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Characterization of late embryonic B cell stages in chicken bursa of Fabricius

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CHARACTERIZATION OF LATE EMBRYONIC B CELL STAGES IN CHICKEN BURSA OF FABRICIUS

By

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STAGES IN CHICKEN BURSA OF FABRICIUS

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B lymphocytes in chicken develop in a special primary lymphoid organ, the bursa of Fabricius. The most important maturation step in the bursa is the immunoglobulin (Ig) gene conversion, a process that is responsible for immunoglobulin repertoire in birds. In this study the late embryonic B lymphocyte stages are investigated, the bursal stem cell stage and the onset of Ig-gene conversion stage. In chapter II proteomic analysis of the two cell stages was performed. Signal transductions pathways were identified that are associated with proliferation, differentiation, cell adhesion and apoptosis. The project identified differences in protein profiles that might explain hanges in B lymphocyte physiology and bursal microenvironment at the initiation of Ig-gene conversion. In chapter III the antigen recognized by a bursal secretory-dendritic cell specific antibody, GIIF3 was cloned. The antigen was identified as smooth muscle gamma-actin. Futher work will investigate what role the gene plays in dendritic cell function.

DEDICATION

I would like to dedicate this work to my beloved fiancé, Tunde.

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CHAPTER I

INTRODUCTION AND LITERATURE OVERVIEW

Overview of the avian B cell concept

Studies with the avian immune system have played a key role in formation of our current understanding of immunological functions. The concept of humoral immunity was developed from the early work of Bruce Glick (1955), who discovered that lymphocytes, responsible for antibody production arise from the chicken bursa of Fabricius. The bursa is known to provide a unique microenvironment for B-cell repertoire development by immunoglobulin (Ig)-gene conversion (McCormack *et al.*, 1989). In mammals, the bone marrow is considered to be the functional equivalent to the bursa, but the two systems radically differ from each other. Differences are not only present between mammalian and avian systems, but among mammals too. Cattle, rabbit and swine possess homologous gut associated lymphoid organs to the bursa, and are able to undergo Ig-gene conversion (Mage *et al*., 1999; Diaz *et al.,* 1998), while primates, mice and rats only rely on bone marrow. In bone marrow B cells develop under sterile and external antigen free conditions, while in avian species B cells are exposed to environmental and yolk antigens/immune-complexes in post hatch and late embryonic stages respectively (Ekino *et al.,* 1992, 1995; Felfoldi *et al.*, 2005). Stimulus from antigens or immune-complexes is required for normal B cell development and survival,

but the working mechanisms of the effect is not yet clear. It is thought that B cells require a general stimulus for survival, while antigen specific B cell selection does not take place in the bursa (Felfoldi *et al.* 2005).

Although the basic concept of T and B lymphocytes is very similar in mammals and birds, the T cell compartments are highly homologous, but the B cell compartment of the birds is organized differently, and therefore it requires a separate nomenclature for the different stages of developing B-cells. The discovery that all B-cells in the chicken express CD5, a surface marker of human and mouse B-1a cells (Koskien *et al.,* 1998), suggested that chicken B-cells may represent a homolog to B-cells from the gut-associated compartment of the mammalian lymphoid system. Moreover, the avian B-cells are functionally similar to both mammalian B-1 and B-2 cell types (Weil *et al.,* 2005). B cell development in chicken is limited in time and space. After sexual maturity the bursa undergoes involution, a process that eliminates all primary lymphoid activity. In the later period of life, the B cells that entered the periphery prior to bursal involution maintain a self renewing population in secondary lymphoid organs (Kothlow *et al.,* 2007).

B cell development in chicken

In chicken embryo the first hematopoietic stem cells (HSC) emerge from the para-aortic region at embryonic day 2.5 (ED 2.5) (Dieterlen-Lievre and Martin, 1991). These HSCs colonize the extra embryonic yolk sac in the blood islands, leading to the development of various cell types (Fig. 1). The first committed B-cell precursors, the prebursal stem cells, appear in the yolk sac from ED 4 and have already undergone D to J rearrangements at the heavy chain locus (Reynaud *et al.,* 1991). The prebursal stem cells

then migrate into the embryo proper and undergo a second wave of rearrangement (V_H to DJ_H and V_λ to J_λ) at the time of bursal colonization at ED8-14 (LeDouarin *et al.,* 1975; Weill *et al.,* 1986). The immunoglobulin (Ig) gene rearrangement process uses the same molecular mechanisms with Rag1/Rag2 enzymes as in mammals, but the low number of V, D and J gene segments results in a very limited repertoire, which is further expanded by Ig-gene conversion as described below. The prebursal stem cells are characterized by the expression of surface IgM **(**sIgM**),** CD45, the chicken B-cell marker Bu-1 and the sialyl Lewis ^{X} carbohydrate epitope (Masteller *et al*., 1995). The prebursal stem cells that enter the embryonic bursal mesenchyme cross the basement membrane of the double-layered epithelial lining that separates the mesenchyme from the bursal lumen (Olah *et al.,* 1986). The epithelial buds represent precursors to the bursal follicles (Olah *et. al.*, 1986), and generally contain only 3–4 prebursal stem cells (Pink *et al.*, 1985).

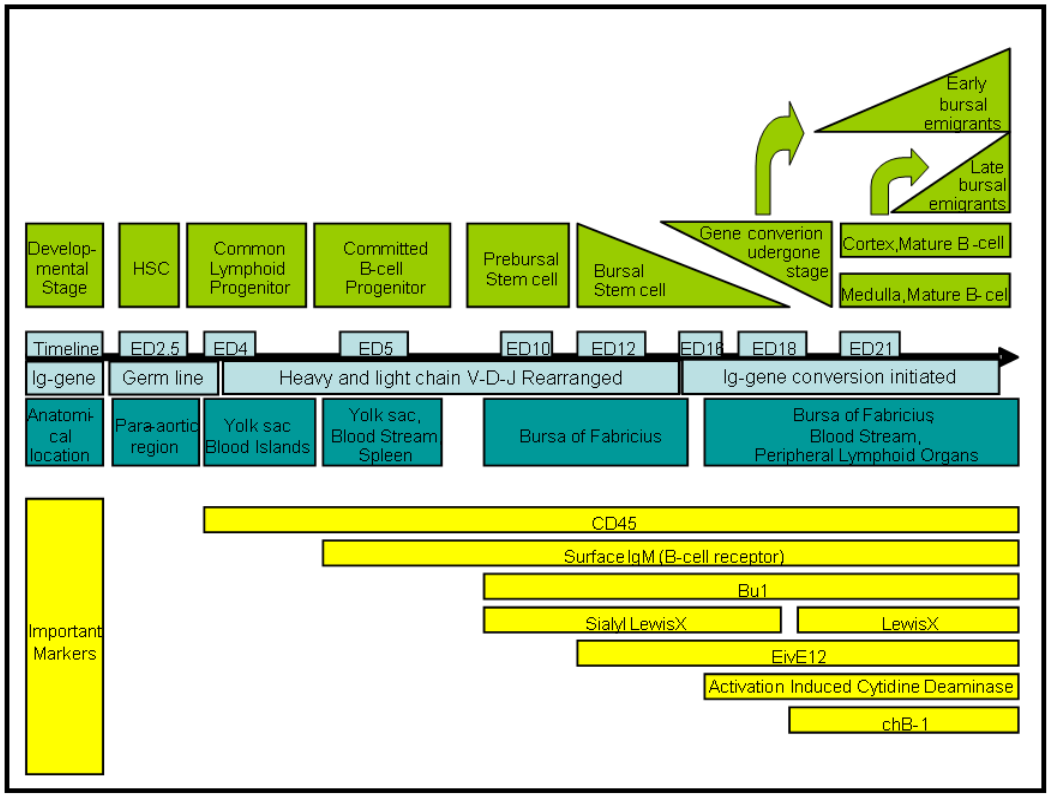

Figure 1. Schematic diagram of B cell developmental stages in embryo and young chicken.

