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Expression of the Ephrin Receptor B2 in the Embryonic Chicken Bursa of Fabricius

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Abstract: Chicken B-cells develop in the bursa of Fabricius. To understand the bursal microenvironment guiding B-cell development, previous studies identified ephrin (Eph) receptor B2 (EphB2) gene transcripts in the embryonic bursa. We hypothesize that the EphB2 receptors and their ligands could initiate contacts between developing B-cells and epithelial cells in the embryonic bursal follicle. To address this hypothesis, additional basic studies were conducted to determine if EphB2 is expressed in the embryonic bursa. Therefore, EphB2 gene expression was examined with reverse transcriptase-PCR and western blotting in the bursa on embryonic days (ED) 15 and 18. The RT-PCR experiments with bursal cDNA amplified transcripts from the EphB2 gene, which was confirmed by nucleotide sequencing. The anti-EphB2 antibody recognized a protein of the expected 120 kDa molecular mass and gave an acceptable signal to noise ratio on whole tissue protein western blots from the ED15 and ED18 bursa. This information can be used to design experiments for determining the cell type (s) expressing the EphB2 protein in the embryonic bursa.

Key words: Embryo, bursa of Fabricius, ephrin receptor B2

INTRODUCTION

The bursa of Fabricius is an appendage of the chicken cloaca that is responsible for development of B-lymphocytes (Glick *et al*., 1956; Cooper *et al*., 1965). In the embryonic period, prebursal stem cells enter the epithelium of the bursal anlage and proliferate, forming the nascent lymphoid follicles between ED 8-14. During the period of ED15-18, a developmental event occurs which marks the beginning of immunoglobulin gene conversion in developing B-cells (Masteller *et al*., 1997). The long-term goal of our laboratory is to characterize the receptors and ligands directing B-cell differentiation in the embryonic bursal follicle. In previous studies, we examined receptor tyrosine kinase (RTK) gene expression during an early B-cell differentiation event (ED15 to ED18 transition) in the embryonic bursa to identify gene candidates that are important for the developing B-cell interactions with the follicular microenvironment. The RTK genes influence important events during embryonic development (Cornett *et al*., 2013; Eagleson *et al*., 2013; Hayes *et al*., 2013) and could, therefore, be important for B-cell development in the bursal follicles. In that study, we obtained cDNA for different members of the ephrin (Eph) receptor subfamily of RTK using degenerate primers (Pharr *et al*., 2009).

Based on the specificity of ligand binding, the Eph receptors are organized into separate sets termed EphA and EphB (Egea and Klein, 2007). The Eph receptor-ephrin interactions are important for the arrangement of cells within tissues as well as maturation of cells in embryonic tissues of mammalian and avian species (Pasquale, 2005, 2008). Our hypothesis is that Eph receptors and their ligands are responsible for communication between developing B-cells and reticular epithelial cells in the embryonic bursal follicle (Pharr *et al*., 2009). To address this hypothesis, we need additional basic studies on EphB expression in the embryonic bursa. In the present study, we evaluated EphB2 receptor expression at the cDNA and protein level in the bursa during the period of ED15-18.

MATERIALS AND METHODS

Chicken embryos: Ross X Ross 308 broiler embryos (Peco Farms, Gordo AL, USA) were used for preparation of whole tissue protein extracts. High Antibody Selected (HAS) line embryos were used for preparation of whole tissue RNA.

Preparation of whole tissue RNA: Trizol reagent was used to prepare total RNA from whole bursal tissue

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according to the manufacturer's instructions (Invitrogen, Carlsbad CA, USA). Total RNA was also extracted from the DT40 chicken bursal B-cell line (generously provided by Dr. Henry Hunt, USDA/ARS Avian Disease and Oncology Laboratory, East Lansing MI, USA).

Reverse transcriptase polymerase chain reaction: The Superscript III kit (Invitrogen, Carlsbad CA, USA) was used for cDNA synthesis. The total RNA was divided into three treatments for cDNA synthesis as described (Glew *et al*., 1995; Pharr *et al*., 2009). Briefly, treatment B consisted of 1 µg RNA in the absence of DNase and reverse transcriptase; treatment D consisted of 1 µg RNA with DNase only and treatment E consisted of 1 µg RNA incubated with DNase, followed by the addition of reverse transcriptase. The cDNA samples B, D and E were then used to amplify cDNA from the EphB2 gene. For PCR amplification of cDNA, the GoTaq polymerase kit (Promega, Madison WI, USA) was used with the thermocycler program of 94°C for 3 min and then 30 cycles of 94°C for 30 sec, 55°C for 30 sec, followed by 72°C for 2 min. The samples were then incubated for 5 min at 72°C prior to analysis.

