Identification of the *LB-FABP* promoter as a liver specific promoter via the generation of transgenic quail expressing eGFP within their liver cells.

THESIS

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By

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Abstract

The liver is a multifunctional organ found in vertebrates. The identification of liver specific genes and their promoters are valuable for delineating the functional roles of specific target genes in genetically engineered animals. In the present study, through the comparison of gene expression in different tissues by microarray analysis, the liver specificity of liver basic fatty acid binding protein (LB-FABP) expression was identified in chickens. Reverse transcription-PCR (RT-PCR) further confirmed the liver specificity of *LB-FABP*. An analysis of *cis*-acting elements in chicken, turkey, brown kiwi, and zebra finch *LB-FABP* promoter regions revealed conserved binding sites within a 2.1 kb promoter region for transcription factors hepatocyte nuclear factor 1 (HNF1), hepatocyte nuclear factor 6 (HNF6), and CCAAT-enhancer-binding proteins (C/EBP). A lentiviral vector containing the 2.1kb *LB-FABP* promoter and the *eGFP* gene was constructed. A packaging cell line derived from human embryonal kidney cells (293FT cells), were used to package the lentiviral particles. The lentivirus was then tested to ensure its functionality using HepG2 cells (hepatoblastoma cell line) prior to microinjection into fertilized quail eggs. While over 270 birds were screened from seven G0 (generation zero) lines, no transgenic offspring were identified from the chimeric founders. In conclusion, microarray data and RT-PCR confirmed the liver specificity of *LB-FABP*. However, further studies are needed to confirm the functionality of the constructed vector *in vivo*.

Similarly, the comparison of gene expression in different tissues by analysis of a microarray database, the intestinal specificity of mucin 2 (MUC2) expression was identified in mice and humans, and further confirmed in chickens by RT-PCR (reverse transcription-PCR) analysis. An analysis of cis-acting elements in avian *MUC2* gene

promoters revealed conservation of binding sites, within a 2.9 kb proximal promoter region, for transcription factors such as caudal type homeobox 2 (CDX2), GATA binding protein 4 (GATA4), hepatocyte nuclear factor 4 α (HNF4A), and transcription factor 4 (TCF4) that are important for maintaining intestinal homeostasis and functional integrity. By generating transgenic quail, we demonstrated that the 2.9 kb chicken *MUC2* promoter could drive green fluorescent protein (GFP) reporter expression exclusively in the small intestine, large intestine, and ceca. Fluorescence image analysis further revealed GFP expression in intestine epithelial cells. The GFP expression was barely detectable in the embryonic intestine but increased during post-hatch development. The spatiotemporal expression pattern of the reporter gene confirmed that the 2.9 kb *MUC2* promoter could retain the regulatory element to drive expression of target genes in intestinal tissues after hatching. This new transgene expression system, using the *MUC2* promoter, will provide a new method of overexpressing target genes to study gene function in the avian intestine.

Dedication

To my friends, family, and students at The Ohio State University

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Field of Study

Major Field: Animal Sciences

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List of Abbreviations

aFABP:	Adipocyte fatty acid binding protein
CDX2:	Caudal type homeobox 2
C/EBP:	Ccaat-enhancer-binding proteins
GATA4:	GATA binding protein 4
GEO:	Gene Expression Omnibus
GDS:	GEO DataSet
eGFP:	Enhanced green fluorescence protein
HNF1:	Hepatocyte nuclear factor 1
HNF4A:	Hepatocyte nuclear factor 4 alpha
HNF6:	Hepatocyte nuclear factor 6
I-FABP:	Intestinal fatty acid binding protein
LBFABP:	Liver basic fatty acid binding protein
MUC2:	Mucin 2
PCR:	Polymerase chain reaction
RPS13:	Ribosomal protein S13
RT-PCR:	Reverse transcription Polymerase chain reaction
TCF4:	Transcription factor 4

SV40: Simian virus 40

ESC: Embryonic stem cells

Chapter 1: Aims

1.1 Aims

Aim 1: To confirm liver specificity of the *LB-FABP* promoter.

