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# Research Article
# Protein Tyrosine Kinase Gene Expression Profiles in the Embryonic Bursa of Fabricius of Chicken Lines Selected for High and Low Antibody Responses

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## Abstract

**Background and Objective:** The chicken lines selected for high (HAS line) and low (LAS) antibody responses to antigenic challenge represent a valuable model for identifying genes controlling the development of humoral immunity. In this study, we evaluated the expression of receptor tyrosine kinase (RTK) and non-receptor tyrosine kinase (nRTK) genes in the developing bursa from both chicken lines. These genes have a critical role in cell survival, migration and differentiation. In addition, we examined bursas from embryos exposed to testosterone, to detect changes in the expression of these genes when normal bursa development was inhibited. **Materials and Methods:** cDNA was synthesized from whole bursa RNA and PCR amplified with degenerate primers. The nucleotide sequence of the PCR products was analyzed with the NCBI database. **Results:** A total of 172 cDNAs from the HAS and 183 cDNAs from the LAS lines were sequenced. The most abundant nRTK and RTK cDNAs were from the Fyn-related kinase (FRK) and Ephrin A1 receptor (*EphA1*) genes and the expression levels differed significantly between the HAS and LAS bursas. Embryonic exposure to testosterone increased the number of cDNAs from the FRK gene. **Conclusion:** The significant differences in transcription of the FRK and *EphA1* genes due to genetic selection may result in differences in bursal development between the HAS and LAS lines. Testosterone treatment significantly modulated FRK expression, which suggests a role for this gene in the differentiation and organization of the epithelium in the developing bursa.

Key words: Bursa of Fabricius, receptor tyrosine kinase genes, high antibody line, low antibody line, fyn-related kinase

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The high (HAS) and low (LAS) antibody lines established by selection for antibody responses to a thymus-dependent antigen, sheep red blood cells, serve as a good model for identifying genes controlling humoral immune responses in chickens[1]. Several loci that are linked to antibody responses have been located on eight different chromosomes in the HAS and LAS lines[2]. While the alleles at each locus have yet to be identified, it is possible that the selection for humoral immune responses could have modified certain alleles of genes that are important for bursal function. Previous studies have shown that bursal weights, which are directly correlated with the number of developing B-cells, are greater in the HAS line than in the LAS line[3]. Therefore, the evaluation of gene expression in the bursa from the HAS and LAS lines could reveal genes that are central to B-cell development.

Our long-term goal is to identify and characterize B-cell differentiation events in the embryonic bursa. Phenotype analysis and functional studies have characterized an important differentiation event between embryonic day 15 (ED15) and ED18, which is coincident with the onset of repertoire development by immunoglobulin (Ig)-gene conversion[4]. Understanding this differentiation event requires characterization of both developmental B-cell stages, as well as the underlying bursal microenvironment supporting this development. One approach to this goal is to survey gene expression in the bursa at ED15 and ED18 to identify candidate genes for controlling the progression from one developmental stage to the next. Multiple genes from the receptor tyrosine kinase (RTK) gene superfamily were expressed in bursal B-cells at ED15 in a previous study from our laboratory[5,6]. The RTK genes are known to regulate numerous developmental processes including control of cell survival, cell migration and differentiation[7,8]. Therefore, the purpose of this study was to determine the full complement of RTK genes expressed in the bursa at ED15. To do this, we used degenerate PCR primers that are complementary to conserved regions of the tyrosine kinase catalytic domain. The primers would therefore amplify all RTK and non-receptor tyrosine kinase (nRTK) gene cDNAs[9]. We also evaluated ED15 bursas from HAS and LAS embryos that had been treated with testosterone propionate for comparison to bursas from untreated embryos to aid in the identification of RTK that could be important in bursal B-cell development. The treatment of chick embryos with androgens such as testosterone propionate will inhibit the normal function of the bursa by suppressing bursal B-cell development[10]. We theorize that some of the RTK genes which are important in bursal B-cell development may be suppressed in testosterone propionate-treated bursae and would be detected by comparison to bursae from untreated embryos.

## MATERIALS AND METHODS

**Experimental units:** Fertile eggs were obtained from both the Virginia High (HAS) and Low (LAS) antibody lines[1]. These lines have undergone long term selection for antibody response to sheep red blood cells (SRBC). At ED3, fertile eggs from both lines at generation 37 of selection were exposed to testosterone propionate (tp) in ethanol or vehicle (ethanol only) and incubated until ED15. Bursae were dissected from the embryos and placed in RNAlater tissue storage solution at ED15 (Invitrogen, Carlsbad, CA, USA).

