 s at 60 °C. A melting curve analysis was performed from 60 °C to 95 °C to detect potential nonspecific products. Standard templates of each cDNA were prepared by RT-PCR, purified, and quantified spectrophotometrically. Four serial dilutions of cDNA templates were used in separate real-time reactions to determine their threshold cycle values. By plotting the threshold cycle versus the dilution factor, a standard curve was generated and the amount of each experimental sample was determined. Each assay was performed in quadruplicate, and each sample was analyzed at least in triplicate. Each primer set was designed based on the cDNA sequence obtained in this study. The size of amplicon is 70, 110, 109, 111, 104 and 113 bp for *Luciferase*, Maj, SPV, *dSPEF2*, *PRLR* and *dPRLR*, respectively.

2.8. Statistical analysis

The results are expressed as mean ± SEM, and one-way analysis of variance (ANOVA) was performed followed by comparison with the control group by Tukey's test. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Identification of mRNAs of the K locus fusion gene

To obtain insights into the mode of action of the *K* allele, we attempted to identify transcripts of the *dSPEF2/dPRLR* fusion gene by 3′RACE analyses. By using a gene-specific forward primer corresponding to exon 2 of the *dSPEF2* and *SPEF2* genes, we successfully identified three novel transcripts with a poly(A) tail expressed in primary follicles of 8-day-old LF chicks (male, Broiler). The transcripts were 290, 346, and 392 bp in length and were predicted to encode different truncated SPEF2s with lengths of 61, 63, and 70 aa, whereas intact SPEF2 is 1410 aa in length. We then designed a PCR reverse primer corresponding to a region common to these three transcripts, and performed RT-PCR to examine whether these transcripts are really from the *dSPEF2* gene. As shown in Fig. 1, multiple bands of cDNA were detected in LF chicks, but not in EF chicks. Sequence analysis revealed that these cDNAs represent multiple *dSPEF2* mRNAs generated by the alternative splicing of eight exons. Four of them were exons 1–4 of the *dSPEF2* gene, and the other four exons, which we named A to D, were novel exons located within the *dPRLR* gene on the opposite strand. We also identified four *dPRLR* mRNAs with different polyadenylation sites that were located within intron 4 of the *dSPEF2* gene on the opposite strand, and 234, 401, 433, and 623 bp downstream from the translation stop codon TAA of the *dPRLR* gene. The exon compositions of the *dSPEF2* mRNAs as well as alternative poly(A) sites of *dPRLR* mRNA are schematically shown in Fig. 2. Exons A and B were located between exons 6 and 7, and exons 1I and 2, respectively, of the *dPRLR* gene. Exons C and D, in contrast, spanned 144–198 bp and 818–973 bp, respectively, upstream of exon 1G, indicating that transcription of the *dSPEF2* gene occurs beyond all coding exons of the *dPRLR* gene on the opposite strand in the *K* allele. The sequences of the mRNAs identified here are available in the DDBJ/GenBank databases with accession numbers LC311580, LC311581, and LC222290 to LC222295 for *dSPEF2* mRNAs, and LC222296 to LC222299 for *dPRLR* mRNAs.

3.2. Effect of dSPEF2 gene expression on the dPRLR mRNA level

Since the transcription unit of the *dSPEF2* gene overlaps with the *dPRLR* gene in the *K* allele fusion gene, *dPRLR* gene expression may be repressed by transcription of the *dSPEF2* gene. It has been demonstrated that the coding region of the *dPRLR* gene has an exon–intron organization identical to that of the original *PRLR* gene (except for exon 16 truncation) (Bu et al., 2013b; Elferink et al., 2008); moreover, the promoter regions driving the expression of the *dPRLR* gene have been

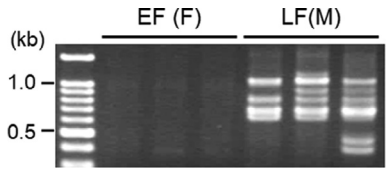

Fig. 1. Expression of *dSPEF2* mRNA in primary flight feather follicles in chicks. Representative electrophoretic RT-PCR patterns of cDNAs from the primary flight feather follicles of 8-day-old male and female Broiler chicks are shown. Males (M) and females (F) show late- (LF) and early-feathering (EF) phenotypes, respectively. The three lanes in each column show the results from three different experimental animals. Left lane is the 100-bp ladder used as a molecular marker. The primers used were P12 and P13, and 35 cycles were applied.

