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Expression Patterns of Germ Cell-specific Phosducin-like 2 during Testicular and Ovarian Development in Chickens*

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ABSTRACT : Phosducin (PDC) is a photoreceptor cell-specific protein that is phosphorylated by cyclic nucleotide-dependent protein kinase. PDC and PDC-like proteins (PDCL, PDCL2, and PDCL3) are members of a conserved family of small thioredoxin-like proteins that modulate the β- and γ-subunits of G-proteins. In mammals, *Pdc*, *Pdcl*, and *Pdcl3* genes show ubiquitous expression; however, *Pdcl2* gene expression is limited to the testis and ovary. The aim of the present study was to examine the expression patterns of chicken *Pdcl2* (c*Pdcl2*) during testicular and ovarian development. Protein sequence comparisons performed using the CLUSTAL X program revealed that the amino acid sequences and potential phosphorylation sites of cPDCL2 and mammalian PDCL2 proteins were highly conserved. Quantitative real-time PCR analysis revealed that c*Pdcl2* was differentially expressed in the testis and ovary. Specifically, c*Pdcl2* expression was detected at low levels in the ovary at all time points. In the testis, c*Pdcl2* expression was detected at low levels until 5 weeks of age. At 8 weeks of age, however, c*Pdcl2* showed increased expression levels in the testis. Using *in situ* hybridization, we detected high levels of c*Pdcl2* expression in the testis, particularly in the spermatocytes and round spermatids. In summary, our data describe expression patterns of germ cell-specific *Pdcl2* during testicular and ovarian development in chickens. (**Key Words :** Chickens, *In situ* Hybridization, *Pdcl2*, qRT-PCR)

INTRODUCTION

Phosducin (PDC) is a photoreceptor cell-specific protein that is phosphorylated by cyclic nucleotide-dependent protein kinase. PDC is a known G-protein regulator. Specifically, PDC modulates visual phototransduction by binding with the β- and γ-subunits of the heterotrimeric G-protein (Gβγ) transducin (Kuo et al., 1989; Bauer et al., 1992). PDC and PDC-like proteins (PDCL, PDCL2, and PDCL3) are members of a conserved family of small thioredoxin-like proteins. PDC family members share an N-terminal helical domain, a central thioredoxin-like fold, and a charged C-terminal extension (Stirling et al., 2007). PDC family members have been identified in many different species, including humans, chimpanzees, cattle, rats, mice, chickens, zebrafish, *Drosophila*, and yeast. PDCL and PDC share extensive amino acid sequence homology, and both inhibit Gβγ (Thibault et al., 1999). PDCL2 and PDCL3 also share amino acid sequence homology with PDC, but their binding efficiencies with G-protein are lower (Lopez et al., 2003; Wilkinson et al., 2004; Stirling et al., 2006). In mammals, *Pdc* expression is not limited to the retina and pineal gland; it is ubiquitously expressed in a series of adult tissues (Bauer et al., 1992). *Pdcl* and *Pdcl3* also show ubiquitous expression (Thibault et al., 1999; Wilkinson et al., 2004). In contrast, *Pdcl2* expression is restricted to male and female germ cells (Lopez et al., 2003).

In the present study, we investigated the germ cell-specific protein *Pdcl2* in chickens. We used the CLUSTAL X program to perform a comparative analysis of predicted chicken PDCL2 (hereafter, cPDCL2) protein sequences with known PDCL2 protein sequences found in humans, chimpanzees, cattle, dogs, rats, and mice. Expression patterns of c*Pdcl2* during testicular and ovarian development in chickens were examined by quantitative

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real-time PCR (qRT-PCR), Northern blot hybridization, and *in situ* hybridization. Our data describe the expression patterns of the germ cell-specific gene c*Pdcl2* during testicular and ovarian development in chickens.

MATERIALS AND METHODS

Animals

White Leghorn chickens were used in the present study. Animal management, reproduction, and experimental procedures were performed in accordance with standard protocols of the Division of Animal Genetic Engineering, Seoul National University. All experimental data reported here were from at least three independent experiments.

