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Expression Patterns of Prp Gene During Chicken Embryo Development

¹Diao Xiao-Long, ^{1,2}Wu Run, ¹Liu Lei, ¹Zhao Chun-Lin, ¹Wang Xiong, ¹Guan Hong-Wei and ¹Luo Peng

¹College of Veterinary Medicine, Gansu Agricultural University, Lanzhou 730070, China

²Gansu Key Lab of Crop Improvement and Germplasm Enhancement, Lanzhou 730070, China

Corresponding Author: Wu Run, College of Veterinary Medicine, Gansu Agricultural University, Lanzhou 730070, China

ABSTRACT

In mammals, PrP^C is widely distributed in the Central Nervous System (CNS) and many peripheral tissues. This study demonstrated the expression pattern of ChPrP in the CNS throughout chicken embryo development by real-time quantitative PCR. Chicken PrP gene is expressed in brain as early as embryonic day 5. The expression level gradually elevated to a peak on day 15, then stabilized. The levels of ChPrP mRNA in different tissues and organs were detected after 15 day-old chicken embryos. The highest level of ChPrP mRNA was observed in the brain and lower levels were observed in the following tissues: heart, lung, stomach, intestine, kidney. The expression of ChPrP was not observed in liver. The results therefore raise the possibility that ChPrP plays an essential role in chicken embryo neurons development but the precise and mechanism in this processes remain to be clarified.

Key words: RT-PCR, expression, ChPrP, chicken embryo, function

INTRODUCTION

The mammalian prion protein (PrP) is a cellular glycolipid-anchored protein of unknown function. The altered isoform of prion (PrP^{Sc}) is a component of the infectious particle thought to be responsible for Transmissible Spongiform Encephalopathies (TSE) in humans and animals (Prusiner *et al.*, 1987). PrP^C was widely distributed in the Central Nervous System (CNS) and many peripheral tissues in mammals (Horiuchi *et al.*, 1995). The special organization and cellular localization of PrP^C would be consistent with a number of different functions, including antioxidant activity, copper homeostasis, signal transduction, cell adhesion, anti-apoptosis, differentiation and neuronal synaptic transmission (Roucou *et al.*, 2004; Du *et al.*, 2005; Chiesa and Harris, 2009). As an ancient protein, all the mammalian PrP^C have about 90% sequence identity. Although, the Chicken Prion Protein (ChPrP) and shared only 30% identity with the mammalian prion amino acid sequence. The three-dimension structure of chicken PrP^C was found to be very similar to the mammalian PrP^C (Calzolari *et al.*, 2005). Moreover, the major biochemical and cellular properties of the chicken and mammalian PrP^C are similar (Ji and Zhang, 2007). Conservation of these properties in chicken and mammalian PrP^C would suggest that they have similar roles. But there is only a few researches about the ChPrP.

This study aimed to characterize chicken PrP^C during development by examining the temporal and spatial expression patterns of PrP in different development stage of chicken embryos. Furthermore the PrP expression of different tissues was examined in the late development

stage of and chicken embryos, including in brain, heart, lung, stomach, intestine and kidney. The evidence will provide new insights into and histological and auxanology data relevant to understanding the functions of PrP in vertebrates.

MATERIALS AND METHODS

Study was carried out in the College of Veterinary Medicine of Gansu Agricultural University during 2009-10.

Chicken embryo tissue samples: Four day-old chicken embryos were obtained from Lanzhou chicken farm in China and reared to 20 day-old in a laboratory incubator at 37°C. Every age of embryos were collected and sacrificed for tissue sampling. For 5-15 day-old chicken embryo samples, only nerve tissues were collected. After 15 day-old chicken embryo samples, including lung, stomach, intestine, kidney and nerve tissues were collected and immediately frozen in liquid nitrogen for 1 h and then stored at -70°C until RNA extraction was performed.

RNA extraction and reverse transcription: Total RNA was independently extracted in triplicate from each sample (50-100 mg) using the Trizol Reagent (Invitrogen, USA). Each sample was homogenized in 1 mL Trizol Reagent for 5 min at room temperature. Chloroform (200 µL) was then added and vortexed for 15 sec, centrifuged at 12,000×g for 15 min at 4°C. The upper aqueous phase was transferred in a tube containing an equal volume of isopropanol. Mixtures were thoroughly vortexed and centrifuged at 12,000×g for 10 min at 4°C. Supernatants were discarded and the precipitated RNA pellets were washed using 1 mL of 75% ethanol. RNA pellets were centrifuged at 12,000×g for 5 min at 4°C. After discarding supernatants, pellets were allowed to air-dry for 10-15 min, then resuspended in DEPC-treated water. Trace genomic DNA was removed from total RNA by DNase I treatment. Total RNA purities were checked using OD260/OD280 ratios on a spectrophotometer (GE, USA) and by confirming integrity using 1% agarose gel electrophoresis. RNA (1 µg) from each sample was reverse-transcribed to cDNA using a random hexamer.