The bursal follicle starts to form as the epithelial layers further separate when the prebursal stem cells proliferate, possibly in response to intrinsic signals from sIgM (Sayegh *et al.,* 1999), creating a large pool of candidates with productively rearranged H-chain and L-chain genes for diversification by Ig–gene conversion (McCormack *et al.*, 1989). The process of Ig-gene conversion initiates in the proliferating pool of developing B-cells between ED15 and ED17, and continues until bursal involution (Thompson and Neiman, 1987; Reynaud *et al.*, 1987)**.** By ED18 most of the bursal B-cells undergo at least one round of Ig-gene conversion, further rounds are possible until the bursa undergoes involution (24-weeks post hatch), and in splenic germinal centers upon

activation by antigens (from 2 weeks post hatch) (Ekino *et al*., 1995). The Ig-gene conversion can either be monitored by the analysis of the rearranged Ig gene sequences or phenotypic changes. The few markers described on developing B-cells undergoing Ig-gene conversion are the switch from sialyl-Lewis ^{X} to Lewis ^{X} antigen, and the expression of chB1 lectin, which is a pro-apoptotic receptor (Masteller *et al*., 1995 a, b; Goitsuka *et al.,* 1997). From ED18 to the time of hatching no phenotypic changes have been described (Masteller *et al.,* 1995 a, b; Goitsuka *et al.,* 1997). During this developmental time period, the major event in the bursa is a high rate proliferation, expanding the sizes of follicles to fill up all available space, and this is the time when the first wave of immigrant cells can be detected in the blood and spleen (Cooper *et al*., 1969). After hatching a structural rearrangement takes place, some B-cells migrate out from the follicles to form the cortical area, and the original embryonic follicle becomes the medullary area (Grossi *et al*., 1974) (Fig. 2).

The second role the bursa plays is the selection of the B cells. After hatching a large number of developing B-cells are eliminated by apoptosis (Motyka *et al.,* 1991). Recent studies suggest that sIgM is critical for positive selection of developing B-cells by recognition of environmental antigens (Sayegh *et al.,* 1999; Sayegh and Ratcliffe, 2000). After hatching the bursal lumen is exposed to the microflora and yolk sac proteins from the gut, which are transported into the medulla by the follicle-associated epithelium found on the luminal side of the follicle (Bockman and Cooper, 1973; Sorvari *et al.,* 1975; Felfoldi *et al.,* 2005). In addition, immune complexes consisting of IgG and antigens derived from the gut flora are found in the medulla of the follicle, in association with bursal secretory dendritic cells (Olah *et al.,* 1991; Yasuda *et al.,* 2002). As

developing B-cells have been shown to express only IgM in the bursa, the immune-complexes must therefore consist of maternal antibody (Ekino *et al.,* 1995). Taken together, it has been proposed that low affinity recognition of immune complexes by sIgM is responsible for stimulating B-cell migration across the CM border into the follicular cortex after hatching (Sayegh and Ratcliffe, 2000; Arakawa *et al.,* 2002). Those cells which lose expression of sIgM, or that fail to make low affinity recognition of immune complexes would presumably fail to be positively selected and may undergo apoptosis (Paramithiotis *et al.,* 1995; Sayegh *et al.,* 2000). The final maturation of B cells occurs in the peripheral lymphoid organs, after activation by high affinity antigen binding. Activated B cells within germinal centers undergo affinity maturation with further rounds of Ig-gene conversion, and isotype class switch, and then develop into plasma cells and memory B cells. The vast majority of cell types in the bursa belong to different B cell developmental forms, but other cell types also play a role in follicle formation. The non lymphoid subset of bursal cells contains BSDC, and three types of epithelial cells (Fig. 2). Epithelial cells are derived from gut epithelium that originally became the bursal anlage (Olah *et al.,* 1992). Interfollicular epithelium (IFE) covers the bursal lumen between follicles, and is equivalent of gut epithelium.

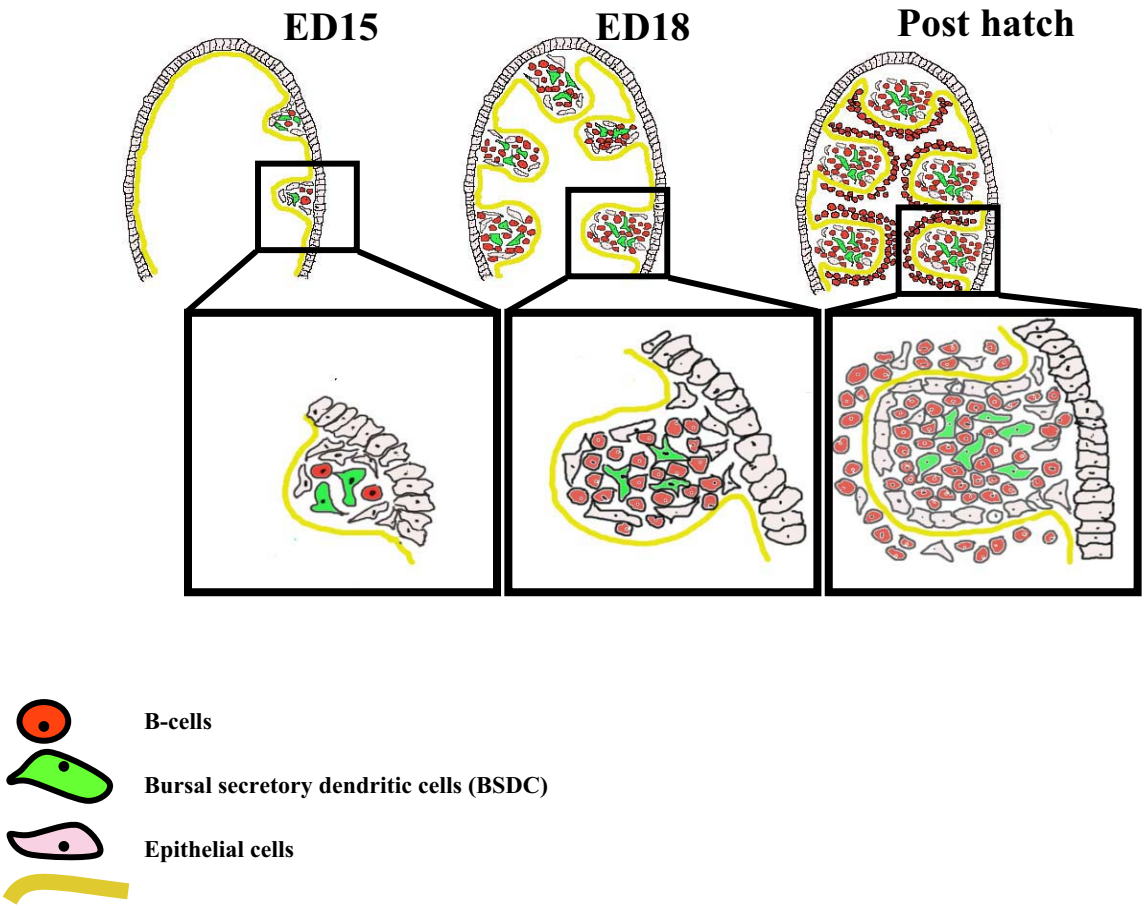

Figure 2. The major events in the development of the bursal follicle. Follicles start to form after bursal colonization when B cells and BSDCs cross the basement membrane and form clusters among epithelial cells. By ED18 the follicles expand in number and size, filling up all the available space in the bursa. After hatching mature B cells cross the basement membrane and form the cortical area that is the source of bursal emigrant cells.

Follicle associated epithelium (FAE) is a transformation of IFE at places where follicles align with the lumen. IFE cells are responsible for antigen transport between bursal lumen and the medulla of the follicles, and are functionally and anatomically closely related to M cells in Peyer's patches. The structural integrity of the follicle is maintained by reticular epithelial cells forming a scaffold system. The most important non lymphoid cell type is the BSDC. Originally described with histological techniques (Olah *et al.*,

1978), BSDC is thought to be regulating the B cell development processes in the bursa, providing survival and differentiation signals to B cells (Glick *et al.,* 1993).

Objectives

The goal of this study is to conduct protein profile analysis of bursal B-cells at ED15 ($SLEX^+$) and ED18 (LEX^+). We hypothesize that various receptors for cytokines, chemokines and cell-adhesion proteins would be identified, as these kinds of proteins would be important candidates for receiving bursal microenvironmental signals that would guide the major B-cell differentiation event that is responsible for the onset of Ig repertoire development; the $SLEX^+$ to LEX^+ transition between ED15 and ED18. Previous research indicated the importance of this process in B cell development, but still little is known about how it is regulated. Based on previous studies by others it is expected, that the two stages express different sets of proteins controlling cell adhesion, differentiation and apoptosis (Lampisuo *et al.,* 1997) The characterization of the B cell stages should reveal expression of genes in ED 15 and ED18 B cells that help to understand the regulation of embryonic developmental processes in the B cell compartment of the chicken.

The study involves two projects aimed to provide data on the late embryonic bursal microenviromnent. The first project (Chapter II) is an approach to gather the gene expression profile on the protein level of ED15 and ED18 B cells in order to detect gene candidates that would be expected to play a role in B cell development regulation. This information is critical for designing future studies to understand the bursal microenvironment guiding B-cell development.