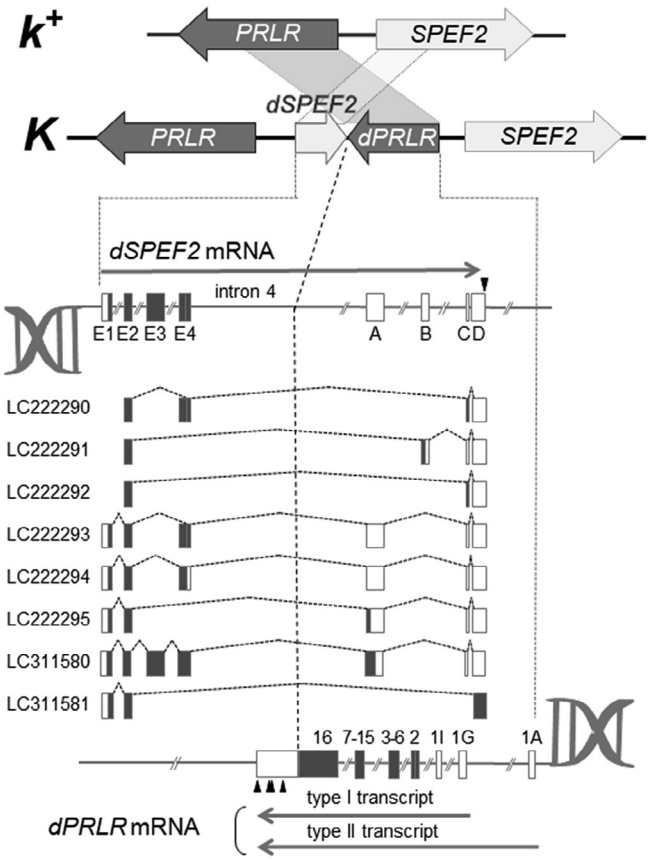

Fig. 2. Schematic representation of the *K* allele fusion gene and its mRNA structure. The *dSPEF2* and *dPRLR* genes, eight species of *dSPEF2* mRNA with different exon compositions, and 4 poly (A) sites of *dPRLR* mRNAs are shown. In the *dPRLR* gene, only major exons are shown. *PRLR* type I transcript and *PRLR* type II transcript contain exons 1G and 1A, respectively, as the first exon. Exons 1G and 1A are located approximately 9 and 72 kb, respectively, upstream of the translation initiation codon. Exons of each gene are denoted by boxes and coding regions are indicated by solid boxes. Arrowheads indicate the locations of poly (A) sites.

duplicated from the original *PRLR* gene (Bu et al., 2013a,b). Therefore, without such repression, *dPRLR* mRNA might be expressed as much as *PRLR* mRNA. We compared the expression levels between *dPRLR* mRNA and *PRLR* mRNA by applying competitive RT-PCR using a mixture of primers including reverse primers specific for each mRNA and a common forward primer. As shown in Fig. 3A, the intensity of the band of *dPRLR* cDNA was clearly low compared with that of *PRLR* cDNA in PCR with total RNA from primary follicles of 1-week-old LF chickens (male, Broiler). Similar results were obtained in all tissues confirmed to express *dSPEF2* mRNA (Fig. 3B). Although it cannot exclude the possibility that amplification efficiencies of *dPRLR* cDNA and *PRLR* cDNA are different, the expression of the *dPRLR* gene might be inversely related to *dSPEF2* gene expression. These results are consistent with the previous finding that the abundance of *dPRLR* mRNA was only 2.8% of that of *PRLR* mRNA in the skin at the tips of LF chicken wings when measured by RT-qPCR (Luo et al., 2012).