Real-time quantitative RT-PCR analysis of ChPrP expression: A pair of primers: 5'-AGAAGGGCAAAGGCAAACCCAGTGG-3' (forward) 5'-CCTTGACCCCAGCCTGGGTAACCTG-3' (reverse) was designed to specifically amplify a 208 bp fragment of ChPrP gene by PCR. The PCR product was cloned into pMD18-T Vector and sequenced. The recombinant plasmid DNA was extracted and purified using the AxyPrep™ Plasmid Miniprep Kit (Axygen, USA). Then the plasmid DNA concentration was estimated using OD260/OD280 ratios on a spectrophotometer and the copy number was calculated from the molecular weight of the construct plasmid. For construction of a standard curve and determination the sensitivity of the Real-time quantitative RT-PCR assays, five 10-fold serial dilutions of plasmid DNA ranging from 10⁸ to 10⁷ molecules were then prepared.

Real-time quantitative PCR was performed on a Stratagene Mx3005P (Stratagene, USA) using SYBR® Premix Ex Taq™ II with ROX (TaKaRa, Japan). The primers above were used to amplify cDNA of the samples, negative controls and five plasmid DNA standards. PCR reactions were performed in triplicate and threshold cycle numbers were averaged. The 20 µL reaction mixture ROX Reference Dye II (0.4 µL) and reverse transcribed total RNA or plasmid DNA (2 µL). The Real-time PCR thermocycles were run as follows: after denaturation at 95°C for 5 min, 40 PCR cycles were performed including 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec. Fluorescence was

read following each annealing and extension phase. A melting-curve analysis between 72 and 95°C was performed after each PCR to check the specificity of the amplification. Finally amount of ChPrP mRNA was calculated using an absolute standard curve method. The cDNA samples were amplified in parallel with plasmid standards in each run and their Ct values were plotted together with the standard curves, from which the normalized ChPrP mRNA copy numbers were determined.

Statistical analysis: Data entry and statistical analysis were done with the Statistical Program for Social Sciences (SPSS). An independent sample t-test was applied to analyze differences in mRNA expression between different organs. P values of <0.05 were considered significant difference.

RESULTS

Quantification of ChPrP expression: Qualities of total RNA extracted from samples were examined by A260/A280 ratio and 1% formaldehyde gel electrophoresis. Total RNAs revealed an average A260/A280 ratio of 1.9 and a well-resolved thick 18S rRNA band and a thin 28S rRNA band in gels. The verified result shows good quality of total RNA for downstream experiments.

The efficiency and sensitivity of ChPrP real-time quantitative PCR were assessed by repeated testing of serial logarithmic dilutions of recombinant standard (covering a range of 5 logs from 10^3 to 10^7). The amplification efficiency of our real-time quantitative PCR assays was 3.6 defined by the standard curve slope. The correlation coefficient (RSq) was 0.993 which indicates a high linearity of the Ct values plotted in the standard curves (Fig. 1). The lowest standard dilution consistently detectable in replicate reactions was 10^8 copies/reaction which shows the limit detection of this assay was 10^8 copies/reaction. Dissociation curves of amplicons of the recombinant standard and samples showed a single peak (data not shown) and all PCR reactions produced specific amplification products without primer-dimer formation. The specificity of real-time quantitative PCR products was documented with high resolution gel electrophoresis and resulted in a single product with the desired length (not shown).

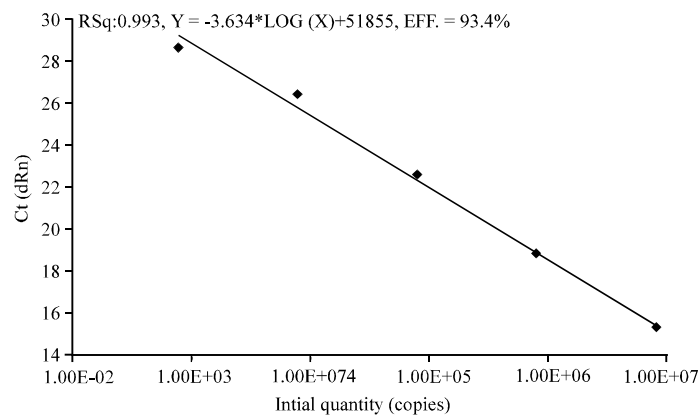


Fig. 1: Standard curve of ChPrP DNA quantitation with real-time PCR. The equation of the slope is $y = -3.634 \cdot \log(X) + 51.55$ when the Ct value is variant y and the value of ChPrP DNA concentration is variant x. It was acquired from serial dilution of standard samples

Differential expression of ChPrP transcripts: We analyzed the expression profile of ChPrP transcripts in brain by real-time quantitative PCR during the development of chicken embryo. Results showed that ChPrP gene is expressed in brain as early as embryonic day 5 and the expression level gradually elevate to a peak on day 15. In early development stage of chicken embryo, ChPrP expression level was very low. The value of ChPrP mRNA copy number per ng of total RNA was only 1828 on day 5. However, it was increased gradually in development process of chicken embryo to 10277 on day 15 (Table 1).