Objective 1. Utilize microarray data and RT-PCR to confirm tissue specificity of *LB-FABP*.

Aim 2: To generate transgenic quail expressing GFP in liver tissue.

Objective 2. Construct a lentiviral vector with *LB-FABP* promoter and *GFP* gene.

Objective 3. Confirm lentivirus efficiency via cell culture tests.

Aim 3: To confirm the liver specific expression of eGFP within transgenic quail.

Objective 4. Perform Western blot analysis, immunohistochemistry, and gross observations to confirm liver specific eGFP expression.

Chapter 2: Literature Review

2.1. Transgenics

A transgenic organism is one that has had foreign DNA introduced into its genome. The first transgenic animal was generated in 1974, when researchers used microinjection of the simian virus 40 (SV40) to generate transgenic mice (Jaenisch et at., 1974). Since this pioneering work, many target genes have been expressed in a wide variety of transgenic animals through several different methods. Those methods include DNA microinjection, embryonic stem cell-mediated gene transfer, and retrovirus-mediated gene transfer (Wheeler, 2003).

This technology, which has expanded tremendously over the past few decades, has had a major impact in multiple fields of research. The genetic manipulation of animals, and other organisms, has allowed for increased animal production as well a greater understanding of many biological processes, gene functions, growth and development, and diseases (Houdebine, 2005).

2.2. Application of transgenic technology

The application of transgenic technology to animals has the potential to benefit multiple facets of society. The first transgenic farm animals were produced nearly a decade after the first transgenic mouse was generated (Hammer et al., 1985). Transgenic technology can be used to improve animal production by increasing feed efficiency, growth, reproduction of livestock, and more. For example, transgenic pigs overexpressing myostatin pro domain and pigs overexpressing growth hormone have been generated (Whyte and Prather, 2011).

Transgenics also plays a significant role in research. Transgenic zebrafish are commonly used to gain a better understanding of vertebrate development (Pyati et al., 2005; Lin et al., 2005; Higashijima, 2008). Also, transgenic animals are a useful tool in studying human disease. For

example, transgenic mouse models have been used to study Alzheimer's, cardiovascular disease, obesity, and other debilitating illnesses (Carmeliet and Collen., 2000; Elder et al., 2010; Lutz and Woods., 2013). Transgenic technology could further advance human medicine through the generation of bioreactor systems.

Bioreactors are genetically modified animal systems that have the potential to reduce the production cost and improve production efficiency of pharmaceutically relevant recombinant proteins. This has been documented in a wide variety of animal species including goats, cattle, and chickens (Huang et al., 2007; Yang et al., 2008, Table 2). These transgenic animals secrete a target protein into milk, egg whites, blood, or other bodily fluids, which is then purified and ready for human use (Wang et al., 2013). Over the past few decades, avians have become an increasingly desirable animal model for bioreactor production systems because recombinant proteins can be laid down in the egg whites and collected with the egg (Ivarie, 2006).

Additionally, transgenic technology has the potential to allow for the generation of more environmentally friendly animals. One notable success is the Enviropig^{TM}, which was generated at the University of Guelph. These Yorkshire pigs overexpress phytase within their salivary glands, allowing them to better digest plant phosphorus (Forsberg et al., 2013). The smaller phosphorus output could reduce the number of algae blooms due to run off into water sources. Algae blooms negatively impact the fish and other aquatic life within the affected water sources. While many transgenic animal systems have been developed for a wide variety of purposes, very few have gained legislative approval.