3.3. Effect of dSPEF2 gene expression on RNA processing of dPRLR transcripts

As *dSPEF2* mRNAs contain no antisense sequence of PRL receptor mRNAs, it seems unlikely that *dSPEF2* mRNA leads to RNA interference or influences the translation of the PRL receptor mRNAs. Heterogeneous nuclear RNA of the *dSPEF2* gene, however, contains the antisense sequence of PRL receptor genes, and thus may interfere with

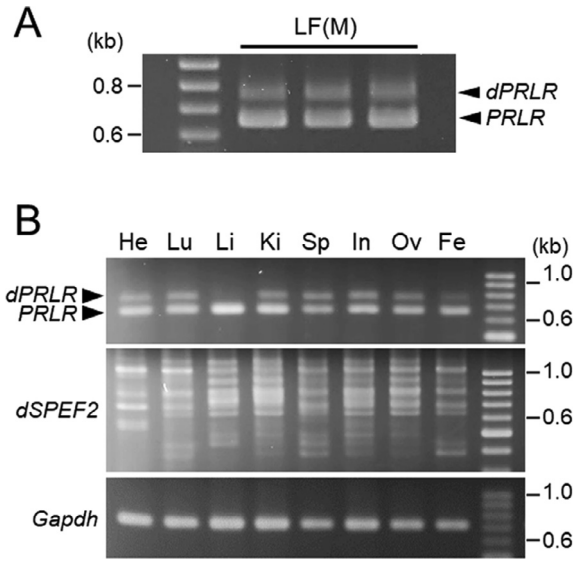

Fig. 3. Comparison of the expression of *PRLR* and *dPRLR* mRNAs in LF chickens. (A) Expression of *PRLR* and *dPRLR* mRNAs in the primary flight feather follicles of Broiler LF chicks. Representative electrophoretic competitive RT-PCR patterns of cDNAs from 7-day-old male (M) chicks are shown. The three lanes in each column show the results from three different experimental animals. Left lane is the 100-bp ladder used as a molecular marker. The PCR primers used were P14, P15, and P16, and 35 cycles were applied. (B) Expression of mRNA for *PRLR*, *dPRLR*, and *dSPEF2* in various tissues of adult White Leghorn chickens with the late-feathering (LF) phenotype. Representative electrophoretic competitive RT-PCR patterns (top panel) and RT-PCR patterns (middle and bottom panels) are shown. *Gapdh* is an internal control. Right lanes are the 100-bp ladder used as a molecular marker. The primers used for competitive PCR were P14, P15, and P16, and 35 cycles were applied. The primer sets for amplification of *dSPEF2* and *Gapdh* cDNAs were P12 and P13, and P17 and P18, respectively. The cycle numbers of those RT-PCRs were 35 and 18, respectively. He, Lu, Li, Ki, Sp, In, Ov, and Fe denote heart, lung, liver, kidney, spleen, small intestine, ovary, and feather follicle, respectively.