**Preparation of total RNA:** Four bursae were pooled from each of the control groups (HAS and LAS) and each of the testosterone propionate – treated groups (HAStp and LAStp). Total RNA was extracted from bursal tissue with the Trizol reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

**Preparation of cDNA and polymerase chain reaction:** Reverse transcription reaction was performed with the Superscript III kit (Invitrogen, Carlsbad, CA, USA) as described[6]. Degenerate primers[9] were used to amplify cDNA from RTK genes by PCR as described previously[6]. To identify RTK gene expression, total RNA was extracted from the bursae of HAS, LAS, HAStp and LAStp. The cDNA derived from the total RNA was PCR amplified in two separate reactions with the following pairs degenerate primers: forward primer TKI with reverse primer TKII and forward primer TKI with reverse primer TKIII[9]. The 210 bp PCR product was ligated into a plasmid vector and transformed into *E. coli*. Ampicillin-resistant colonies, propagated in broth cultures, were used for preparation of plasmid DNA for nucleotide sequencing.

**Data analysis:** The cDNA sequences were identified with manual database BLAST search (NCBI database www.ncbi.nlm.nih.gov). The number of RTK and nRTK gene cDNAs identified from the two separate PCR reactions were summed and those numbers are listed in Table 1-3. The data were analyzed with the Audic-Claverie Distribution (ACD Test)[11,12] [http://www.igs.cnrs-mrs.fr/acdtool/] to determine the significance of differential cDNA expression between HAS and LAS (Table. 1), HAS and HAStp (Table 2) and with LAS and LAStp (Table 3).

Table 1: Protein tyrosine kinase cDNA expressed in the HAS line ED15 bursa compared with the LAS line ED15 bursa

| Tyrosine kinase gene cDNA (subfamily) | cDNA (n)[a] HAS | LAS | p-value[b] |
|---|---|---|---|
| FRK (Src) | 8 | 20 | 0.0183 |
| IGFR (Ins) | 0 | 5 | 0.0188 |
| EphA1 (Eph) | 28 | 16 | 0.0225 |
| MERTK (Axl) | 1 | 7 | 0.0243 |
| PDGFRa (PDGF) | 49 | 34 | 0.0272 |
| c-Kit (PDGF) | 17 | 9 | 0.0439 |
| RYK (Ryk) | 5 | 12 | 0.0628 |
| EphB2 (Eph) | 0 | 3 | 0.0706 |
| CSK (CSK) | 7 | 14 | 0.0879 |
| TIE1 (Tie) | 2 | 0 | 0.1137 |
| SYK (SYK) | 2 | 0 | 0.1137 |
| FGFR4 (FGF) | 0 | 2 | 0.1370 |
| EphA4 (Eph) | 9 | 6 | 0.1912 |
| EphB1 (Eph) | 1 | 0 | 0.2347 |
| EphB6 (Eph) | 1 | 0 | 0.2347 |
| PTK7 (PTK7) | 1 | 0 | 0.2347 |
| PTK2 (FAK) | 1 | 0 | 0.2347 |
| Jak 2 (Jak) | 5 | 8 | 0.2476 |
| erbB2 (ErbB) | 0 | 1 | 0.2657 |
| MATK (CSK) | 0 | 1 | 0.2657 |
| Fer (Fes) | 0 | 1 | 0.2657 |
| MAP2K5 (MAPK) | 0 | 1 | 0.2657 |
| SRMS (Src) | 0 | 1 | 0.2657 |
| BTK (Tec) | 0 | 1 | 0.2657 |
| Jak 1 (Jak) | 26 | 32 | 0.2940 |
| EphA3 (Eph) | 3 | 2 | 0.3152 |
| Flk-1/VEGFR-2 (VEGF) | 4 | 5 | 0.4156 |
| EGFR (ErbB) | 1 | 1 | 0.4768 |
| v-abl (Abl) | 1 | 1 | 0.4768 |
| Total cDNAs | 172 | 183 | |

[a]Number of cDNAs identified from two independent PCR reactions. [b]Probability that differential gene expression occurred by random biological variation

Table 2: Protein tyrosine kinase cDNA expressed in the HAS line ED15 bursa compared with the testosterone-treated HAS line (HAStp) ED15 bursa