The second project (Chapter III) was aimed to identify a BSDC specific marker, an antigen that is recognized by the monoclonal antibody GIIF3 (Nagy *et al.*, 2001). As the number of available markers for embryonic bursal microenvironmental research are low, and therefore every new antibody offers significant improvement to understand the B cell development process. The antigen recognized by GIIF3 monoclonal antibody is selectively expressed by BSDC in the bursa during the late embryonic age was expected to be a factor that contributes to the bursal microenvironment guiding B cell development.

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CHAPTER II

PRELIMINARY PROTEIN PROFILE ANALYSIS OF THE LATE EMBRYONIC B-CELL STAGES IN THE CHICKEN BURSA OF FABRICIUS.

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Abstract

The bursa of Fabricius serves as a primary lymphoid organ for the development of a diverse repertoire of B-cells. The embryonic bursa is colonized during embryonic days (ED) 8-14 by stem cells expressing the sialyl Lewis^X carbohydrate (SLEX). At ED13-15, cells with the SLEX phenotype initiate proliferation leading to the development of the bursal follicle. By ED15-17, a key differentiation event occurs resulting in the onset of repertoire development by immunoglobulin gene-conversion. This differentiation event is defined by a phenotypic transition in cell surface glycosylation from SLEX to a related

carbohydrate structure termed Lewis^X (LEX). The goal of this study was to identify functional groups of genes in the two stages that might be involved in critical biological processes as proliferation, differentiation, apoptosis and cell adhesion, explaining the observed functional differences. We found that before the immunoglobulin gene conversion B cells express genes related to ephrin receptor signaling, epidermal growth factor receptor (EGFR) signaling and Wnt signaling. B cells undergoing immunoglobulin gene conversion express genes from TNFR signaling and both stages were found to express members of Wnt signaling, integrin signaling and EGFR signaling pathways. The differentially expressed pathways agree with previous observations, offering explanation to signals leading to proliferation, differentiation and apoptosis in the two B cell stages.

Key words: Bursa, B cell, immunoglobulin gene conversion, development, proliferation, mass spectrometry.

Introduction

The importance of the chicken as a model for studying humoral immunity was established with early studies of a gut-associated lymphoid tissue, the bursa of Fabricius (Glick *et. al*., 1956). The bursa is known to provide a unique microenvironment for B-cell repertoire development by immunoglobulin (Ig)-gene conversion. In chicken embryo, the first hematopoietic stem cells (HSC) emerge from the para-aortic region at embryonic day 2.5 (ED 2.5) (Dieterlen-Lièvre, 1975). These HSCs colonize the extra embryonic yolk sac blood islands, leading to the development of various blood cell types (Dieterlen-Lièvre and Martin, 1981). The first committed B-cell precursors, the prebursal stem cells, appear in the yolk sac from ED4 and have undergone D to J rearrangements at the heavy chain locus. The prebursal stem cells then migrate into the embryo proper and undergo a second wave of rearrangement (V_H to DJ_H and V_λ to J_λ) at the time of bursal colonization, ED8-14 (LeDouarin *et al.,* 1975; Weill *et al.,* 1986; Mansikka *et al*., 1990; Reynaud et al., 1992). The prebursal stem cells are characterized by the expression of surface IgM (sIgM), CD45, the chicken B-cell marker Bu-1 and the sialyl $Lewis^X$ (SLEX) carbohydrate epitope **(**Houssaint *et al.,* 1989, Masteller *et a*l. 1995b). The prebursal stem cells that enter the embryonic bursal mesenchyme cross the basement membrane of the double-layered epithelial lining that separates the mesenchyme from the bursal lumen (Olah *et al.,* 1986) and express a surface marker recognized by the EIVE12 monoclonal antibody (Pharr *et al.,* 1995). The epithelial buds represent precursors to the bursal follicles (Olah *et al*., 1986) and generally contain only 3 – 4 prebursal stem cells (Pink, 1985). The bursal follicle starts to form as the epithelial layers further separate when the prebursal stem cells proliferate, possibly in response to intrinsic signals from

sIgM (Sayegh *et al*., 1999a), creating a large pool of candidates with productively rearranged H-chain and L-chain genes for diversification by Ig – gene conversion (McCormack *et al.*, 1989). The process of Ig-gene conversion initiates in the proliferating pool of developing B-cells between ED15 and ED17 and continues until bursal involution (Thompson and Neiman; 1987; Reynaud *et al.,* 1987).

By ED18 most of the bursal B-cells undergo at least one round of gene conversion, further rounds are possible until the bursa undergoes involution (24-weeks post hatch) and in splenic germinal centers upon activation by antigens (from 2 weeks post hatch) (Arakawa *et al*., 1996). The Ig-gene conversion can be monitored either by the analysis of immunoglobulin gene sequences or phenotypic changes. The markers described on developing B-cells undergoing Ig-gene conversion are: the switch from sialyl-Lewis ^{X} to Lewis ^{X} (LEX) antigen and the expression of chB1 lectin, which is a pro-apoptotic receptor (Goitsuka *et al.,* 1997). From ED18 to the time of hatching there is a high rate of B-cell proliferation and an expansion in the sizes of follicles. Also at this time, the first wave of immigrant cells can be detected in the blood and spleen (Cooper *et al*., 1969). After hatching a structural rearrangement takes place; some B-cells migrate out from the follicles to form the cortical area and the original embryonic follicle becomes the medullary area (Grossi *et al*., 1974).

The second role the bursa plays is the selection of the B cells. After hatching a large number of developing B-cells are eliminated by apoptosis (Motyka and Reynolds, 1991). Recent studies suggest that sIgM is critical for positive selection of developing B-cells by recognition of environmental antigens (Sayegh *et al*., 1999b; Sayegh and Ratcliffe, 2000). After hatching, the bursal lumen is exposed to the microflora and yolk

sac proteins from the gut, which is transported into the medulla by the follicle-associated epithelium found on the luminal side of the follicle (Bockman and Cooper, 1973; Sorvari *et al.,* 1975; Pike *et al*., 2004; Felfoldi *et al*., 2006). In addition, immune complexes consisting of IgG and antigens derived from the gut flora are found in the medulla of the follicle, in association with bursal secretory dendritic cells (Olah *et al*., 1991; Yasuda *et al.,* 2002). As developing B-cells have been shown to express only IgM in the bursa, the immune-complexes must therefore consist of maternal antibody (Ekino *et al*., 1995). Therefore, it has been proposed that low affinity recognition of immune complexes by sIgM may be responsible for stimulating B-cell migration across the cortico-medullary (CM) border into the follicular cortex after hatching, where further selection (Sayegh and Ratcliffe, 2000; Arakawa *et al*., 2002a). Those cells that lose expression of sIgM, or that fail to make low affinity recognition of immune complexes would presumably fail to be selected and may undergo apoptosis (Paramithiotis *et al*., 1995; Sayegh and Ratcliffe, 2000).

Our long-term goal is to contribute a mechanistic understanding of the differentiation events occurring in the bursal follicles prior to hatching. Accomplishing this goal requires a thorough characterization of developing B-cells expressing the SLEX epitope at ED15 and the LEX epitope at ED18. The central hypothesis of this project is that cellular proteins differentially expressed between the SLEX and LEX developing B-cell populations will represent candidate proteins involved in the SLEX to LEX transition. In this manuscript, we report the proteomic comparison of the ED15 and ED18 B-cell stages in chicken bursa of Fabricius, representing the pre and post Ig-gene conversion developmental stages (Palojoki *et al.,* 1995, Masteller *et al.,* 1995b), to further

our understanding of the biological processes occurring during this period of bursal B-cell development.

Materials and Methods

Experimental Animals

The embryos used were the first cross of $15I_5$ and 7_1 highly inbred White Leghorn chicken lines (Avian Disease and Oncology Laboratory, East Lansing, Michigan USA). Embryos were incubated under standard conditions (100°F, 60% relative humidity, regular rocking).

Preparation of Cell Lysates

Single cell suspension of B-cells was prepared in RPMI cell culture medium (Mediatech Inc, Herdon VA) from dissected bursas of ED15 and ED18 embryos as described (Glick and Schwartz, 1975). B-cells were then purified using Histopaque (1.077, Sigma, St. Louis, MO) gradient centrifugation. Whole cell protein lysates were then prepared in Radioimmunoprecipitation (RIPA) Buffer from bursal B-cells isolated from ED15 (92 embryos) and ED18 (56 embryos). Nuclear proteins were isolated using an SDS buffer as described (McCarthy *et al.,* 2006). Equal amounts of protein (200ug) from each developmental stage were digested with trypsin and then the reaction products were desalted with a MacroTrap reverse-phase HPLC column for evaluation with mass spectrometry as described (McCarthy *et al.,* 2006).