Tissues and cDNA synthesis

In total, 23 tissues were collected during sexual development in male and female chickens as follows: male and female gonads on embryonic days E6.0, E8.0, and E12.0; testis and ovary at 1 day (hatch), 5 weeks, 8 weeks, 10 weeks, 12 weeks, and 24 weeks; and brain, liver, muscle, spleen, and kidney at 24 weeks. Sex was determined on E4.0 by PCR using W chromosome-specific primers (5'-CTATGCCTACCACATTCCTATTTGC and 5'-AGCTGG ACTTCAGACCATCTTCT; Ogawa et al., 1997). Total RNA was extracted using Trizol reagent (Invitrogen), and approximately 0.5 μg total RNA was reverse-transcribed using the Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol.

Multiple sequence alignment and phylogenetic tree analysis

Predicted mRNA sequences (XM_420702) and protein sequences (XP_420702) of c*Pdcl2* were obtained from a BLAT search of the Chicken Genome Database at the University of California, Santa Cruz (Karolchik et al., 2008), and National Center for Biotechnology Information (NCBI; Benson et al., 2004). PDCL2 protein sequences from chickens, humans (NP_689614), chimpanzees (XP_526622), cattle (NP_001035641), dogs (XP_532378), rats (XP_573585), and mice (NP_075997) were compared using the CLUSTAL X program and edited with the BioEdit program (Lee et al., 2008). The conserved functional domains and potential phosphorylation sites of the cPDCL2 protein were searched in Pfam-A family matrices and the MOTIF search program, respectively (Finn et al., 2006; Zhang et al., 2007). The percent identity between PDCL2 proteins from chickens, humans, cattle, and mice was determined using the NCBI blastp engine (Tatusova and Madden, 1999).

A phylogenetic tree of PDC family members was constructed using the MEGA program via the neighbor-joining method (Lee et al., 2008). PDC, PDCL, and PDCL3 protein sequences from humans (NP_002588, NP_005379, and NP_076970), chimpanzees (XP_524997, XP_528422, and XP_001161637), cattle (XP_615567, XP_001250725, and NP_001069113), dogs (NP_001003076, XP_852231, and XP_531782), rats (NP_037004, NP_071583, and NP_001020880), mice (NP_001153202, NP_080452, and NP_081126), and chickens (XP_426634, XP_001234493, and NP_001025983) were obtained from NCBI to construct the phylogenetic tree.

qRT-PCR

qRT-PCR was performed using an iCycler PCR detection system (Bio-Rad Laboratories) to examine the expression patterns of c*Pdcl2* during testicular and ovarian development. The PCR reaction mixture contained 2 μl PCR buffer, 1.6 μl 2.5 mM dNTP mixture, 10 pmol each of the forward and reverse primers of c*Pdcl2* or c*Gapdh* (Table 1), 2 μl cDNA, 1 μl EvaGreen (Biotium), and 1 U Taq DNA polymerase in a final volume of 20 μl. PCR was performed using an initial incubation at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. PCR was terminated by a final incubation at the dissociation temperatures of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. Threshold cycles of c*Pdcl2* were normalized with c*Gapdh*. Relative c*Pdcl2* expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1. Primers used for c*Pdcl2* and c*Gapdh* examined by qRT-PCR, Northern hybridization and *in situ* hybridization in chickens

Gene	Accession No.	Primer sequences[a]	Size
c*Pdcl2*	XM_420702	F: 5'- GAGGAGGCAAAAGTATGGGG R: 5'- CCTGGCTAAGTGGCTGAGGT	160 bps
		F: 5'- ATGCTCCTGAGGATGTTTGG R: 5'- TTTCAGCACGTCACTGCTTT	404 bps
c*Gapdh*	NM_204305	F: 5'- ACACAGAAGACGGTGGATGG R: 5'- GGCAGGTCAGGTCAACAACA	193 bps
		F: 5'- CACAGCCACACAGAAGACGG R: 5'- CCATCAAGTCCACAACACGG	443 bps

[a] First primer pairs were used for qRT-PCR, and second primer pairs were used for the synthesis of hybridization probes.