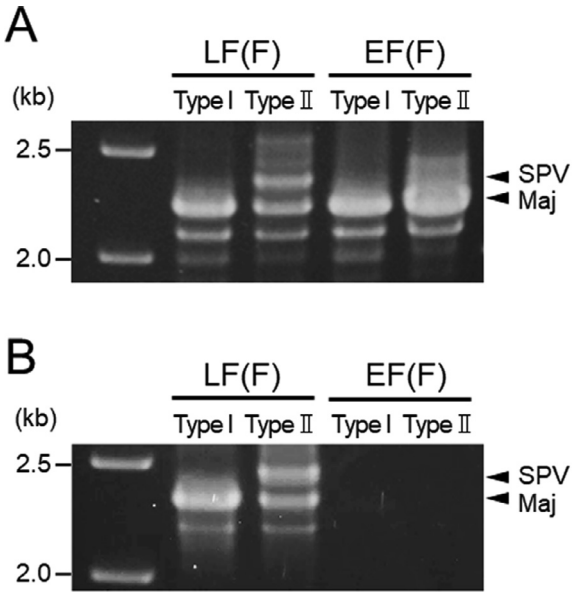

Fig. 4. Comparison of *dPRLR* and *PRLR* mRNAs between EF and LF chickens. RT-PCR analysis of type I and type II transcripts of *PRLR* (A) and *dPRLR* (B) in the kidney of adult female White Leghorn chickens of MK line (LF) and MB line (EF). Representative electrophoretic patterns of RT-PCR patterns are shown. Left lanes are the 1-kb ladder used as a molecular marker. The primers used for the amplification of type I and type II transcripts of *PRLR* were P19 and P21, and P20 and P21, and those of *dPRLR* were P19 and P22, and P20 and P22, respectively. 35 cycles were applied. The primers P19 and P20 are located in exons 1G and 1C, respectively. The primers P21 and P22 are located in exon 16 of *PRLR* and *dPRLR* genes, respectively. The name of the exons of *PRLR* and *dPRLR* genes used in this paper is based on the report of Bu *et al.* (Bu et al., 2013b).

their RNA processing. To test this possibility, we amplified nearly full-length cDNA of *PRL* receptor mRNAs by RT-PCR and compared the results between LF and EF chickens. The chicken *PRLR* mRNA can be classified into two classes, type I and *PRLR* type II transcripts (Bu et al., 2013b). Their transcription is controlled by the P1 promoter responsible for tissue-specific expression in the kidney and small intestine, and the P2 promoter responsible for ubiquitous expression, respectively (Bu et al., 2013b). As kidneys express both type I and type II transcripts, we first used total RNA from the kidney of LF and EF chickens. Differences in electrophoretic patterns were observed in type II transcripts of both PRL receptor genes, but not in type I transcripts; specifically, they featured a decrease in the intensity of the major band and the appearance of an additional band (Fig. 4). Sequence analysis revealed that the additional band represented a splicing variant (SPV) containing exon 1E in the 5′ untranslated region (5′UTR) of PRL receptor mRNAs. The major transcripts (Maj) and the SPV corresponded to T2a and T2b, respectively, of the recently identified 5′UTR isoforms of *PRLR* mRNA type II transcripts (Bu et al., 2013b).

If the appearance of this SPV was the result of the interference of *dSPEF2* transcripts, then the SPV should be observed in other tissues including the feather follicles in LF chickens. To examine this, we carried out RT-PCR with a primer corresponding to exon 1E as a forward primer. As expected, in two breeds of the White Leghorn and Broiler, the SPV was detected as two bands with higher intensity in LF chickens than in EF chickens (Fig. 5). Sequencing revealed that the two bands represent the SPV with or without exon 3 of the PRL receptor genes. These results suggest that *dSPEF2* transcripts act as trans-acting RNA to interfere with splicing of the PRL receptor transcripts,

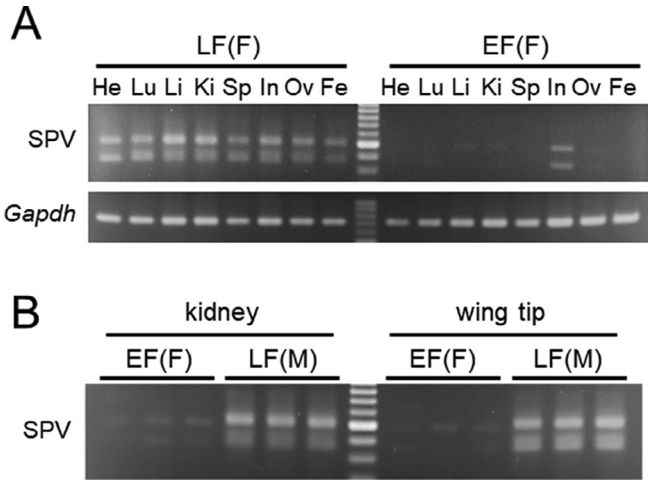

Fig. 5. Expression of *dPRLR/PRLR* mRNA 5′UTR splice variant in EF and LF chickens. (A) RT-PCR analysis of SPV in various tissues of adult female White Leghorn chickens of the MK line (LF) and MB line (EF). Representative electrophoretic RT-PCR patterns are shown. *Gapdh* is the internal control. He, Lu, Li, Ki, Sp, In, Ov, and Fe denote heart, lung, liver, kidney, spleen, small intestine, ovary, and feather follicle, respectively. (B) RT-PCR analysis of SPV in the kidney and wing tip of 2-day-old male (M) and female (F) Broiler chicks. Representative electrophoretic RT-PCR patterns are shown. The three lanes in each column show the results from three different experimental animals. The PCR primers used for detecting SPV were P23 and P24, which are located in exon 1E and exon 3, respectively and35 cycles were applied.