The 549 bp cDNA from the chicken chemokine CXCR4 gene was amplified as a positive control to test the quality of the cDNA (Koskela *et al*., 2003):

1: Forward primer 5'-GGACGGCCCGGACCTACTCG-3'
2: Reverse primer 5'-GGCAGCCAGACACCCACATAC ACA-3'

The PCR primers specific for the EphB2 gene cDNA were designed at the Saccharomyces Genome Database (http://seq.yeastgenome.org/cgi-bin/web-primer):

EphB2(F) 5'-AAGCTTAACATCCTGGTCAACAGCAA-3'
HindIII

Primer EphB2(F) starts at nucleotide 91,199 of exon 12 (Accession No. NC_006108):

EphB2(R) 5'-GAATTCAGCTGCCTGTCTCTGATCCA-3'
EcoRI

Primer EphB2(R) starts at nucleotide 97,755 of exon 16 (Accession No. NC_006108).

The EphB2 gene-specific primers amplify a 819 bp cDNA PCR product.

Genomic DNA amplification with the primers would give a 6,599 bp PCR product.

Data analysis: The nucleotide sequence data were analyzed by a BLAST search at the NCBI database (www.ncbi.nlm.nih.gov). The nucleotide sequence alignments were conducted using the Clustal W program (Thompson *et al*., 1994).

Preparation of whole tissue extracts: Whole tissue protein extracts were prepared by mincing bursas in a glass-Teflon homogenizer in the presence of radioimmunoprecipitation assay buffer as described by Felfoldi *et al*. (2008).

Western blot analysis: A total of 50 µg of each protein extract was electrophoresed on 10% SDS-PAGE and then transferred to nitrocellulose membranes as described (Wan *et al*., 2004). The membranes were blocked overnight and then incubated with the antibodies (Wan *et al*., 2004). Western blot experiments were repeated twice.

Antibodies: The mouse anti-EphB2 monoclonal antibody (IgG_1 isotype) [Invitrogen Corporation, Carlsbad CA, USA, No. 37-1700] was raised against a peptide synthesized from the C-terminus of the chicken EphB2 protein. The mouse monoclonal was used at a concentration of 3 µg/mL. A mouse myeloma IgG_1 protein (3 µg/mL) [Sigma-Aldrich, St. Louis, MO, USA, No. M9035] was used as a control antibody for western blots. The mouse anti-EphB2 monoclonal antibody was recognized using goat anti-mouse immunoglobulin, alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL, USA, No. 1010-04) at a 1:1250 dilution.

RESULTS

In previous studies, we examined receptor tyrosine kinase gene cDNA expression in partially purified developing B-cells collected from the bursa at ED15 and at ED18 (Pharr *et al*., 2009). In that study we obtained cDNA evidence for EphB2 expression. In the present study, we describe additional work to confirm that the EphB2 receptor is expressed in the embryonic bursa.

Reverse transcriptase-polymerase chain reaction: The RT-PCR experiments gave products in the expected molecular mass range of 819 bp for the EphB2 cDNA in the chick embryo brain cDNA, which served as a positive control (Fig. 1, lane 11). The 819 bp product was also observed in cDNA treatment E samples from ED15 and ED18 bursa samples (lanes 6 and 10). The absence of a 6,599 bp PCR product from the EphB2 gene in control sample D (lanes 5 and 9) suggests that contaminating genomic DNA in bursal RNA preparations was reduced below the level of detection by the DNase treatment of RNA prior to reverse transcription. The 819 bp EphB2 cDNA was not detected in the cDNA synthesized from the chicken DT40 B-cell line (lane 12).