| Tyrosine kinase gene cDNA (subfamily) | cDNA (n)[a] HAS | HAStp | p-value[b] |
|---|---|---|---|
| FRK (Src) | 8 | 28 | 0.0003 |
| EphA4 (Eph) | 9 | 3 | 0.0471 |
| c-Kit (PDGF) | 17 | 10 | 0.0950 |
| Flk-1/VEGFR-2 (VEGF) | 4 | 1 | 0.1107 |
| CSK (CSK) | 7 | 3 | 0.1151 |
| FGFR4 (FGF) | 0 | 2 | 0.1239 |
| IGFR (Ins) | 0 | 2 | 0.1239 |
| YES1 (Src) | 0 | 2 | 0.1239 |
| TIE1 (Tie) | 2 | 0 | 0.1260 |
| Jak 2 (Jak) | 5 | 2 | 0.1464 |
| PDGFRa (PDGF) | 49 | 59 | 0.1615 |
| EphA1 (Eph) | 28 | 21 | 0.1662 |
| Jak 1(Jak) | 26 | 20 | 0.1963 |
| SYK (SYK) | 2 | 4 | 0.2241 |
| CSF1R (PDGF) | 0 | 1 | 0.2485 |
| TYK2 (Jak) | 0 | 1 | 0.2485 |
| SRMS (Src) | 0 | 1 | 0.2485 |
| EphB1 (Eph) | 1 | 0 | 0.2514 |
| EphB6 (Eph) | 1 | 0 | 0.2514 |
| EGFR (ErbB) | 1 | 0 | 0.2514 |
| PTK2 (FAK) | 1 | 0 | 0.2514 |
| RYK (Ryk) | 5 | 5 | 0.4960 |
| EphA3 (Eph) | 3 | 3 | 0.4968 |
| MERTK (Axl) | 1 | 1 | 0.4978 |
| PTK7 (PTK7) | 1 | 1 | 0.4978 |
| v-abl (Abl) | 1 | 1 | 0.4978 |
| Total cDNAs | 172 | 171 | |

[a]Number of cDNAs identified from two independent PCR reactions. [b]Probability that differential gene expression occurred by random biological variation

## RESULTS AND DISCUSSION

The data were derived from a total of 172 plasmids sequenced for HAS and 183 plasmids for LAS shown in Table 1; 171 plasmids for HAStp (Table 2) and 173 plasmids for LAStp (Table 3). The RTK gene cDNAs were divided into 10 subfamilies for the HAS and LAS lines based on nucleotide sequence identities and then analyzed with the Audic-Claverie Distribution test to determine significance in the number of cDNAs identified between the HAS and LAS lines (Table 1) as well as in control or testosterone propionate-treated samples within lines (Table 2 and 3). The Fyn-related kinase (FRK) and the *EphA1* genes represented the largest number of cDNAs cloned. Interestingly, the number of cDNAs identified from the FRK and *EphA1* genes also differed significantly between the HAS and LAS lines ($p = 0.0183$ and $p = 0.0225$, respectively) (Table 1) and therefore will be discussed in relation to their potential functions in the bursa of the developing chicken embryo.

The FRK gene cDNA was significantly higher in the LAS bursa than in the HAS bursa (Table 1). To our knowledge, this is the first demonstration of FRK gene expression in the bursa during embryonic development. In mammals FRK gene expression is restricted to epithelial tissues, with the highest expression in the liver and kidney[13]. Only background levels of transcripts from the FRK gene were observed[14] in a previous bursal B-cell transcriptomics study, suggesting that FRK expression may be specific to nonlymphoid cells possibly epithelial cells, in the embryonic bursa. FRK is a Src non-receptor protein tyrosine kinase family member found in different subcellular locations[15] because it lacks a myristoylation site that is present in other Src-related members. The FRK gene displays either tumor suppressor or tumor promoting activity depending on the specific tissue. In the mammalian brain, FRK functions as a tumor suppressor because FRK protein expression is significantly reduced in glioma tumors, in contrast to healthy brain tissue. Moreover, enforced expression of FRK in glioma cell lines significantly reduced cell migration[16]. Breast cancer cell lines have reduced FRK expression compared with normal epithelial cells. However, like glioma tumors, the enforced expression of FRK

Table 3: Protein tyrosine kinase cDNA expressed in the LAS ED15 bursa compared with the testosterone-treated LAS (LAStp) ED15 bursa

| Tyrosine kinase gene cDNA (subfamily) | cDNA (n)[a] LAS | LAStp | p-value[b] |
|---|---|---|---|
| Jak 1 (Jak) | 32 | 13 | 0.0040 |
| PDGFRa (PDGF) | 34 | 54 | 0.0083 |
| CSK (CSK) | 14 | 4 | 0.0131 |
| IGFR (Ins) | 5 | 0 | 0.0184 |
| MERTK (Axl) | 7 | 1 | 0.0238 |
| PTK7 (PTK7) | 0 | 4 | 0.0271 |
| RYK (Ryk) | 12 | 20 | 0.0596 |
| EphA3 (Eph) | 3 | 0 | 0.0698 |
| EphB1 (Eph) | 0 | 2 | 0.1147 |
| EphA4 (Eph) | 6 | 3 | 0.1958 |
| FGFR4 (FGF) | 2 | 4 | 0.2041 |
| EphB2 (Eph) | 3 | 1 | 0.2055 |
| EphB3 (Eph) | 0 | 1 | 0.2361 |
| Ron (Met) | 0 | 1 | 0.2361 |
| RET (Ret) | 0 | 1 | 0.2361 |
| TIE1 (Tie) | 0 | 1 | 0.2361 |
| SYK (SYK) | 0 | 1 | 0.2361 |
| Jak 2 (Jak) | 8 | 5 | 0.2441 |
| FRK (Src) | 20 | 23 | 0.2614 |
| EGFR (ErbB) | 1 | 0 | 0.2642 |
| erbB2 (ErbB) | 1 | 0 | 0.2642 |
| SRMS (Src) | 1 | 0 | 0.2642 |
| YES1 (Src) | 1 | 0 | 0.2642 |
| BTK (Tec) | 1 | 0 | 0.2642 |
| c-Kit (PDGF) | 9 | 11 | 0.2859 |
| EphA1 (Eph) | 16 | 17 | 0.3684 |
| Flk-1/VEGFR-2 (VEGF) | 5 | 4 | 0.4119 |
| v-abl (Abl) | 1 | 1 | 0.4789 |
| Fer (Fes) | 1 | 1 | 0.4789 |
| Total cDNAs | 183 | 173 | |