Mass Spectrometry

2D LC ESI MS^2 was done as described elsewhere (McCarthy *et al.,* 2005; Lee *et al.,* 2006). Proteins were identified and analyzed as previously described (McCarthy *et al.,* 2005, Lee *et al.,* 2006). The search term Gallus gallus was searched against the organism field of the National Center for Biotechnology Information (NCBI) protein database to create a chicken-specific protein database. TurboSEQUEST (Bioworks Browser 3.1; ThermoElectron) was used to apply in silico trypsin digestion to the chicken database and mass changes due to cysteine carbamidomethylation and methionine oxidation were included.The chicken non-redundant protein database was used to search tandem mass spectra using a peptide (MS precursor ion) mass tolerance of 1.5 Da, and a fragment ion (MS2) mass tolerance of 1.0 Da. Peptide matches were considered valid if they were ≥7 amino acids with X correlation values of 1.5, 2.0 and 2.5 (+1, +2, and +3 ions, respectively) and Delta Cn values >0.1.

Data Analysis

Identified proteins were categorized into functional groups with manual database search (www.expasy.org, and the NCBI database www.ncbi.nlm.nih.gov). Proteins falling into the functional groups of proliferation, apoptosis, cell adhesion and differentiation were subjected to additional searches to find proteins associated with canonical signal transduction pathways (www.biocarta.com).

Results

The first analysis step included combining the results from nuclear and soluble samples from the ED15 and ED18 stages, respectively. After removing duplicates and unknown proteins the ED15 sample resulted in 496 identified proteins, and ED18 sample resulted in 834 identified proteins. The proteins were sorted into functional groups, such as basic cell functions, proliferation, apoptosis, cell adhesion and differentiation, based on manual selection (Table 1).

Further analysis identified functional signal transduction pathways among proteins from the ED15 and ED18 samples. Only pathways with at least three identified members were considered functional. The ED15 sample contained members of the following pathways: B-cell receptor pathway (data not shown), integrin signaling, and ephrin receptor signaling, Wnt signaling and epidermal growth factor receptor (EGFR) signaling. The ED18 sample contained members of the following pathways: B-cell receptor pathway (data not shown), integrin signaling, tumor necrosis factor receptor (TNFR) type 1 signaling and Wnt signaling. The qualitative differences in gene expression between ED15 and ED18 bursal B-cells are presented in Tables 2 and 3.

Discussion

Our results add information to explain functional changes related to one of the critical developmental steps in embryonic chicken B-cell development (Fig. 1), the onset of repertoire development. The importance of the process is that only developing B-cells that have undergone successful Ig gene-conversion events are capable of continuing development in the bursa. This development involves giving rise to early bursal emigrant B-cells (from ED17 to hatch) (Cooper *et al.,* 1969) and differentiation into the cortical and medullary populations of developing B-cells in post hatch bursal follicle, that produce late bursal emigrant B-cells (from hatch to 16 weeks of age) (Paramithiotis and Ratcliffe, 1994).

The most important difference between the B-cells at ED15 and ED18 appeared in the apoptosis related genes. The ED18 B-cells express most of the TNFR1 pathway components, suggesting that these cells are highly susceptible to apoptosis. TNFR1 signaling is one of the best-characterized apoptosis inducer pathways (reviewed by Vermeulen *et al.*, 2005). TNFR1 can initiate apoptosis by recruiting the caspase cascade or initiate signal transduction leading to activation of NF-κβ transcription factor (reviewed by Hehlgans and Pfeffer, 2005). Ig-gene conversion results in a high number of non-functional or self-specific B-cell receptors that are eliminated from the repertoire (McCormack *et al.,* 1989). Previous observations indicate that large numbers of bursal B-cells are removed by apoptosis (Motyka and Reynolds, 1991). Based on our findings, it may be possible that bursal B-cells are susceptible to apoptosis while undergoing Ig-gene conversion. The involvement of the TNF super family in the initiation of bursal B-cell apoptosis was shown before (Abdalla *et al.,* 2004).

Ephrin receptor signaling was first described in regulation of axon guidance during nervous tissue development (Wang and Anderson, 1997). Ephrin receptors, belonging to the receptor tyrosine kinase family, were found to be expressed on different lymphocytes that have a migratory phenotype (Aasheim *et al.,* 2000; 2004)**.** Lymphocytes were reported to express various types of both ephrins and ephrin receptors (Shimoyama *et al.,* 2002, Muñoz *et al.,* 2002). Ephrins may be important at the early stage by regulating the homing of prebursal B-cells to the bursa and possibly at the later stage of development for cell movements associated with formation of the mature bursal follicle. Our findings may be similar to the study of Aasheim *et al.,* (1997), where the ephrin receptor family member Hek11 receptor tyrosine kinase (later classified as ephrin type-A receptor 7, EphA7) was reported to be expressed in pre and pro B-cells but not in more mature stages in human bone marrow. We expect similar differences in ephrin receptor expression in different developmental stages in bursal B-cell development. Further work is needed to identify chicken ephrin genes and follow their expression in B-cells.

Integrins are receptors for different extracellular matrix (ECM) components depending on different combinations of alpha and beta chains. When bound to their ligands, integrins transmit intracellular signals affecting cytoskeletal organization, cell motility and cell cycle. In B-cells integrin signaling is related to cell migration (Terol *et al.,* 1999) and cluster formation at germinal centers in spleen (Ambrose *et al.,* 2004). In bursal B cell development, migration is needed when prebursal stem cells enter the bursal mesenchyme and cross the basement membrane to enter the developing follicles. Integrins would be important after hatch when B-cells are exported from the bursa to peripheral lymphoid organs. The bursal B-cells might utilize integrins for interaction with

the ECM in the developing follicle. We found the expression of the α8 and the β1 subunits at both ED15 and ED18 (Tables 2 and 3), which could form an integrin heterodimer with specificity for the ECM (Shnapp *et al.,* 1995). Another possible heterodimeric combination could involve the α11 and β1 chains forming a receptor for collagen (Humphreys *et al.,* 2006). Most of the integrin adhesion molecules are shared on ED15 and ED18 B-cells, there was no major difference found in integrins and other cell adhesion molecules. The expression of similar integrin chains in the ED15 and ED18 samples suggests that the ECM microenvironment in the bursal follicle does not change during this period of embryonic bursal development. Significant changes in cell adhesion proteins would therefore be expected at earlier time-points during the homing of prebursal stem cells to the bursa, and later, with export of B-cells to peripheral lymphoid organs begins.

Other cell adhesion molecules, belonging to the cadherin superfamily of showed differences between ED15 and ED18 B-cells (Tables 2 and 3). As cadherins mediate monophylic cell-to-cell type interactions, we speculate that in the bursal system cadherins play a role in cluster formation of B-cells. The different cadherin expression pattern in different stages suggests that B-cells prefer to interconnect with cells that are in the same developmental stage.

Members of the wingless (Wnt) signaling pathway are expressed in diverse genera ranging from Drosophila to human. The members of this pathway are highly conserved due to the important role they play in development (reviewed by Clevers, 2006). The different combinations of Wnt growth factors and Frizzled receptor subtypes lead to two major pathways: the canonical pathway, through beta-catenin and the non-

canonical pathway, through Janus kinase (Clevers, 2006). In mature T lymphocytes Wnt signaling induces transmigration, increasing malignancy in lymphomas where it is activated (Wu *et al.,* 2007). In B-cell development, WNT is considered to provide a proliferation signal to immature B-cell stages in bone marrow (Reya *et al.,* 2000). Other studies found Wnt signaling important in regulation of developmental processes including proliferation, differentiation and apoptosis (Døsen *et al.,* 2006; Ranheim *et al.,* 2005). The Wnt signaling pathway was found to be active in both ED15 and ED18 B-cells; as both cell stages undergo differentiation and proliferation (Ratcliffe, 1989). The components identified in ED15 stage are related to the non-canonic Wnt pathway, while the components found at ED18 are related to the canonic type. The difference between the two pathways is not well characterized, so we cannot make conclusions about the differences between ED15 and ED18 differentiation based on the Wnt signaling pathway.

Epidermal Growth Factor Receptors belong to the receptor tyrosine kinase protein super family. EGFR signaling is essential in all multicellular organisms and regulates a high number of cellular functions, as well as organ development and pattern formation (reviewed by Edwin *et al.,* 2006). In mammalian B-cells, the EGFR signal was found to provide a survival and proliferation signal in the bone marrow microenvironment (Spengeman *et al.,* 2005). Both ED15 and ED18 B-cells express components of the pathway, in agreement with reports that both stages proliferate at a high rate (Ratcliffe, 1989). Therefore, it may be possible that proliferative signals are provided to the B-cells through EGFR. The role of EGFR signaling was investigated in chicken ovarian follicle development (Volentine *et al.,* 1998; Wang *et al.,* 2007) and feather pattern development, as examples of epithelial-mesenchymal interaction (Atit *et al.,* 2003). In the bursal

system EGF, signaling might play two roles: it can provide a differentiation signal, regulating the epithelial-mesenchymal interaction at follicle formation as seen in other systems; and EGF signaling might be one source of proliferation signal for both ED15 and ED18 B-cells.