We next investigated the tissue distribution of ChPrP mRNA after 15 day-old chicken embryos. Real-time quantitative PCR revealed that and the highest level of ChPrP mRNA was observed in the brain and lower levels were observed in the following tissues: heart, lung, stomach, intestine, kidney. The expression of ChPrP was not observed in liver (Table 2). At the same embryonic day, we found that ChPrP expression in the brain was significantly higher ($p < 0.05$) than that in other tissues and a hierarchy of ChPrP expression was present in peripheral tissues with the higher level of ChPrP mRNA in the stomach and intestine, the lower level in heart, lung and kidney, no in liver.

Table 1: Quantitative real-time PCR results from different stages in chick development

Embryonic day	RNA (ng)	CV (%)	Copy/RNA (X±SE)	CV (%)
5	949	2.3	1828±135	7.4
6	1272	5.2	2161±444	20.5
7	1533	33.9	2659±329	12.4
8	2627	18.5	2900±516	17.8
9	1606	30.3	4122±169	4.1
10	3542	15.3	4561±524	11.5
11	816	1.1	4968±549	11.1
12	1878	13.5	5092±434	8.5
13	1594	8.7	6946±789	11.4
14	1873	3.8	7993±886	23.6
15	2106	19.1	10277±1201	11.7
16	1278	11.1	9020± 887	9.8
17	2736	5.2	6611± 414	6.3
18	2630	13.4	5597± 298	23.7
19	1613	4.4	6726± 364	11.1
20	1626	8.7	5798±71	13.4

Parameters of quantification. RNA (ng), yield of RNA in 1 mg of tissue; CV, coefficient of variation; copy/RNA, number of PrP mRNA copies in 1 ng of total RNA; X±SE, Mean±SE

Table 2: Detection ChPrP mRNA in different tissues by quantitative real-time PCR using an start curve

Tissue	Different embryonic day ChPrP mRNA (copies/ng RNA ¹)				
	16	17	18	19	20
Brain	9020±887	6611±414	5597±298	6726±364	5798±71
Heart	7851±492	4407±766	3269±127	2950±211 ^a	2985±754 ^a
Lung	2997±105 ^a	3332±44 ^a	2510±408 ^a	3855±362 ^a	4226±434
Stomach	5012±120 ^a	3338±44 ^a	3569±870	2486±639 ^a	4150±556
Kidney	2504±534 ^a	2674±119 ^a	1756±196 ^a	1293±34 ^a	2854±634 ^a
Intestine	4792±980 ^a	6302±33	3238±116	5426±307	5169±357
Liver	-	-	-	-	-

^aMean values for analyses in a row with a common superscript are significantly different ($p < 0.05$); ChPrP mRNA was not detected in this tissue, or was below detection levels; ¹Mean the number of ChPrP mRNA copies in 1 ng of total RNA

DISCUSSION

The results presented here demonstrate that ChPrP gene expression patterns are highly tissue-specific and developmental stage-dependent. The mRNA of ChPrP is widely distributed in the central nervous system and several peripheral tissues of chicken embryo. This expression pattern is in line with that found in mammalian tissues and including human and mouse (Prusiner *et al.*, 1987), in which PrP is also expressed mainly in the central nervous system (Zhang *et al.*, 2006). In addition, our study reveals that the amount of ChPrP mRNA increases gradually with the development of chicken embryo. These results are consistent with previous Northern blot analyses and in situ hybridization studies (Harris *et al.*, 1993). These evidences suggested that ChPrP gene expression may be associated with the development of chicken embryo. It is probable that ChPrP plays a role in regulation of the central nervous system development. But to date, there is no biochemical evidence show that they are identical molecules.

Results show that ChPrP mRNA are widespread distribution throughout the chicken embryonic somatic tissues. The localization of ChPrP is consistent with findings in mammals which reveals that the protein is highly and conserved during evolution. PrP^C may subserve a more general and widespread function. And the level of PrP^C expression in a particular tissue is important as it may determine its potential function. PrP^C is attached to the cell surface by a glycosylphosphatidylinositol anchor (GPI) and specific fragments of the molecule are released into the extracellular medium and (Graner *et al.*, 2000). These biochemical properties suggest a role in cell attachment, recognition, or intercellular signaling (Daggett, 1998; Sakudo and Ikuta, 2009). Considerable evidences indicate that the biochemical properties and distribution patterns of ChPrP and mammalian PrP are similar; it is likely that ChPrP play an important role in signal transduction, cell adhesion, differentiation during the development of chicken embryo.

Therefore, the identity and the extent to which various cells and tissues express ChPrP may provide suggestions for further studies into the function of this unusual and enigmatic protein. And chicken may represent a suitable species to further investigate the normal physiological function of PrP^C.

CONCLUSION

In this research results demonstrate the correlation of ChPrNP and the development process of chicken embryo and the expression and distribution ways of PRNP between chicken embryo and mammals was similar which suggested that ChPrP may be involved in nervous system development and maintaining functions and plays an important physiological functions in the process of embryo development.

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