2.3. Food and Drug Administration (FDA) approvals

The FDA approved the first transgenic animal system in 2009. A biotherapeutics company engineered transgenic goats, whose milk contained human antithrombin, a 432-amino acid glycoprotein naturally produced in the liver (Sullivan et al., 2014). In 2014, the FDA approved a pharmaceutical protein, produced from transgenic rabbits, that could treat hereditary angioedema (Editorial staff, 2014). In 2015, chickens producing human lysosomal acid lipase within their egg whites were approved by the FDA (Sheridan, 2016). Later that year, the first transgenic animal, the AquaAdvantage® salmon were approved from human consumption (Waltz, 2016). AquaAdvantage® salmon, which contain a gene and promoter from two other fish species (the Chinook salmon and the Ocean Pout) allowing them to grow faster than their wildtype counterparts (Waltz, 2016).

As transgenic technology is being applied to the production of livestock for human consumption, there are a number of ethical and health concerns from the general public. Some common concerns include: risk to human health, accidental gene transfer to non-targeted species, and environmental risks (Levi et al., 2014). This push back from the consumer could lead to a delay or completely stop these products from ever entering the market. This rapidly growing, yet controversial, technology is also commonly applied to avians.

2.4. Avian transgenics

The first recorded transgenic bird was generated in 1986, well after its mammalian counterpart (Salter et al., 1987). The generation of transgenic avian species has been more challenging than the generation of transgenic mammalian species, likely due to avians' unique reproductive systems (Sang, 2004). Many bird species, such as chickens, zebra finch, and Japanese quail, have been used to generate transgenic birds for varying research studies.

Chickens have been used in a wide variety of studies, including the production of pharmaceutically relevant proteins (Table 2). Along with the use of chickens as bioreactors, transgenic chickens have also been generated to increase the health and growth of the birds, with target genes including Leukosis resistance, H5N1 resistance, LacZ, and bovine growth hormone (Forabosco et al., 2013).

Transgenic technology is commonly applied to songbirds, such as the zebra finch, to study communication deficiencies and disorders (Agate et al., 2009; Abe et al., 2015; Tanaka et al., 2016). A 2015 study developed transgenic songbirds expressing the human mutant huntingtin (mHTT) gene. This particular protein is the cause for the neurological issues in Huntington's disease. The researchers found that the transgenic birds had many vocal issues (Liu et al., 2015).

Fluorescently-labeled Japanese quail have been generated to improve research methods for the study of avian embryogenesis, growth, and development (Huss et al., 2015). Transgenic quail, on multiple occasions, have also been used to confirm the tissue specificity of target genes. Both Mucin 2 (MUC2) and RBP7 have had their tissue specificity confirmed via the generation of transgenic Japanese quail (Ahn et al, 2015, Woodfint et at., 2017). In these studies, a lentiviral vector was constructed containing the tissue specific promoter of interest and eGFP (enhanced green fluorescent protein) downstream of the promoter.

2.5. Green Fluorescent Protein (GFP)

GFP is a bioluminescent protein that was first extracted and then purified from the *Aequorea victoria* jellyfish in the early 1970's (Morise et al., 1974). In 1985, an *A. Victoria* cDNA library was used to obtain a portion of the GFP sequence (Prasher, McCann, and Cormier et al., 1985). The structure and full sequence of GFP, however, was not determined until 1992 (Prasher

et al., 1992). GFP has the ability to fluoresce without the aid of cofactors, making it a useful reporter gene (Day and Davidson, 2009). The codon usage has also been changed within the GFP sequence to allow for better expression within mammalian cells. This altered version of GFP has been termed enhanced green fluorescent protein (eGFP) (Tsien, 1999). Many studies have been published using GFP as a reporter, including studies involving transgenic mice, zebrafish, and quail (Mignone et al., 2004; Lin et al., 2005; Scott and Lois, 2005). In these studies, GFP expression was driven by the nestin promoter, CD41 promoter, and human synapsin gene 1 promoter respectfully. Two previous studies from our lab identified liver and intestinal specific genes promoters, where GFP was used as the reporter gene (Ahn et al., 2015, Woodfint et al., 2017).