Conclusion

This is the first study to evaluate protein expression at the ED15 and ED18 B-cell stages in the chicken. The proteins revealed signal transduction pathways that differ between the two stages and refer to observed functional differences. The information derived from this work should give insight into the kinds of microenvironmental signals that developing B-cells receive in the bursal follicles. The reported pathways help to explain possible sources of developmental (Wnt, ephrin receptor), proliferative (Wnt, EGFR), apoptotic (TNFR) signals and interactions with the microenvironment (integrins, cadherins) in the B-cell stages. Future studies will confirm and extend MS data using alternative methods and identify specific receptor-ligand pairs in the bursal system associated with the reported signal transduction pathways.

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Table 1. Number of expressed gene products identified in ED15 and ED18 samples, categorized into functional groups.

Stage	ED18	ED15
Total	834	496
Basic cell functions	589	321
Proliferation	26	18
Apoptosis	18	5
Cell adhesion	9	6

Table 2. Proteins involved in cellular processes in ED15 developing B-cells.

Symbol	Gene Name	GI number
Ephrin receptor signaling pathway members in ED15 sample		
ABL1	Abelson murine leukemia viral oncogene homolog 1	50751126
GNAQ	Guanine nucleotide binding protein q polypeptide	22651974
ITGA11	Integrin, alpha 11	50753262
ITSN1	Intersectin 1 (SH3 domain protein)	50745113
JAK2	Janus kinase 2	45382379
RAC1	Ras-related C3 botulinum toxin substrate 1	45384330
Integrin signaling pathway members in ED15 sample		
Symbol	Gene Name	GI number
ABL1	Abelson murine leukemia viral oncogene homolog 1	50751126
ACTN2	Actinin, alpha 2	46048687
ITGA8	Integrin, alpha-8	124950
RAC1	Ras-related C3 botulinum toxin substrate 1	45384330
RAC3	Ras-related C3 botulinum toxin substrate 3	45384328
TSPAN7	Tetraspanin 7	50752761
ITGA11	Integrin alpha 11	50753262
Other cell adhesion molecules ED15 sample		
Symbol	Gene Name	GI number
PCDH7	Protocadherin-7	50747110
PCDH12	Protocadherin12	50754824
DSCAM	Down syndrome cell adhesion molecule 5	0729979
Wnt signaling pathway members in ED15 sample		
Symbol	Gene Name	GI number
WNT10A	Wingless type 10A	54260406
JAK1	Janus kinase 1	45382379
ARD30A	Ankyrin Repeat Domain 30A	50728154
EGFR signaling pathway members in ED15 sample		
Symbol	Gene Name	GI number
Jak1	Janus kinase 1	45382379
MAPK3	Mitogen-activated protein kinase kinase kinase 3	50754317
EPS15R	Epidermal growth factor receptor substrate EPS15R	50761168

Table 3. Proteins involved in cellular processes in ED18 developing B-cells.

TNFR signaling members in ED18 sample

Symbol	Gene Name	GI number
P33	Arginine-specific ADP-ribosyltransferase target protein 1	258401
ARHGDIB	D4 GDP dissociation inhibitor	50728568
TNFRAP1	TNFR associated protein1	57525126
TRADD1	TNFRSF1A-associated via death domain	50753615
TNFR8	Tumor necrosis factor receptor superfamily, member 8	45383273
MKP1	MAP kinase phosphatase-1	50764356

Integrin signaling pathway members in ED18 sample

Symbol	Gene Name	GI number
ACTN2	Actinin, alpha 2	46048687
ITGA8	Integrin, alpha-8	124950
RAC1	Ras-related C3 botulinum toxin substrate 1	45384330
ITGA11	Integrin, alpha 11	50753262
ITGB2	Integrin, beta 2	46048728
ITGB1	Integrin, beta 1	86129418

Other cell adhesion molecules ED18 sample

Symbol	Gene Name	GI number
PCDHB20	Protocadherin-beta20 (Protocadherin-beta T)	50762730
TCAD-2	T-Cadherin 2	386363
CAD13	Cadherin 13 (H-cadherin)	48976117
FLM1	Flamingo 1	40287630
DSG4	Desmoglein 4	50737406

Wnt signaling pathway members in ED18 sample

Symbol	Gene Name	GI number
FLM1	Flamingo 1	40287630
WNT5A	Wingless type MMTW integration site protein 5A	45382433
sFRP-2	Secreted frizzled-related protein 2 precursor	61216846

EGFR signaling pathway members in ED18 sample

Symbol	Gene Name	GI number
STAT3	Signal Transducer and Activator of Transcription 3	71896343
PI3K	Phophatidylinositol 3 kinase	50761547
MEK2	Dual specificity mitogen-activated protein kinase kinase 2	2499630
MK2	MAP kinase-activated protein kinase 2	50760337

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CHAPTER III

IDENTIFICATION OF THE CHICKEN BURSAL SECRETORY DENDRITIC CELL SPECIFIC ANTIGEN RECOGNIZED BY GIIF3 MONOCLONAL ANTIBODY

Introduction

In chicken, B cell development is strictly regulated by signals from bursal microenvironment. Bursal secretory dendritic cells (BSDC) are considered to provide crucial survival, developmental and proliferative factors for developing B cell forms. B cells removed from the bursa are very susceptible to apoptosis and cannot be maintained in culture for more than few days. The communication between bursal B cells and dendritic cells is not well understood, only a few factors are known on molecular level, and antibodies are available against some antigens that are not characterized yet. Identification of such factors may lead us to understand what signals might contribute to proliferation or development.

Many monoclonal antibodies (mAb) were produced for chicken research and used without identifying the reacting antigen. This project was part of an an international cooperation with Dr. Nandor Nagy and Dr. Imre Olah (Department of Human Morphology and Developmental Biology at Semmelweis University, Hungary), aimed to identify the antigen, recognized by the mAb GIIF3. The antibody was produced against guinea fowl spleen cells, but showed positive staining in other avian tissue samples on

histological staining. In chicken the antibody stains BSDC in the bursal follicles throughout embryonic development. It also stains all three muscle types expressed in the embryo (Fig. 3), but after hatching only smooth muscle shows positivity. Western blot analysis showed that the molecular weight of the antigen was in the range of actins (42 kDa and a secondary band at 50 kDa) (Nagy et al., 2001). However, additional experiments indicated that the staining patterns of mAb GIIF3 and a commercially available alpha and beta actin-specific polyclonal antibody were different. These immunohistological experiments showed that GIIF3 positive cells appear from embryonic day 2 in heart, somites, and smooth muscle cells, two days earlier than other alpha- and beta-actin markers. The GIIF3 positivity appeared early in embryonic development in various myogenic types, but after hatching only smooth muscle cells continued to express it (Nagy *et. al.,* 2001a).

Additional studies using immunohistochemistry with tissue sections of the bursa of Fabricius identified cells of mesenchymal origin expressing the antigen recognized by mAb GIIF3 early in bursal development (Nagy *et. al.,* 2001b). The immunohistochemical study of serial sections revealed the migration of GIIF3-reactive mesenchymal cells across the basement membrane of the double-layered epithelial lining that separates the mesenchyme from the bursal lumen. The GIIF3-reactive cells were then observed on the luminal side of the epithelium where the cells differentiate into the follicle-associated epithelium (FAE). After hatching, the lumen of the bursa is exposed to the intestinal flora, which is transported into the bursal follicle by the FAE (Bockman and Cooper, 1973; Sorvari et. al., 1975). Recent studies have shown that components of the intestinal flora is required for driving bursal B-cell development by the time of hatching (Sayegh

and Ratcliffe, 2000; Arakawa *et. al*., 2002). Therefore the FAE plays a critical function in the development of humoral immunity in the Aves with the transport of luminal contents into the lymphoid follicles of the bursa.

The hypothesis of this study was that the antigen recognized by mAb GIIF3 is a gene product that plays an important role in bursal B cell development as well as muscle development. The time regulated expression if the antigen in the bursa coincides with the late embryonic stage when Ig-gene conversion is activated (Nagy et al., 2001). During embryonic development the antigen is expressed from ED2 in all muscle types until hatching when it is down regulated in heart and somatic muscles but continued in smooth muscle. The regulated expression patterns and the differences from alpha and beta actin staining patterns on histological sections gave the option to expect a factor that plays a role in developmental regulation processes.