Nucleotide sequence analysis: The EphB2 PCR products were ligated into a plasmid vector. A plasmid from the EphB2 ligation (termed clone B2) was characterized by complete nucleotide sequencing. The

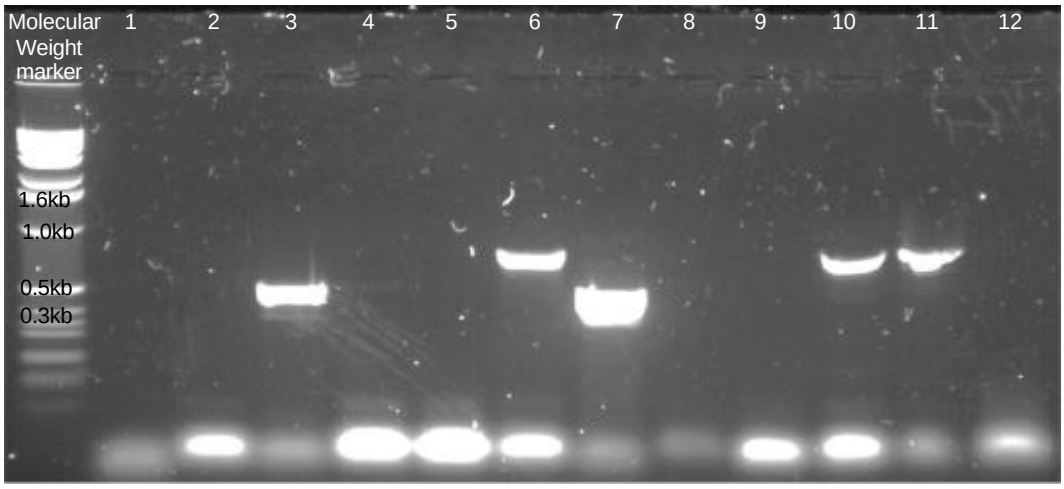

Fig. 1: Gel Electrophoresis of PCR products obtained from ED15 or ED18 bursal cDNA amplified with EphB2 specific primers.
Lane 1: CXCR4 primers, no DNA.
Lane 2: EphB2 primers, no DNA.
Lane 3: CXCR4 primers, ED15 cDNA treatment E.
Lane 4: EphB2 primers, ED15 cDNA treatment B.
Lane 5: EphB2 primers, ED15 cDNA treatment D.
Lane 6: EphB2 primers, ED15 cDNA treatment E.
Lane 7: CXCR4 primers, ED18 cDNA treatment E.
Lane 8: EphB2 primers, ED18 cDNA treatment B.
Lane 9: EphB2 primers, ED18 cDNA treatment D.
Lane 10: EphB2 primers, ED18 cDNA treatment E.
Lane 11: EphB2 primers, chick embryo brain (positive control cDNA).
Lane 12: EphB2 primers, DT40 cDNA treatment E.
B: RNA incubated without DNase, without reverse transcriptase.
D: RNA treated with DNase, incubated without reverse transcriptase.
E: RNA treated with DNase and then incubated with reverse transcriptase

nucleotide sequence data are aligned with the chicken EphB2 transcript from the database (Fig. 2). The two mismatches in the EphB2 plasmid (clone B2) at nucleotide positions 698 and 761 most likely represent PCR-induced mutations. The nucleotide sequence data suggest that the PCR products represent bonafide cDNAs and that the EphB2 gene is transcribed in the embryonic bursa.

Western blotting analysis: In agreement with previous studies by others (Pasquale, 1991; Kalo *et al*., 2001), the EphB2-specific antibody gave a signal in the 120 kDa range with the chick embryo cerebellum protein lysate (Fig. 3B, lane 3) which was absent on the control blot treated with an isotype-matched unrelated antibody (Fig. 3A). A 120 kDa signal was also detected in the bursa samples (Fig. 3B, lanes 1 and 2).

DISCUSSION

The long-term goal of our laboratory is to understand the microenvironment of the bursa of Fabricius that supports B-cell development in the late embryonic period. Our results suggest that the EphB2 protein is expressed in the embryonic bursa across an important B-cell developmental event associated with immunoglobulin gene diversification which was first described by Craig Thompson and coworkers in the mid-1990s (Masteller *et al*., 1995a,b).