Hybridization probes

Testis cDNA from 24-week-old chickens was amplified using c*Pdcl2*- and c*Gapdh*-specific primers (Table 1). The amplified fragments were separated by gel electrophoresis and cloned into a pGEM-T Easy vector (Promega), respectively. After sequence linearization, recombinant plasmids containing c*Pdcl2* and c*Gapdh* were amplified with T7- and SP6-specific primers, respectively (Rengaraj et al., 2008a). The recombinant DNA was labeled with a digoxigenin RNA labeling kit (Roche) to prepare sense and antisense cRNA probes for Northern blotting and *in situ* hybridization.

Northern blot hybridization

Tissue-specific c*Pdcl2* expression was examined in the brain, liver, muscle, spleen, kidney, testis, and ovary of 24-week-old chickens by Northern blot using a digoxigenin Northern Starter Kit (Roche; Holtke et al., 1995). Total RNA was electrophoresed on a 1% formaldehyde agarose gel, capillary-blotted onto a Hybond membrane (Amersham), and UV cross-linked. Blots were hybridized with c*Pdcl2* and c*Gapdh* antisense cRNA probes. The membrane was then rinsed three times with 2×SSC and 0.2% SDS at room temperature, and rinsed twice with 0.1×SSC and 0.2% SDS at 68°C. After nonspecific binding was blocked, the membrane was incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) for 1 h at room temperature. The signal was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in a buffer containing 0.1 M NaCl, 0.05 M $MgCl_2$, and 0.1 M Tris-HCl (pH 9.5) for 5 h in the dark.

***In situ* hybridization**

Localization of c*Pdcl2* mRNA was examined during testicular and ovarian development in 1-day-, 5-week-, 10-week- and 24-week-old chickens using *in situ* hybridization as described previously (Rengaraj et al., 2008b). Briefly, frozen sections mounted on slides treated with 3-aminopropyltriethoxysilane (Sigma) were dried at 50°C and fixed in 4% paraformaldehyde for 1 h at room temperature. The sections were incubated with a hybridization mixture containing 50% formamide, 5× SSC (pH 7.0), 10% dextron sulphate sodium, 0.02% BSA, 250 μg tRNA, and sense or antisense cRNA probes of c*Pdcl2* at 55°C for 18 h. After nonspecific binding was blocked with 1% blocking reagent, the sections were incubated overnight with a sheep anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche). The mRNA signal was visualized using nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and levamisole. The sections were counterstained with 1% methyl green (Sigma), and photographs were taken under an Axiophot light microscope (Carl Zeiss).

RESULTS

Multiple sequence alignment and phylogenetic tree analysis

Predicted gene sequences of c*Pdcl2* were obtained from a University of California, Santa Cruz, BLAT search of NCBI GenBank sequences from the chicken genome. c*Pdcl2* was mapped to chromosome 4 in the chicken genome database and was found to encode a 244-amino acid protein. Searches for conserved domains of cPDCL2 in Pfam-A with the E-value cut-off level set at 0.05 had a specific hit with a PDC domain consisting of 232 amino acids (E-value: 1.4e-06; Figure 1).

Structurally, the cPDCL2 protein consisted of three potential phosphorylation sites for casein kinase II (S/T-X-X-D/E, where X represents a variable amino acid; Pinna, 1990), one potential phosphorylation site for tyrosine kinase (R/K-X-X-D/E-X-X-X-Y, where X represents a variable amino acid; Cooper et al., 1984), one potential phosphorylation site for N-glycosylation (N-X-S/T-X, where X represents a variable amino acid but not Proline; Pless and Lennarz, 1977; Bause, 1983), and two potential phosphorylation sites for N-myristoylation (G-X-X-X-S/T/A/G/C/N-X, where charged residues (EDRKHPFYW) at position 2 and Proline at position 6 are not allowed; Towler et al., 1988). Of these, two phosphorylation sites for casein kinase II, two phosphorylation sites for N-myristoylation, and one phosphorylation site for N-glycosylation were highly conserved in most of the species (Figure 1).