Materials and Methods

Cell Lysate Preparation

Gizzards were dissected from 42 day old Ross broiler chicken. 100 µg muscle tissue was added to 1000 µl radioimmunoprecipitaion (RIPA) buffer with 10 µl Protease Inhibitor Cocktail (Sigma) and PMSF added (Ferguson *et. al.,* 1994) and incubated on ice for 30 minutes. After 10 minutes centrifugation at 15000 x g supernatant was saved and quantified with 2D Quant Kit (Amersham Biosciences). The protein lysate was tested for a positive reaction with mAb GIIF3 with Western blot analysis.

Immunoprecipitation

100 µg of the protein lysate was precleared with 50 µl protein G-agarose micro bead slurry (Sigma). The GIIF3 antibody (100 µg) was added and incubated 2 hours at room temperature, and then 50 µl protein G-agarose beads were added and incubated overnight. After 2 minutes centrifugation at 12000xG supernatant was discarded and precipitated protein was released with reducing sample preparation buffer at 95^{o}C.

Mass Spectrometry

At least 20 µg of immunoprecipitated protein was resolved on a 10% SDS-PAGE gel and the protein band of 42 kDa was excised for in gel trypsin digestion as described (Collier *et. al.,* 2006). Tryptic peptides were desalted with a MicroTrap reverse-phase

HPLC column for evaluation with 1D-LC-MS/MS at the Mississippi State University Life Sciences and Biotechnology Institute.

Cloning and Recombinant Protein Production

Based on NCBI chicken smooth muscle gamma actin sequence (NCBI Genbank AF012348), PCR primers were designed to amplify the gene from bursal cDNA using the primer pair:

forward: 5'-CGGATCCGATGTGCGAGGAGGGACCCC-3'
(underlined: inserted BamHI restriction site)

reverse: 5'ACTCGAGTTAGAAGCACTTTCGGTGGAC-3'
(underlined: inserted XhoI restriction site)

The PCR product was ligated into PCR TOPO 2.1 vector (Invitrogen) and transfected into chemically competent *E. coli* K-12 cells. Plasmids were isolated with Qiaprep Spin Miniprep kit (Qiagen), and cycle sequencing was performed using Big Dye Terminating kit (Applied Biosystem). The sequencing reaction products were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosciences). The nucleotide sequence data were analyzed with CLUSTAL 2.0.5 multiple sequence alignment program (www.clustal.org). Plasmid DNA from clones with lowest number of PCR-induced mutations was selected and restriction digested with BamHI and XhoI restriction enzymes and then ligated into the pET-32b(+) prokaryotic expression vector (Novagen) with Fast-Link ligation kit

(Novagen). Purified plasmid was used to transform recipient *E. coli* BL21(DE3)pLysS cells (Novagen). Bacteria were grown in LB broth with Ampicillin (50μg/ml) and Kanamycin (50μg/ml) at 37°C until reaching $O.D._{600}$ = 0.8. Protein expression was initiated with 3 μM IPTG and further incubated for 3 hours. Bacteria were collected with centrifugation and lysed with sonication in PBS with 1μl/ml, bensonase and Protease Inhibitor Cocktail (Sigma) added.

Western Blotting

5μg of each protein sample was run on 10% SDS-PAGE gels at 100V for 1.5 hours and transferred to nitrocellulose membrane as described previously (Wan et. al., 2004). Blots were blocked in TBS with 5% nonfat dry milk and 5% Tween20 (Sigma). Two step staining was performed using GIIF3 supernatant as primary and anti-mouse-IgG-AP conjugate (Southern Biotech) as secondary antibody. Signal was visualized with NBT/BCIP alkaline phophatase substrate (Sigma).

Results

Mass Spectrometry

Mass spectrometry analysis identified 10 separate peptides that matched with chicken actin proteins in the non-redundant protein database (Table 4.). Each peptide was considered genuine based on Delta Cn values >0.1, the presence of a C-terminal lysine or arginine, and the fact that multiple copies of each peptide was identified in the sample.

To determine the actin specificity of the peptides, each peptide was aligned with chicken smooth muscle and skeletal muscle α-actin isoforms, and the non-muscle β- and γ-actin isoforms (Kovacs and Zimmer, 1993). The location of the peptides within the actin proteins is shown underlined in Figure 4 and the corresponding amino acid position is listed in Table 4. The sequence of six of the peptides could be found in all three actin isoforms, whereas two peptides show specificity for only the α- or γ-actin isoforms. However, the β-actin - specific peptide GYSFTTTAER (amino acid positions 199-208) differs from peptide GYSFVTTAER by a conservative change from a threonine to a valine at amino acid position 200 (Table 4 and Fig. 4). The peptide PEYDEAGPSIVHR (amino acid positions 362-374) is found only in the γ-actin isoform sequence. Therefore, the mass spectrometry analysis of the immunoprecipitated protein suggests that mAb GIIF3 recognizes an actin-specific epitope expressed within the β- and γ-actin isoforms.

In previous studies GIIF3 was compared to commercially available alpha and beta actin antibodies on histological sections. As the staining patterns were different, alpha and beta actin were not considered to be candidates for GIIF3 antigen. Therefore, to

test the hypothesis that GIIF3 monoclonal antibody recognizes gamma actin, we cloned and expressed the smooth muscle gamma actin gene. The gamma actin gene that gave the most peptides with the mass spectrometry analysis is listed under the accession number AF012348 in the NCBI Genbank database (Kovacs et al., 1998).

Sequence Analysis of Cloned Gamma-Actin Gene

The complete sequence of the cloned smooth muscle gamma-actin cDNA was constructed from the use of internal primers. The 1151 bp ORF was aligned with reference chicken gamma-actin sequence (Fig. 5). The selected clone showed 4 mismatches compared to the reference sequence (Kovacs et al., 1998). The low number of PCR induced mismatches enabled this clone to be introduced into a prokaryotic expression vector to produce recombinant protein.

Western Blotting

The recombinant smooth muscle gamma actin protein was tested with mAb GIIF3 (Fig. 6). A positive signal was observed at 66 kDa indicating that the gene was expressed successfully. The pET32b(+) vector codes a thioredoxin protein with 23 kDa molecular weight, which would result in an increase over the calculated 42 kDa weight of the gamma actin protein. The Western blot analysis confirmed that the antigen reacting with mAb GIIF3 is smooth muscle gamma actin.

Discussion

The present study showed that antigen recognized by mAb GIIF3 is encoded by the chicken actin gene. Previous studies by North and Coworkers (1994) investigated actin isoform expression in the chicken gizzard. In gizzard smooth muscle the β-actin fibrils are linked together by α-actinin and therefore represent a portion of the cytoskeleton. On the other hand, γ-actin is associated with the contractile myosin filaments of the muscle cell. The α-actin isoforms are not expressed in the gizzard (North *et. al.,* 1994). Therefore, with relevance to our study the six peptides found in regions conserved within all three actin isoforms most likely originated from the gizzard β- and γ-actin isoforms (Table 4 and Fig. 4). Future studies will require testing of recombinant protein derived from the α- and β-actin isoforms to confirm and extend the present results. The role the smooth muscle gamma actin plays in BSDC needs to be clarified, it is expectedly associated with cell migration and cell polarization, as the BSDC is considered to be a migratory cell type. In summary, the mAb GIIF3 represents a new marker in avian research that can be utilized in research focused on embryonic muscle cell development. Additionally, the staining of the FAE of the bursal follicle by GIIF3 (Nagy *et. al.,* 2001a, b) suggests that the cytoskeletal β- and γ-actin isoforms may be important in FAE function involving endocytosis and vesicle trafficking (Yarar *et. al.,* 2005).

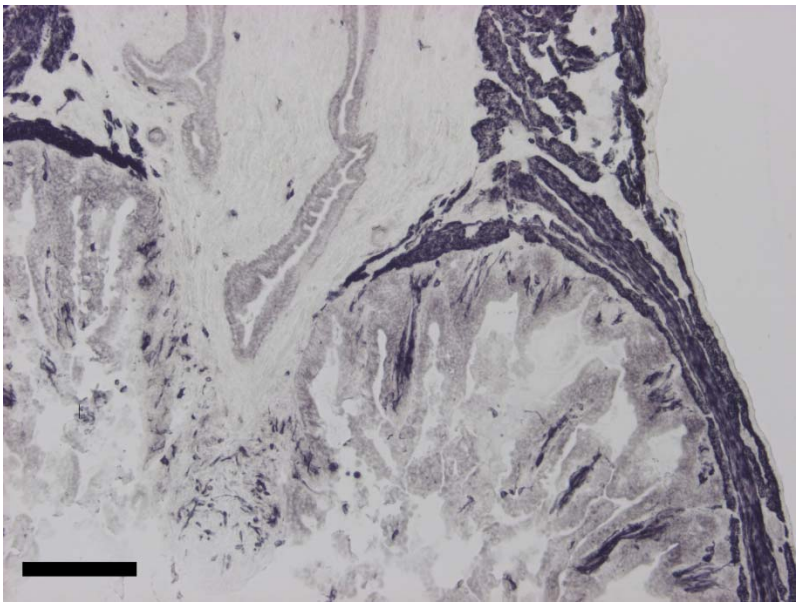

Figure 3. Immunohistochemical staining with GIIF3 monoclonal antibody on chicken small intestine at ED18. The antibody strongly stains horizontal and diagonal muscle layers in the wall of the organ, and outlines elements of the circulatory system that contain smooth muscle cells. (Bar represents 300 μm).