2.6. Tissue specific genes

Tissue-specific genes are genes whose expression is limited to a particular tissue or few tissue types. Identification of tissue- and developmental stage-specific gene promoters is valuable for delineating the functional roles of specific genes. The specificity of gene expression for tissue- or cell-types and developmental stages is often regulated by promoters containing transcription factor binding sites, enhancers, and chromatin openers, which are all considered cis-acting elements (Marie et al., 1989). Therefore, identification of tissue- and stage-specific genes and characterization of their promoters is important in order to promote transgene expression in a tissue and stage-specific manner in genetically engineered animals.

Microarray data is a common and efficient way to identify candidate genes for tissue specific studies (Song et al., 2013). Tissue specific genes have been identified in bone marrow, muscle, intestine, liver, adipose tissue, reproductive tissue, skin and more, as delineated by the human protein atlas. The tissue specific genes in the Human Protein Atlas project fell under one

of three potential categories. Tissue-enriched (minimum five-fold higher mRNA level in one tissue compared to other tissues), group-enriched (minimum five-fold higher mRNA level in two to seven tissues compared to other tissues), and tissue-enhanced (minimum five-fold higher mRNA level in one tissue compared to average of other tissues). Of the 19,613 genes analyzed in the human genome project, 2,608 genes were categorized as tissue enriched, 1,139 genes were categorized as group enriched, and 4,157 genes were categorized as tissue enhanced. A previous study performed within our lab confirmed the adipose specificity of the *RBP7* gene via the generation of transgenic Japanese quail (Ahn et al., 2015). A similar study identified the intestinal specificity of the chicken *MUC2* promoter (Woodfint et al., 2017).

2.7. Avian intestinal tissue and intestinal specific genes

The gastrointestinal tract of avians is highly unique, consisting of an esophagus, crop, proventricules, gizzard (or ventriculus), small intestine, paired ceca, large intestine, cloaca, and vent. There is a large amount of variation in the gastrointestinal tract of avian species. For example, the ceca and intestinal length tend to be shorter in carnivorous birds, such as hawks, as compared to herbivores and omnivores, such as the chickens (Klasing, 1998). The expression of certain genes are specific to the intestinal tract.

A few intestinal specific genes have been previously used in transgenic studies, such as I-FABP and Villin. I-FABP is expressed in the small and large intestine from the epithelial cells within the mucosal layer of the intestinal tissue (Lau et al., 2016). The promoter of intestinal fatty acid binding protein (I-FABP) has been used to direct the intestinal expression of transgenes such as growth hormone (GH), cystic fibrosis transmembrane conductance regulator (CFTR) genes and to study the development of the zebrafish intestine (Sweetser et al., 1988, Her et al., 2003, Stotz et al., 2013).

A 12.4 kb promoter-enhancer complex or a 9 kb regulatory region of the mouse Villin gene was used to direct expression of transgenes such as reporter genes, the oncogenic K-ras gene, and the Cre recombinase gene in mice to label the intestine, induce intestinal tumorigenesis, and induce gene knockout within the epithelium (McConnell et al., 2001, Janssen et al., 2002, Madison et al., 2002, El Mariou et al., 2004, Nandan et al., 2008,).

Mucin 2

Mucins are a family of glycoproteins that coat epithelial tissue such as digestive tissue, reproductive tissue, and respiratory tissue. Mucins also coat the skin of some amphibians. In coating these epithelial tissues, mucins create barriers and lubricate the tissues. There are a number of different mucins produced in the body of mammals and birds, and these mucins are categorized as either membrane bound or secreted (Perez-Vilar et al., 1999). MUC2 is secreted by goblet cells of the intestine and is a gel-forming protein that aids in the protection of the gastrointestinal tract (Rousseau et al., 2004).