Percent identity calculations of cPDCL2 with mammalian PDCL2 proteins over the entire alignment indicated extensive similarities: 76% to human and chimpanzee; 75% to cattle; and 72% to dog, rat, and mouse. A phylogenetic tree was constructed using full-length protein sequences of PDC family members (i.e., human, chimpanzee, cattle, dog, rat, mouse, and chicken) by the neighbor-joining method. In the phylogenetic tree, PDC family proteins were grouped into two major clusters. PDC and PDCL proteins showed high similarity and formed one major cluster. PDCL2 and PDCL3 proteins also showed high similarity and formed another major cluster. PDC, PDCL, PDCL2, and PDCL3 proteins of different species were grouped into four minor clusters, respectively. All chicken PDC family members showed high similarity and grouped with the clusters of their mammalian homologues (Figure 2).

Tissue- and duration-specific c*Pdcl2* expression

qRT-PCR was used to examine expression levels of c*Pdcl2* during testicular and ovarian development in

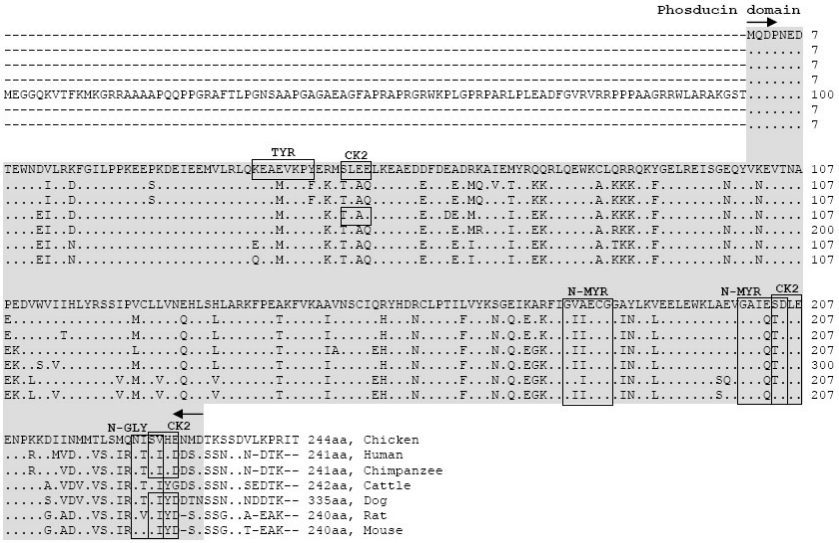

Figure 1. Multiple sequence alignment of phosducin-like 2 (PDCL2) proteins. Predicted protein sequences of chicken PDCL2 was compared to the known PDCL2 proteins from humans, chimpanzees, cattle, dogs, rats and mice using the CLUSTAL X program and edited with the BioEdit program. Dots indicate amino acids identical to chicken sequences and dashes represent gaps in the sequences. Amino acid sequences shown in gray shades represent phosducin domain homology compared to chicken sequences. Amino acid sequences shown in boxes are potential phosphorylation sites for tyrosine kinase (TYR), casein kinase II (CK2), N-myristoylation (N-MYR) and N-glycosylation (N-GLY) identified in chicken sequences and conserved in mammalian sequences.