Table 4. Actin-specific peptides identified with mass spectrometry.

Peptide	Number	Actin	Amino acid position
DSYVGDEAQSK	10 [a]	α, β, γ [b]	53-64 [c]
DSYVGDEAQSKR	20	α, β, γ	53-64
AGFAGDDAPR	20	α, β, γ	21-30
PEYDEAGPSIVHR	12	γ	362-374
GYSFVTTAER	10	α, γ	199-208
AVFPSIVGRPR	10	α, β, γ	31-41
VAPEEHPTLLTEAPLNPK	10	α, γ	98-115
AVFPSIVGR	10	α, β, γ	31-39
SYELPDGQVITIGNER	20	α, β, γ	241-216
GYSFTTTAER	10	β	199-208

[a] Number of times the peptide was identified.
[b] Chicken actin proteins identified with the peptides.
[c] Location of the peptide sequence within the actin proteins aligned in Figure 1.

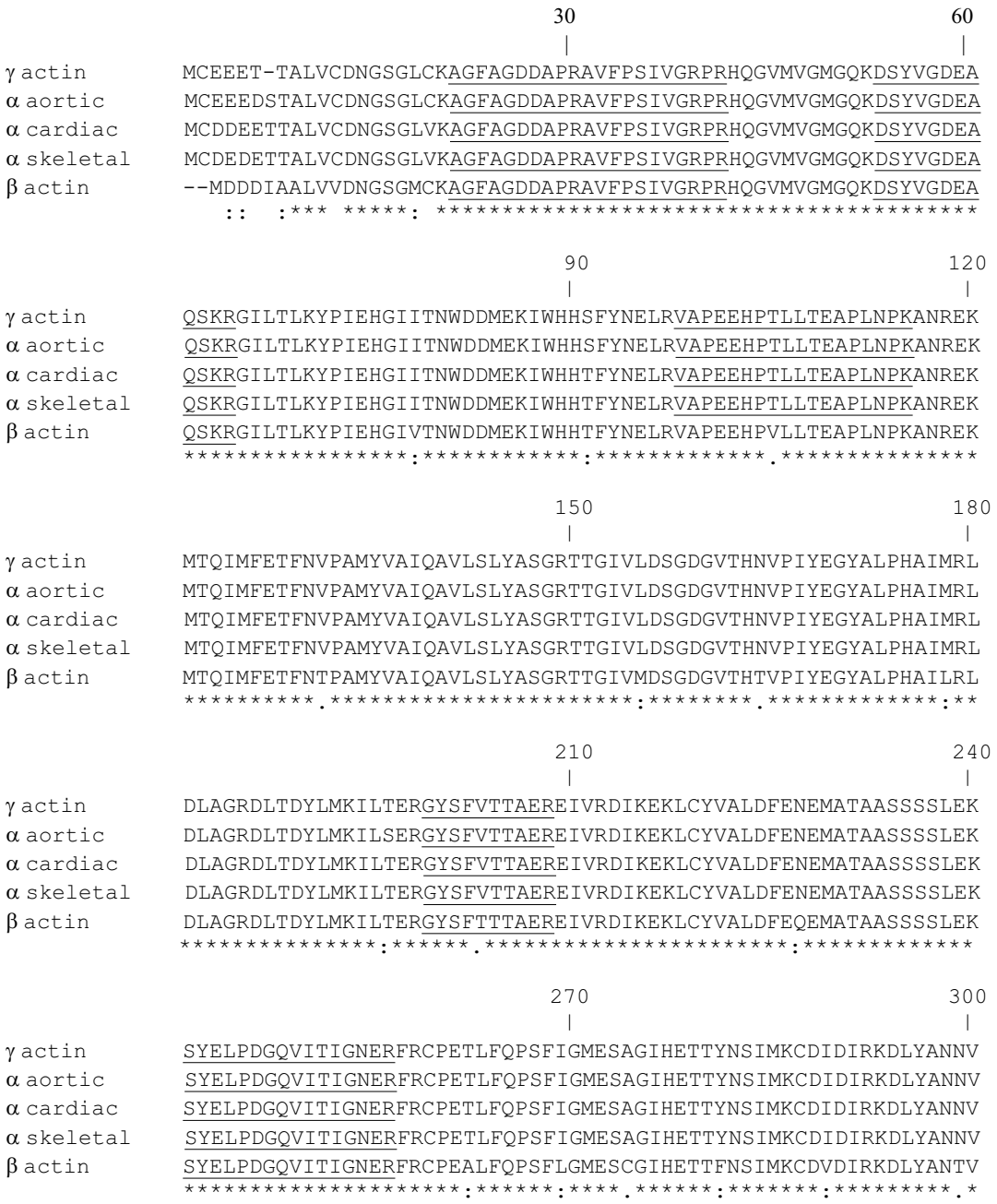

Figure 4. Alignment of chicken actin proteins and location of the peptides identified with mass spectrometry. Identified peptides underlined.

```
                                        330                           360
                                         |                             |
γ actin          LSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWIS
α aortic         LSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWIS
α cardiac        LSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWIS
α skeletal       MSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWIT
β actin          LSGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWIS
                 :************************************************************:

                                 377
                                  |
γ actin          KPEYDEAGPSIVHRKCF
α aortic         KQEYDEAGPSIVHRKCF
α cardiac        KQEYDEAGPSIVHRKCF
α skeletal       KQEYDEAGPSIVHRKCF
β actin          KQEYDESGPSIVHRKCF
                 * ****:**********
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Figure 4. Continued.

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Chicken_Gamma_Actin     ATGTGCGAGGAGGAGACCACCGCCCTGGTCTGCGACAACGGCTCCGGGCTCTGCAAAGCC 60
Cloned_Sequence         ATGTGCGAGGAGGAGACCACCGCCCTGGTCTGCGACAATGGCTCCGGGCTCTGCAAAGCC 60
                        ************************************ ********************

Chicken_Gamma_Actin     GGCTTTGCTGGTGACGATGCCCCCCGCGCCGTCTTCCCCTCCATCGTGGGGCGGCCCCGG 120
Cloned_Sequence         GGCTTTGCTGGTGACGATGCCCCCCGCGCCGTCTTCCCCTCCATCGTGGGGCGGCCCCGG 120
                        ************************************************************

Chicken_Gamma_Actin     CACCAGGGCGTCATGGTAGGCATGGGGCAGAAAGACAGCTACGTGGGCGATGAGGCGCAG 180
Cloned_Sequence         CACCAGGGCGTCATGGTAGGCATGGGGCAGAAAGACAGCTACGTGGGCGACGAGGCGCAG 180
                        ************************************************* *********

Chicken_Gamma_Actin     AGCAAGAGGGGTATCCTCACACTCAAGTACCCCATCGAGCACGGCATCATCACCAACTGG 240
Cloned_Sequence         AGCAAGAGGGGTATCCTCACACTCAAGTACCCCATCGAGCACGGCATCATCACCAACTGG 240
                        ************************************************************

Chicken_Gamma_Actin     GATGACATGGAGAAGATCTGGCACCACTCCTTCTACAACGAGCTGCGCGTGGCCCCCGAG 300
Cloned_Sequence         GATGACATGGAGAAGATCTGGCACCACTCCTTCTACAACGAGCTGCGCGTGGCCCCCGAG 300
                        ************************************************************

Chicken_Gamma_Actin     GAGCACCCCACGCTGCTGACCGAGGCACCGCTCAACCCCAAAGCCAACCGTGAGAAGATG 360
Cloned_Sequence         GAGCACCCCACGCTGCTGACCGAGGCACCGCTCAACCCCAAAGCCAACCGTGAGAAGATG 360
                        ************************************************************

Chicken_Gamma_Actin     ACCCAGATCATGTTCGAGACCTTCAACGTCCCGGCCATGTACGTCGCCATCCAGGCCGTG 420
Cloned_Sequence         ACCCAGATCATGTTCGAGACCTTCAACGTCCCGGCCATGTACGTCGCCATCCAGGCCGTG 420
                        ************************************************************

Chicken_Gamma_Actin     CTCTCCCTTTACGCATCCGGCCGCACCACCGGCATCGTTCTTGACTCTGGGGACGGCGTC 480
Cloned_Sequence         CTCTCCCTTTACGCGTCCGGCCGCACCACCGGCATCGTTCTTGACTCTGGGGACGGCGTC 480
                        ************* ************************************************

Chicken_Gamma_Actin     ACCCACAACGTGCCCATCTACGAGGGCTACGCTCTGCCCCACGCCATCATGCGTCTGGAC 540
Cloned_Sequence         ACCCACAACGTGCCCATCTACGAGGGCTACGCTCTGCCCCACGCCATCATGCGTCTGGAC 540
                        ************************************************************

Chicken_Gamma_Actin     TTGGCCGGCCGCGACCTCACTGACTACCTCATGAAGATCCTCACCGAGAGGGGCTACTCC 600
Cloned_Sequence         TTGGCCGGCCGCGACCTCACTGACTACCTCATGAAGATCCTCACCGAGAGGGGCTACTCC 600
                        ************************************************************

Chicken_Gamma_Actin     TTCGTCACCACCGCCGAGCGGGAAATCGTGCGCGACATCAAGGAGAAGCTCTGCTACGTG 660
Cloned_Sequence         TTCGTCACCACCGCCGAGCGGGAAATCGTGCGCGACATCAAGGAGAAGCTCTGCTACGTG 660
                        ************************************************************

Chicken_Gamma_Actin     GCCCTGGACTTCGAGAACGAGATGGCCACAGCGGCCTCCTCCTCCTCCCTGGAGAAGAGC 720
Cloned_Sequence         GCCCTGGACTTCGAGAACGAGATGGCCACGGCCGCCTCCTCCTCCCTGGAGAAGAGC 720
                        ***************************** ** ****************************

Chicken_Gamma_Actin     TACGAGCTGCCCGATGGGCAGGTCATCACCATCGGCAATGAGCGCTTCCGCTGCCCCGAG 780
Cloned_Sequence         TACGAGCTGCCCGATGGGCAGGTCATCACCATCGGCAATGAGCGCTTCCGCTGCCCCGAG 780
                        ************************************************************

Chicken_Gamma_Actin     ACCCTCTTCCAGCCCTCATTTATCGGCATGGAATCTGCCGGCATCCACGAGACCACCTAC 840
Cloned_Sequence         ACCCTCTTCCAGCCCTCATTTATCGGCATGGAATCTGCCGGCATCCACGAGACCACCTAC 840
                        ************************************************************

Chicken_Gamma_Actin     AACTCCATCATGAAGTGCGACATCGACATCCGTAAGGATCTGTATGCCAACAACGTGCTG 900
Cloned_Sequence         AACTCCATCATGAAGTGCGACATCGACATCCGTAAGGATCTGTATGCCAACAACGTGCTG 900