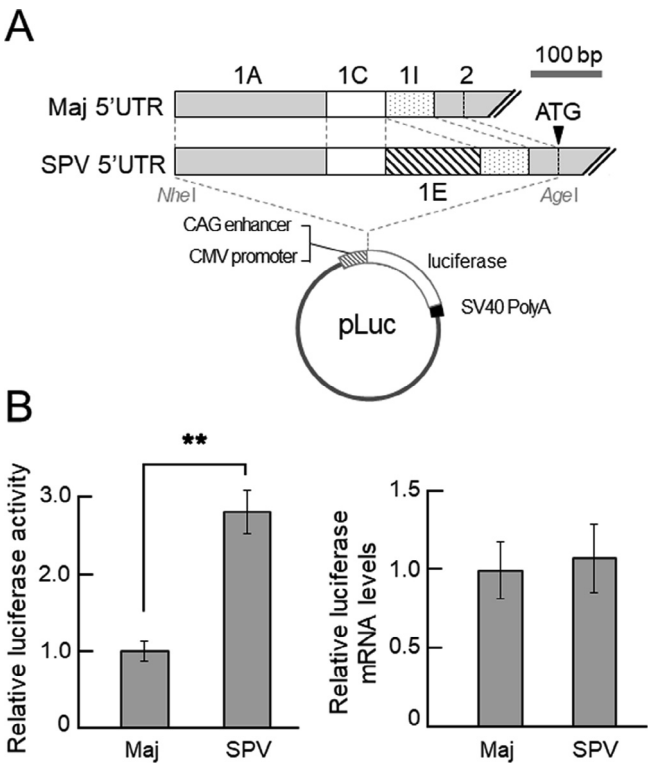

Fig. 6. Differential translation efficiency mediated by 5′UTR of *dPRLR* and *PRLR* mRNAs. (A) Schematic representation of recombinant constructs used for evaluating translation efficiency. (B) Translation efficiency of recombinant constructs evaluated by luciferase activity and luciferase mRNA abundance. Relative luciferase activities (left) measured by luciferase assay and relative luciferase mRNA levels (right) measured by RT-qPCR in HEK293T cells transfected with pLuc-Maj5′UTR (Maj) and pLuc-SPV5′UTR (SPV) are shown. The results are shown as mean ± SEM of quadruplicate determinations. **P < 0.005, significantly different from the group of Maj. The primers used in the RT-qPCR of cDNA for *luciferase* were P34 and P35.

decreasing Maj and increasing SPV.

3.4. Physiological significance of 5′UTR of PRL receptor mRNA

Multiple 5′UTR splice variants of *PRLR* mRNA have been identified to date, but their physiological significance has not been elucidated (Bu et al., 2013b). Therefore, we investigated the effect of different 5′UTRs of PRL receptor mRNAs on translation using an *in vitro* assay system. As shown in Fig. 6A, the 5′UTR of each of Maj and SPV was ligated in front of the ORF of the luciferase reporter gene. Plasmids containing these constructs, named pLuc-Maj5′UTR and pLuc-SPV5′UTR, respectively, were separately transfected into cells of the human embryonic kidney cell line HEK293T and the translational activity of luciferase mRNA was measured by luciferase assay. Luciferase mRNA in those transfected cells was also quantified by RT-qPCR. As shown in Fig. 6B, no difference was detected in the amount of luciferase mRNA in cells transfected with either of the plasmids; however, the luciferase activity was significantly higher in the cells transfected with pLuc-SPV5′UTR than those transfected with pLuc-Maj5′UTR ($p < 0.05$). These results indicate for the first time that the 5′UTR affects the translation rate of chicken PRL receptor mRNAs, and that the SPV is a splicing variant of PRL receptor mRNAs with a higher ability to produce PRL receptors.