It is postulated that cis-acting elements conserved within the promoter of MUC2 contribute to its intestinal specific expression. This includes cis-acting elements such as caudal type homeobox 2 (CDX2), GATA binding protein 4 (GATA4), hepatocyte nuclear factor 4 α (HNF4A), and transcription factor 4 (TCF4). Previous research has indicated the gut specificity of MUC2 via RT-PCR and the generation of transgenic quail (Woodfint et al., 2017).

Furthermore, expression of MUC2 is low during embryogenesis in both broiler chickens and Pekin ducks (Zang et al., 2015). This was shown to also be true of quail (Woodfint et al., 2017). Post-hatch, MUC2 expression increased in all three species according to the same reports.

Unlike MUC2, Villin is not solely expressed from the goblet cells, but from all cells of the intestinal epithelium. It is also expressed from intestinally associated exocrine glands (Wang et al., 2008). This difference in cell expression of these two transgenic models will allow for different studies to take place, using the promoter that is best suited for the study type. Furthermore, the temporal expression differs between MUC2 and Villin. Western blot analysis indicates that there is little to no MUC2 expression pre-hatch (Woodfint et al., 2017). Conversely, Villin is noted to be one of the first genes active in the mouse embryonic intestine (Madison et al., 2002). The same is true of I-FABP, which is observed 3 days post-fertilization within zebrafish embryos. Identifying a suitable gene to drive the expression of a target gene post-hatch could prove very beneficial. For instance, a high amount of target gene expression during embryogenesis could be lethal to developing chicks. Intestinal specific genes such as MUC2 and its promoter could be used to circumvent such an issue. Other tissues, such as the liver, also have tissue specific gene expression.

2.8. Avian liver tissue and liver specific genes

Similar to mammals, avians have large livers located within the abdominal cavity. The liver is a multifunctional organ supplied with blood through the hepatic portal vein. The many functions of the vertebrate liver include blood detoxification, carbohydrate metabolism, protein synthesis, lipid metabolism, and secretion of bile salts (Fox, 2002). In the case of laying hens, the liver is a source of triacylglycerols found within eggs. This is due to a production shift in hepatocytic lipoproteins caused by estrogen (Walzmen et al., 1999).

Previous studies have been conducted in mice to investigate liver specific expression. Albumin is commonly utilized as a strong liver specific promoter within mammals. (Pinkert et al., 1987; Postic et al, 1999; Yakar et al., 1999; and Lee et al., 2003). In two of these studies, a 2.3-kb albumin promoter was used to overexpress preadipocyte factor-1 fused with human

immunoglobulin-γ and Cre to generate glucokinase knock out mice (Lee et al., 2003, and Postic et al., 1999). A third study utilized an 8.5-kb albumin promoter and albumin-Cre gene to generate insulin-like growth factor 1 knock out mice. Researchers were also able to drive the expression of hepcidin in the liver via the transthyretin promoter (Nicholas et al., 2002). In contrast, albumin has been shown to not be liver specific within avians. As such, the identification of avian liver specific promoters is needed in order to successfully generate transgenic birds overexpressing target proteins within the liver.

Liver basic fatty acid binding protein (LB-FABP)

FABPs, or fatty acid binding proteins, are thought to have evolved and undergone their first duplication approximately 930 million years ago (Zheng et al., 2013). Since then, other duplications have occurred leading to orthologs specific to different tissues. FABPs have been identified in both vertebrate and nonvertebrate animals. Tissues that have a higher amount of fatty acid metabolism tend to have a higher amount of these FABPs, such as muscle and adipose tissue (Storch and McDermott, 2009).

Though there are many identified FABPS, they share similarities structure and the patterning of their sequences (Chmurzyńska, 2006). Typically, an FABP gene contains four exons and three introns, which will be spliced out prior to the RNA being translated into a protein. Also, FABPs are all relatively small at approximately 15 kDa.