                        ************************************************************

Chicken_Gamma_Actin     TCCGGAGGCACCACCATGTACCCCGGCATCGCCGACCGCATGCAGAAGGAAATCACAGCG 960
Cloned_Sequence         TCCGGAGGCACCACCATGTACCCCGGCATCGCCGACCGCATGCAGAAGGAAATCACAGCG 960
                        ************************************************************

Chicken_Gamma_Actin     CTGGCCCCCAGCACCATGAAGATCAAAATCATCGCCCCGCCAGAGCGGAAGTACTCGGTG 1020
Cloned_Sequence         CTGGCCCCCAGCACCATGAAGATCAAAATCATCGCCCCGCCAGAGCGGAAGTACTCGGTG 1020
                        ************************************************************

Chicken_Gamma_Actin     TGGATCGGCGGCTCCATCCTGGCGTCCCTCTCCACCTTCCAGCAGATGTGGATCAGCAAA 1080
Cloned_Sequence         TGGATCGGCGGCTCCATCCTGGCGTCCCTCTCCACCTTCCAGCAGATGTGGATCAGCAAA 1080
                        ************************************************************

Chicken_Gamma_Actin     CCCGAGTACGACGAGGCCGGGCCCTCCATCGTCCACCGAAAGTGCTTCTAA 1131
Cloned_Sequence         CCCGAGTACGACGAGGCCGGGCCCTCCATCGTCCACCGAAAGTGCTTCTAA 1131
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Figure 5. Sequence alignment of cloned smooth muscle gamma actin reference sequence and cloned product. Identical bases are marked with asterisk. The cloned product contains four PCR induced mutations compared to the NCBI reference sequence

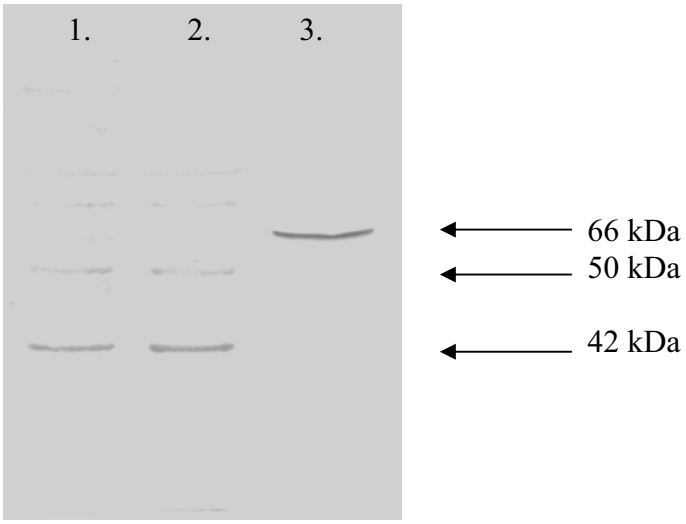

Figure 6. Western blot analysis of recombinant smooth muscle gamma actin protein. ED18 and D42 post hatch gizzard samples (lane 1 and 2) are used as positive control. Recombinant protein sample at lane 3 shows a positive signal at 66 kDa, that was expected as a combined molecular weight of cloned gene sequence and thioredoxin fusion protein from the pET32b(+) vector.

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CHAPTER IV

CONCLUSION

Although the B lymphocyte concept was developed based on avian research, today the mammalian (human and mouse) systems are much better understood. The major reason the research suffers a setback is that several important techniques were not well adapted to avian system. The methods of genetic engineering, such as transgenic technology still present problems in poultry research due to technical difficulties. Also cell cultures of chicken lymphocytes are very difficult to maintain, and results were many times inconclusive. Only one cell line representing a developing bursal B cell is available (DT40), while in mammals most developmental stages of B and T lymphocytes as well as dendritic cells are represented in commercially available cell lines allowing the widespread use of *in vitro* techniques. As a result, large gaps in knowledge persist in understanding immunological functions in avian system. Recently the sequencing of the chicken genome opened new perspectives, and gave us new powerful research tools that were not available earlier. The annotation of sequences and the swift expansion of database resources make available detailed analysis of gene expression profiles in selected cell types.

The objective of Chapter II was to provide an insight into bursal B cell development, focusing on two important cell stages, the bursal stem cell ($SLEX^+$), and

the stage undergoing Ig-gene conversion (LEX^+). The two stages represent very different phenotypes, as well as potentials in humoral immune response. When the bursa is removed from the animals, B cells are only able to develop until bursal stem cell stage resulting in agammaglobulinemia. The organism will only able to produce low affinity IgM type antibodies with a very low repertoire, resulting in an ineffective humoral immune response. Only cells that are activated to the onset of gene conversion are able to develop into fully functional B cells. The characterization of these two cell types was expected to give us information about the microenvironmental signals that are playing a role in developmental processes when gene conversion is initiated. Mass spectrometry was an ideal tool to obtain a list of expressed genes in the interested cell stages. The study resulted in sufficient number of proteins to identify functional groups among them. The data predicted different signal transduction pathways in both ED15 (bursal stem cell stage) and ED18 (onset of gene conversion stage), that provide possible explanations for previously observed phenotypic and functional differences. The identification of signal transduction pathway member genes allows us to create models for further research.

Chapter III was a different approach to identify factors that might play a role in B cell – dendritic cell communication. The antigen, recognized by GIIF3 monoclonal supernatant was a promising candidate for that, as previous research showed its time limited expression in chicken bursa. The observation that the antigen is only expressed by BSDCs in the bursa during the late embryonic period, and down regulated after hatching prompted us to speculate that the GIIF3 monoclonal antibody recognized a BSDC factor that may be associated with B cell development. The results showed that the

antigen, recognized by the GIIF3 monoclonal antibody was smooth muscle gamma actin, a cytoplasmic protein that is probably not involved in microenvironmental regulation processes as expected earlier. However this is the first study to demonstrate BSDCs to express gamma actin and opened new perspectives in the investigation what role the time regulated expression of the gene plays in dendritic cell physiology. The GIIF3 antibody will be a potent tool in future research on bursal processes, as well as muscle development.