Studies in mammalian animal models have shown that Eph and ephrins play an important role in lymphocyte development (Yu *et al*., 2006; Munoz *et al*., 2006, 2011; Alfaro *et al*., 2008, 2011). Experiments using EphB2-deficient mice or mice with an EphB2 incapable of signaling showed that EphB2 is important for the development of thymic epithelial cells and the migration of T-cell progenitors to the different microenvironments

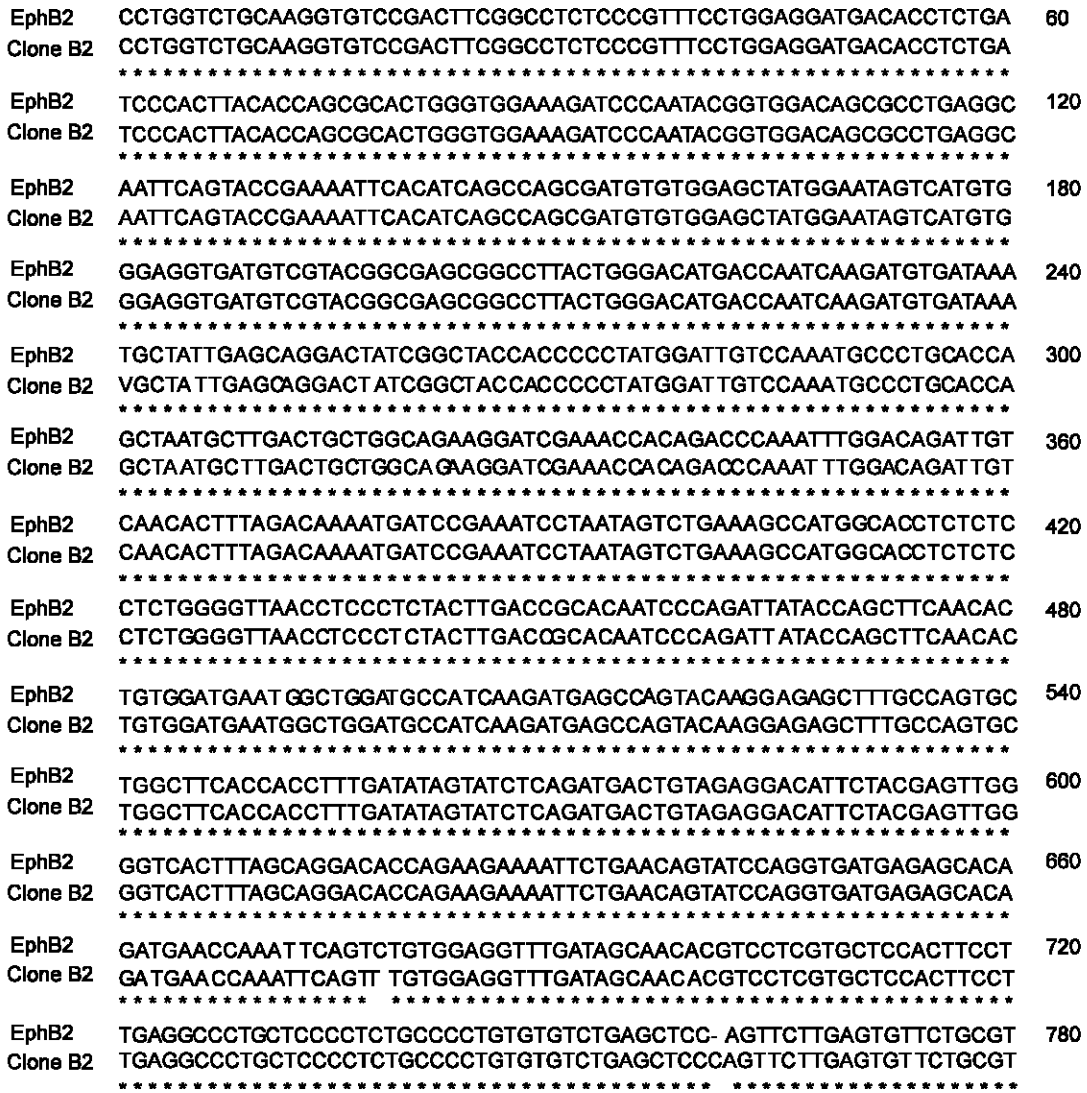

Fig. 2: Nucleotide sequence of PCR products. Nucleotide sequence derived from clone B2 aligned with the chicken EphB2 cDNA sequence (Accession number NM_206951)

within the thymus (Garcia-Ceca *et al*., 2009; Stimamiglio *et al*., 2010).