3.5. Ontogenetic changes in the expression of the mRNAs of the K allele fusion gene

LF chickens have short or no primaries at hatching due to the inhibitory effects of the *K* allele on embryonic feather development; however, their rate of feather growth after hatching is high and it eventually catches up with that of EF chickens. To investigate the relationship between the effects of the *K* allele on feather development and expression of the fusion gene, we examined the expression of *dPRLR* mRNA, *PRLR* mRNA, as well as *dSPEF2* mRNA in the tips of LF chicken wings by RT-qPCR. The expression of *dSPEF2* mRNA (Fig. 7A) as well as *dPRLR* and *PRLR* mRNAs (Fig. 7B, C) tended to increase after hatching. The ratio of *PRLR* mRNA to *dPRLR* mRNA increased significantly as development progressed (Fig. 7D, $p < 0.01$, E20 vs. E15; $p < 0.05$, D1 vs. E15). Likewise, as shown in Fig. 7E and F, the amount of SPV as well as the ratio of SPV to Maj increased significantly ($p < 0.05$, D1 vs. E15). Collectively, these results indicate that *dPRLR* mRNA levels were high in embryos; conversely, SPV was high in hatched chicks, as was *dSPEF2* mRNA.

4. Discussion

The present study identified *dSPEF2* mRNA for the first time and demonstrated that the transcription of the *dSPEF2* gene occurs beyond all coding exons of the *dPRLR* gene on the opposite strand in the *K* allele fusion gene. In addition, it was found that transcription of *dSPEF2* mRNA may repress the expression of *dPRLR* mRNA and alter alternative splicing bias in the 5′UTR of PRL receptor mRNAs to increase translation efficiency. The ontogenetic expression patterns of mRNAs of the *K* allele fusion gene suggested that the amounts of PRLR as well as the ratio of PRLR to dPRLR are low in embryos and high in hatched chicks of LF chickens. Based on these observations, it seems likely that there is a positive correlation between the PRLR level and the rate of feather growth, and conversely that dPRLR has an inhibitory effect on feather development.

PRLR belongs to the class I cytokine receptor superfamily and its signal transduction is mediated by associated signaling proteins, such as Jak2 (Campbell et al., 1994; Lebrun et al., 1994; Rui et al., 1994), Fyn (Clevenger et al., 1994), Grb2/Sos1 (Erwin et al., 1995), Raf (Clevenger et al., 1994), and Vav (Clevenger et al., 1995), which are activated as a consequence of ligand-induced receptor dimerization. The intracellular domain contains three proximal motifs, namely, boxes 1 and 2, and the V box, and an evolutionary conserved tyrosine residue in the C-terminal region (Chang et al., 1998). Structure–function studies have shown that the proximal motifs are sufficient for the activation of Jak2 (Chang et al., 1998), while the C-terminal is thought to be necessary for the activation of other signaling proteins such as Fyn (Kline et al., 1999). Furthermore, it has been shown that the wild-type PRL receptor and C-terminal truncation PRL receptor cannot function as heterodimers, even though they are perfectly capable of functioning as homodimers (Kline et al., 1999).

dPRLR is a C-terminal truncated receptor with a 149-aa deletion, and contains all of the proximal motifs while lacking a C-terminal tyrosine residue (Elferink et al., 2008). It has been demonstrated by *in vitro* assays that dPRLR is a functional receptor and potentially couples to the intracellular JAK/STAT signaling pathway (Bu et al., 2013). Similar to dPRLR, the turkey PRL receptor responsible for the LF phenotype has a 98-aa deletion at the C-terminal (Derks et al., 2018). The chicken and turkey PRL receptors are both 831 aa in length and share 90.24% identity; however, deficiency in feathering is more severe in turkeys than in chickens. LF turkeys generally show poor feathering even later in life, whereas LF chickens show a high rate of feather growth after hatching, eventually catching up with EF chickens (Zakrzewska and Savage, 1997). This is probably due to the presence of the normal *PRLR* gene in the chicken *K* allele.