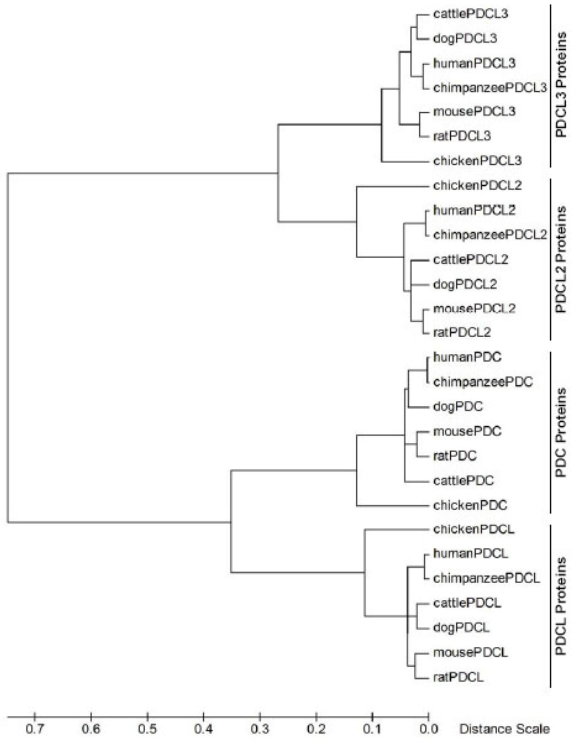

Figure 2. Phylogenetic tree of phosducin (PDC) family proteins. Phylogenetic tree of PDC family proteins (PDC, PDCL, PDCL2 and PDCL3) from humans, chimpanzees, cattle, dogs, rats, mice and chickens was constructed using the MEGA4 program via the neighbor-joining method with the respective full-length protein sequences. The genetic distance was calculated under option with the Poisson correction.

E6.0, E8.0, E12.0, 1-day- (hatch), 5-week-, 8-week-, 10-week-, 12-week-, and 24-week-old chickens. c*Pdcl2* expression was differentially detected during testicular and ovarian development. In females, c*Pdcl2* expression was detected at a low level during all stages of ovarian development. In males, c*Pdcl2* expression was detected at low levels until hatching. After hatching, c*Pdcl2* expression increased slightly until 8 weeks of age and then maintained a constant level until 12 weeks. The relative expression of c*Pdcl2* was developmentally upregulated after 12 weeks and peaked at 24 weeks (Figure 3). Because the relative expression levels of c*Pdcl2* were high in the testis of 24-weeks-old chickens, we further examined the specificity of c*Pdcl2* in several tissues of 24-week-old chickens using Northern blot. c*Pdcl2* mRNA expression was detected only in the testis. No detectable c*Pdcl2* mRNA expression was observed in the brain, liver, muscle, spleen, kidney, or ovary of 24-week-old chickens (Figure 4).

mRNA localization of c*Pdcl2* during testicular and ovarian development

Localization of c*Pdcl2* mRNA during testicular and ovarian development in 1-day-, 5-week-, 10-week- and 24-week-old chickens was performed using *in situ* hybridization. c*Pdcl2* expression was not detected in the ovary during sexual development between hatching and 24 weeks (Figure 5). In the testis, c*Pdcl2* expression was not detected between hatching and 5 weeks. From 10 weeks of age, c*Pdcl2* mRNA expression was detected within the

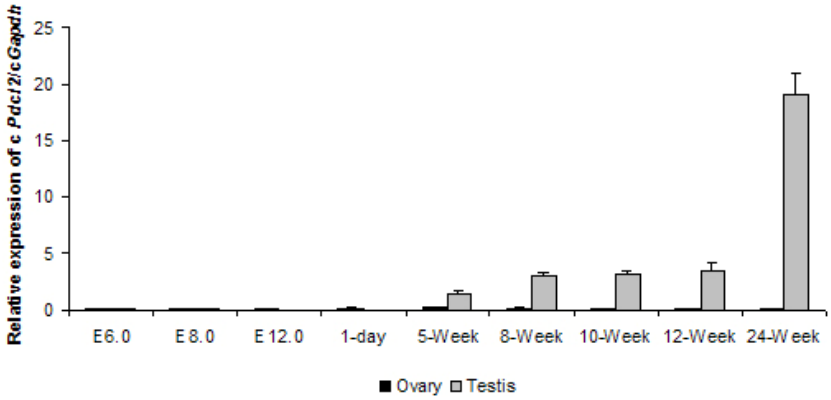

Figure 3. Quantitative real time-PCR analysis of phosducin-like 2 (c*Pdcl2*) during testicular and ovarian development in chickens. The threshold cycle (*C*t) of c*Pdcl2* was normalized with chicken glyceraldehyde-3-phosphate dehydrogenase (c*Gapdh*). Relative c*Pdcl2* expression levels were quantified using the $2^{-\Delta\Delta Ct}$ method. Values on the *y*-axis show the relative expression of c*Pdcl2*/c*Gapdh*. Each bar represents the mean±STDEV of three independent experiments.