Based on the function of EphB2 and ephrin-B ligands in mammalian primary lymphoid tissues (Stimamiglio *et al*., 2010), we hypothesize an important role for these genes in bursal B-cell development. While we did not evaluate EphB2 expression in isolated bursal B-cell populations in this study, the absence of EphB2 gene transcription in the DT40 chicken B-cell line (Baba *et al*., 1985) suggests that bursal B-cells may not express EphB2 (Fig. 1, lane 12). Therefore it is possible that bursal B-cells could express ephrin ligands recognized by EphB2 receptors possibly expressed by the bursal stroma during the embryonic period. Because bursal stromal cells support the architecture of the bursal follicle and also elaborate uncharacterized B-cell differentiation factors, they are, therefore, critical for B-cell development (Obranovich and Boyd, 1996; Nagy *et al*., 2004; Olah and Vervelde, 2008).

Conclusion: Our data suggests that the EphB2 receptor is expressed in the bursa at the ED15 and ED18 developmental time-points. To our knowledge, this is the first such study to identify EphB receptor protein expression in the embryonic bursa. In future studies, we hope to identify the cell type (s) expressing the EphB2 proteins during the embryonic period of bursal B-cell

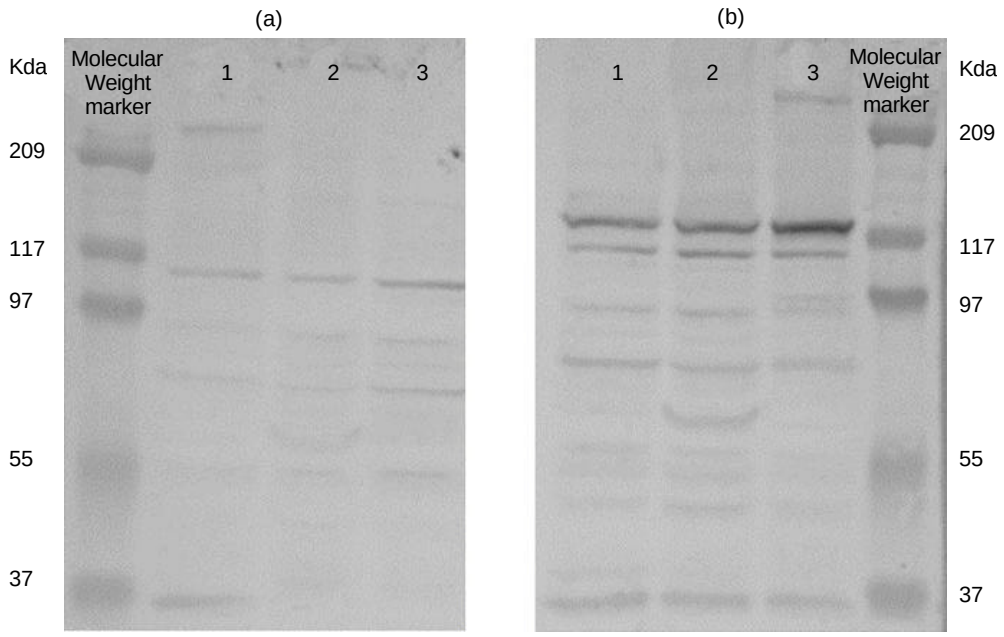

Fig. 3: Western blotting analysis to detect the EphB2 protein.
(a) Western blot with mouse IgG1 isotype control.
Lane 1: Chick embryo cerebellum protein extract.
Lane 2: ED15 bursa protein extract.
Lane 3: ED18 bursa protein extract.

(b) Western blot with monoclonal anti-chicken EphB2.
Lane 1: ED18 bursa protein extract.
Lane 2: ED15 bursa protein extract.
Lane 3: Chick embryo cerebellum protein extract

development. To do this, we plan to conduct immunohistochemistry analysis with bursal tissue sections to confirm the expression of the EphB2 receptor and then determine the cell phenotypes expressing the EphB2 protein with lineage-specific monoclonal antibodies.

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