[a]Number of cDNAs identified from two independent PCR reactions. [b]Probability that differential gene expression occurred by random biological variation

in breast cancer cell lines reduced expression of genes associated with cell survival and invasion[17]. Therefore, FRK mediates tumor suppressor activity through various signal transduction pathways[15].

An additional tumor suppression mechanism is the downregulation of the epidermal growth factor receptor (EGFR) surface expression, which is mediated by FRK kinase activity upon epidermal growth factor (EGF) binding to the receptor[18]. In our study, levels of EGFR and erbB2 cDNAs were low or not observed and did not differ between the HAS and LAS bursas (Table 1). Signals through EGFR play an essential role in epithelial development in mammals[19]. Four mammalian EGFR gene products homodimerize and heterodimerize to bind the ligands EGF and transforming growth factor alpha. The EGF receptors and ligands have an important role in chicken embryo limb development[20] and in maintenance of cells in other specialized tissues such as the avian cochlea[21]. Based on this evidence, it is reasonable to predict that the EGFR and erbB2 heterodimer would be expressed in the developing bursal epithelial cells. Our results suggest that genetic selection for antibody responses modulated the expression of the FRK gene. Given the possible role of FRK-mediated internalization of EGFR upon ligand stimulation[18], this modulation may influence the epithelial differentiation or development in the LAS line bursa. In response to testosterone propionate treatment, the FRK cDNA numbers increased in HAStp to the level observed in LAS (Table 1 and 2). We are unaware of data regarding the effects of sex steroids on FRK gene transcription. The mesenchyme is critical for development and differentiation of the epithelium[22] in the embryonic bursa. After embryonic exposure to testosterone propionate, the epithelium of the developing bursa exhibits vacuolization and a lack of bursal follicle development[10]. The increase in FRK gene transcription in testosterone propionate-treated embryos may be due to the inhibition of bursal epithelial cell development given that communication between the mesenchyme and epithelium may be altered by pharmacological levels of testosterone propionate.

The cDNAs for seven ephrin (*Eph*) receptor subfamily members were identified in both the HAS and LAS lines independent of testosterone propionate treatment (Table 1, 2 and 3). The Eph receptors are divided into two subfamilies based on ligand specificity, such that the Eph A class of receptors bind surface expressed ephrin-A ligands and Eph B class of receptor bind surface expressed ephrin-B ligands[23]. The interactions between Eph receptors and ephrins have a major role in controlling proper cell positioning during embryonic development of tissues[24]. Moreover, the signals transduced by Eph and ephrin interactions can also control cell biology by inducing proliferation or differentiation[25,26]. The largest number of cDNAs expressed in the embryonic bursa was from the *EphA1* gene, which occurred at significantly higher levels in the HAS line (Table 1). In mammals the *EphA1* gene is expressed primarily in epithelial cells of the kidney, skin, uterus, vagina and thymus[27,28]. The chicken *EphA1* gene was originally found in an analysis of RTK gene expression in blastoderm cells[29]. Additional studies with adult chickens showed that the *EphA1* gene was expressed at high levels in the kidney and lung, but at low levels in the thymus and testis[30]. Testosterone propionate treatment had no significant effect on the number of *EphA1* gene cDNAs (Table 2 and 3), suggesting that while *EphA1*-ephrin interactions are not essential for bursal cell maintenance or differentiation, they may contribute to epithelial cell organization in the developing bursa.

## CONCLUSION

This study identified the differential expression of RTK and nRTK genes in the developing bursa between chicken lines selected for high or low antibody response to SRBC. The variation in FRK and *EphA1* gene expression following this genetic selection may shape the differentiation and organization of the epithelium in the developing bursa. Future studies will be necessary to identify the cell types expressing the FRK and *EphA1* genes and the microanatomical differences in bursal development between these chicken lines.

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