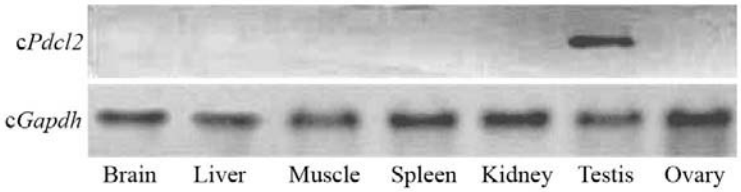

Figure 4. Northern blot analysis of phosducin-like 2 (c*Pdcl2*) expression in chickens. Total RNA from brain, liver, muscle, spleen, kidney, testis, and ovary of 24-week-old chickens were hybridized with antisense probes of c*Pdcl2*. The same RNA samples were hybridized with chicken glyceraldehyde-3-phosphate dehydrogenase (c*Gapdh*) probes for normalization.

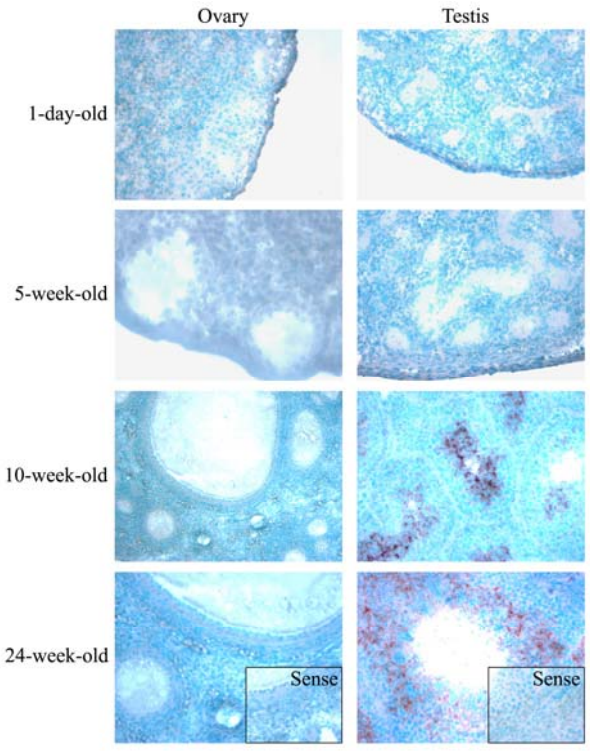

Figure 5. mRNA localization of phosducin-like 2 (c*Pdcl2*) during testicular and ovarian development in chickens. Frozen sections of testis and ovary on 1-day-, 5-week-, 10-week- and 24-week-old chickens were hybridized with antisense cRNA probes of c*Pdcl2*. Inner sections shown on 24-week-old were hybridized with sense cRNA probes of c*Pdcl2*. Original magnification of all pictures was ×200.

adluminal compartment. Specifically, primary spermatocytes and secondary spermatocytes strongly expressed c*Pdcl2* mRNA at 10 weeks. At 24 weeks, high levels of c*Pdcl2* mRNA were detected in primary spermatocytes, secondary spermatocytes, and round spermatids. c*Pdcl2* expression was not detected in Sertoli cells or spermatogonia in the basal compartment or in the basement membrane and interstitial tissues of Leydig cells at either 10 weeks or 24 weeks (Figure 5).