In addition to poor feathering, significant increases in serum glucose levels and plasma sodium concentrations have been reported in LF turkeys (Zakrzewska and Savage, 1997). Furthermore, their production of eggs is adversely affected: some females do not lay any eggs at all while others have few eggs (Zakrzewska and Savage, 1997).

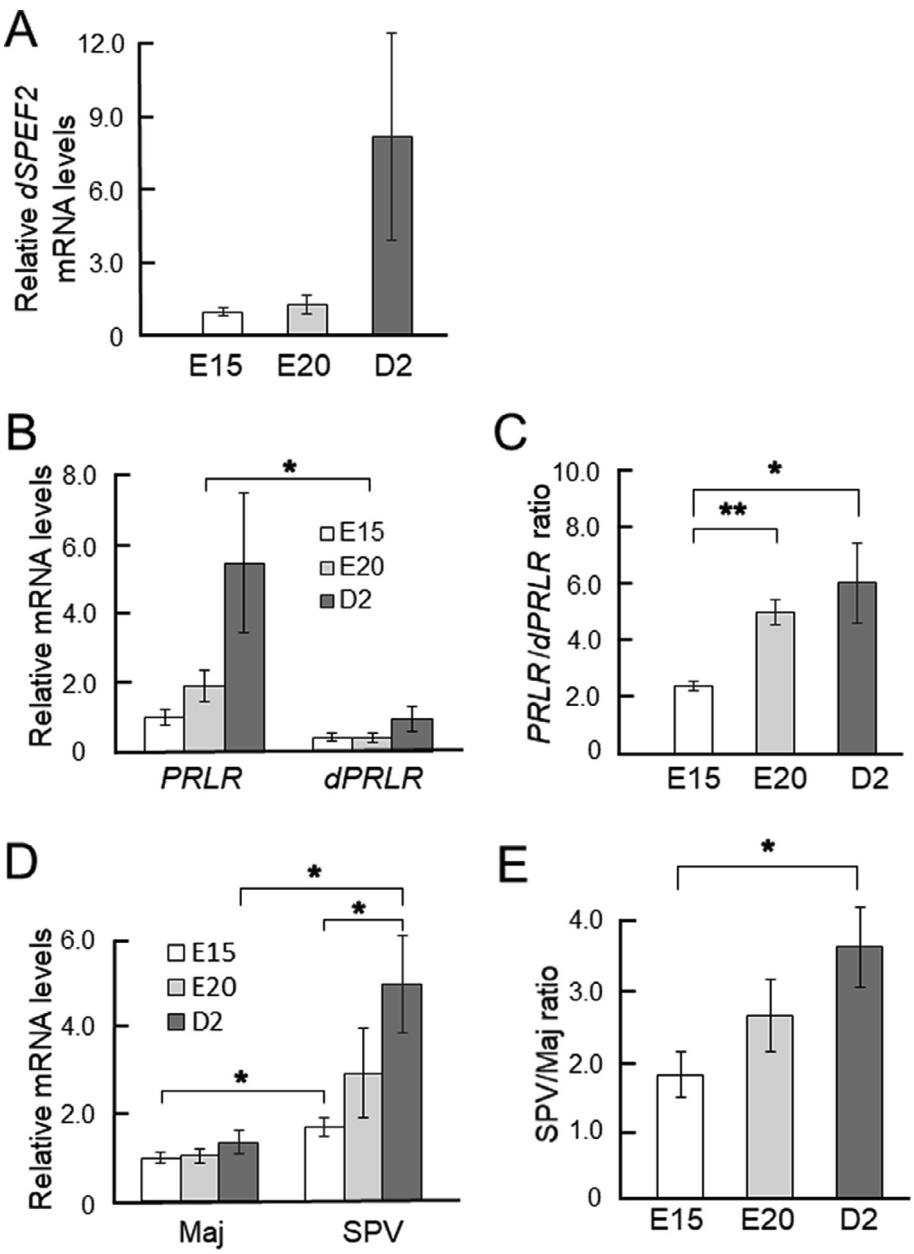

Fig. 7. Ontogenetic profile of the expression of *dPRLR* and *PRLR* mRNAs and their 5′UTR variant, as well as *dSPEF2* mRNA in LF chickens. Relative levels of *dSPEF2* mRNA (A), *PRLR* mRNA (B), and *dPRLR* mRNA (C) in the wing tip of male Broiler chicks and embryos measured by RT-qPCR. (D) The ratio of the amount of *PRLR* mRNA to *dPRLR* mRNA in the wing tip of male Broiler chicks and embryos measured by RT-qPCR. Relative Maj and SPV levels (E) and the ratio of SPV to Maj (F) in the wing tip of male Broiler chicks and embryos measured by RT-qPCR. The results are shown as mean ± SEM of quadruplicate determinations. *P < 0.05 and **P < 0.005, significantly different between the indicated groups. E15, E20, and D2 denote embryonic day 15, embryonic day 20, and day 2 after hatching, respectively. The PCR primers used in the RT-qPCR of cDNA for *dSPEF2*, *PRLR*, *dPRLR*, Maj, and SPV were P29 and P30, P31 and P32, P31 and P33, P25 and P26, and P27 and P28, respectively.

Mammalian PRL stimulates urinary sodium excretion by inhibiting proximal tubular Na^+,K^+-ATPase activity through the renal dopamine system (Ibarra et al., 2005). PRL enhances β-cell proliferation and stimulates insulin gene transcription, and deficiency of PRL receptor causes reductions in islet density and β-cell mass, and higher serum glucose levels after an i.p. glucose load in mice (Huang et al., 2009). In chickens, it has been shown that immunization against PRL slows down ovarian follicular development and reduces the egg-laying performance of hens (Li et al., 2011). Considering these findings along with an accumulated wealth of knowledge on the structure–function relationship of the PRL receptor, it seems likely that the C-terminal truncated PRL receptors affecting feather development in both chicken and turkey show a loss of function to some extent and act in a dominant negative fashion to attenuate the function of the wild-type PRL receptors.

Further analyses focusing on precise quantification of PRLR and dPRLR as well as PRLR homodimers are required. However, our findings provide suggestions on plausible mechanisms by which feather development is regulated in LF chickens; that is, the *K* allele delays feather development at the fetal stage by inhibiting ligand-induced homodimerization of PRLR via the expression of dPRLR as a partner of inactive heterodimers. After hatching, it accelerates feather growth by promoting PRLR homodimerization via increasing PRLR production at the transcriptional and post-transcriptional levels by mechanisms involving *dSPEF2* gene expression. Thus, the chicken LF phenotype may be due to the modulation of PRL receptor signaling by the action of both genes in the *K* allele fusion gene.

The present study also suggests that PRL signaling in each PRLR-expressing tissue may be enhanced or weakened depending on the *dSPEF2* mRNA levels in LF chickens. Therefore, there should be differences in LF phenotypes between chickens and turkeys in which the PRLR signal is inhibited in all tissues. A detailed comparison of the LF phenotype between chickens and turkeys should promote understanding of the PRL system in poultry. Furthermore, we found that alternative splicing of the 5′UTR of PRL receptor mRNA affects translation efficiency, suggesting a novel mechanism of prolactin signal regulation. Further studies on LF chickens should not only elucidate the mechanism of feathering but also shed light on the avian prolactin system.

5. Conclusion

The present study demonstrates that the transcription of the *dSPEF2* gene occurs beyond all coding exons of the *dPRLR* gene on the opposite strand in the *K* allele fusion gene. In addition, the transcription of *dSPEF2* gene possibly represses the expression of *dPRLR* mRNA and alters alternative splicing bias in the 5′UTR of PRL receptor mRNAs to increase translation efficiency. Based on the observation that the ontogenetic changes in the amounts of PRLR as well as the ratio of PRLR to dPRLR correlate well with feather growth in LF chickens, we now propose a new model that can explain the characteristics seen in LF chickens; that is the chicken LF phenotype is due to the modulation of PRL receptor signaling by the action of both genes in the *K* allele fusion gene.

6. Declarations of interest

None.

Acknowledgments

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