DISCUSSION

In mammals, the PDC family consists of four isoforms: PDC, PDCL, PDCL2, and PDCL3. PDC family members are modulators of visual phototransduction, which occurs via binding with the G-protein βγ-subunits. PDC family members also have other important functions. Recent findings suggest that all PDC family members act as co-chaperones with the cytosolic chaperonin complex to assist in the folding of a variety of proteins, including actins, tubulins, and regulators of the cell cycle (Stirling et al., 2007; Willardson and Howlett, 2007). The PDCL2 protein is the least characterized isoform; however, it is necessary for gametocyte transition into the haploid state. In the present study, we investigated the predicted *Pdcl2* gene/protein of the chicken homologue. This is the first study to characterize at least one member of the PDC isoform in chickens.

To better understand the functional similarities, one must characterize the structural features of cPDCL2 and analyze its conservation with mammalian homologues. Searches for conserved domains in the Pfam database have revealed that up to 95% of cPDCL2 protein sequences consist of a PDC domain. PDC is a Gβγ-binding protein that is ubiquitously expressed in several tissues. Furthermore, it is involved in the regulation of the light-dependent movement of the photoreceptor G-protein transducin (Schulz, 2001; Lukov et al., 2004). PDCL2 proteins from chickens, humans, chimpanzees, cattle, dogs, rats, and mice share several potential functional sites-including a casein kinase II phosphorylation site, an N-glycosylation phosphorylation site, and an N-myristoylation phosphorylation site-that may play a role in G-protein signaling. For example, the Gα-subunit can be myristoylated at the N-terminus end for proper plasma membrane localization of Gα, and functional sites can regulate the interactions of Gα with Gβγ, effectors, and G-protein regulators (Lukov et al., 2004). Casein kinase II is a constitutively active and ubiquitously expressed serine-threonine kinase (Humrich et al., 2003). Casein kinase II phosphorylation sites have been identified in other members of the PDC family; casein kinase II phosphorylation is required for normal Gγ translation and Gβγ dimer assembly (Humrich et al., 2003; Lukov et al., 2006). Similarities in structural features between PDCL2 proteins from chickens and mammals suggest that these proteins may have close functional relationships. Moreover, the genetic distances of PDC family proteins in the phylogenetic tree suggest that all PDC family proteins originated from the same ancestral protein.

Mammalian *Pdc*, *Pdcl*, and *Pdcl3* expression was detected in several tissues. The widespread distribution of these proteins is reminiscent of other proteins involved in G-protein-mediated signaling (Bauer et al., 1992; Thibault et al., 1999; Wilkinson et al., 2004). *Pdcl2* expression differs from the expression of other members of the *Pdc* family; mammalian *Pdcl2* expression was detected at high levels in the testis and at a low level in the ovary. In a previous study, mammalian *Pdcl2* was strongly detected in pachytene spermatocytes to early post-meiotic spermatids in the testis (Lopez et al., 2003). In the present study, we found similar expression patterns of c*Pdcl2* in the testis and ovary of chickens using qRT-PCR and *in situ* hybridization analysis. However, Northern blot and *in situ* hybridization failed to detect c*Pdcl2* transcripts in the ovary. The number of gametocytes that undergo meiosis is more limited in the ovary than the testis. This may be a reason why *Pdcl2* expression was not detected in the ovary. In male chickens, the onset of meiosis occurs after the completion of Sertoli cell proliferation (Kirby and Froman, 2000). Using qRT-PCR, we found that c*Pdcl2* expression increased from 8 weeks, which suggests that c*Pdcl2* may be active after the completion of the first meiotic division. *Pdcl2*, which is not necessary for growth, is important for the establishment of the haploid state (Lopez et al., 2003).

In conclusion, we investigated the expression patterns of c*Pdcl2*, which is highly conserved with mammalian *Pdcl2*. *Pdcl2* expression was identified in the testis and ovary by qRT-PCR; however, expression levels were much lower in the ovary at all stages of development. In the testis, c*Pdcl2* expression was elevated after the completion of the first meiotic division and was detected in spermatocytes and early spermatids.

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