LB-FABP, liver basic fatty acid binding protein, is specific to the liver of non-mammalian vertebrates, such as avians. The “basic” aspect of the name refers to the isoelectric point of LB-FABP, which is 9.0 (Nichesola et al., 2004). A 2009 study confirmed the liver specificity of LB-FABP through Northern blot analysis. That study analyzed a chicken tissue distribution which

included brain, heart, lung, proventriculus, gizzard, jejunum, ileum, liver, pancreas, and kidney. A high amount of RNA was detected in the liver sample, and only in the liver sample (Murai et al., 2009).

It has been suggested that LB-FABP should be referred to as FABP10 due to is similarity to zebrafish FABP10 (Hughes and Pointkivska, 2011). Conserved cis acting elements within the chicken, turkey, medium ground finch, and brown kiwi LB-FABP promoter sequences include C/EBP, HNF1, and HNF6 (Figure 3). Two major conserved sites were found between the four sequences, suggesting that these areas are important in the function and tissue specify of the promoter.

2.9. Application of tissue specific transgenic models

Elucidating the tissue specificity of different genes would allow for future studies to gain a better understanding of the physiology, function, and development of individual tissues. Furthermore, the development of transgenic models allows for in vivo studies. Transgenic lines overexpressing enhanced green fluorescent protein (eGFP) could be used as a marker to test for changes in gene expression under varying conditions. Florescent markers could also be used to study embryogenesis if tissue specific genes that are expressed during development are used in similar models. Also, tissue specific promoters could be used to overexpress a wide variety of different proteins to potentially increase production, health, or reduce environmental impact of the animal.

For example, increasing food safety and improving poultry production is of high importance to both consumers and producers. Numerous approaches have been taken in an effort to reduce disease and improve the production of poultry (Vandeplas et al., 2010). As per capita

consumption of poultry continues to increase, it is necessary for the poultry industry to optimize production to accommodate the growing demand, as well as minimize any potential health risks. Currently, genetic modification is being examined as a way to potentially alleviate some of the issues poultry producers face (Van et al., 2008). One such concern to both producers and consumers is contamination of poultry products due to salmonella colonization. If a suitable antimicrobial peptide is identified it could be overexpressed via an intestinal specific promoter, such as MUC2, in an effort to reduce the amount of salmonella colonization within the gut of poultry.

The potential of this model; however, is not solely limited to salmonella reduction. A vast amount of other possible uses for this technology exist. Other peptides could be over expressed within the gut to control feed intake, reduce obesity, regulate hormones and digestion, improve immunology, effect gut motility, or simply gain a better understanding of intestinal tissue both under normal and diseased conditions.

Similarly, the combination of transgenic technology and tissue specific genes could further delineate functions and processes of the liver. The liver is an organ responsible for a large number of functions. These functions include blood detoxification, carbohydrate metabolism, protein synthesis, lipid metabolism, and secretion of bile salts (Fox, 2002). Knowledge of these processes under both homeostasis and diseased conditions would increase our understanding of liver functions from an animal model standpoint and potentially be translatable to human medicine. Equally as interesting would be the overexpression of target proteins within the liver to either improve or alter function. In the case of birds, the liver is a source of triacylglycerols found within eggs. The overexpression of target proteins within the liver via a liver specific promoter, could affect the composition of the egg laid by hens. Overall, there are numerous potential avenues to be

explored with this technology, as such, the identification of tissue specific genes is needed for the continued growth of multiple scientific fields.

Chapter 3: Identification of the *LB-FABP* promoter as a liver specific promoter via the generation of transgenic quail expressing eGFP within their liver cells

Abstract

The liver is a multifunctional organ found in vertebrates. The identification of liver specific genes and their promoters are valuable for delineating the functional roles of specific target genes in genetically engineered animals. In the present study, through the comparison of gene expression in different tissues by microarray analysis, the liver specificity of liver basic fatty acid binding protein (LB-FABP) expression was identified in chickens. Reverse transcription-PCR (RT-PCR) further confirmed the liver specificity of *LB-FABP*. An analysis of *cis*-acting elements in chicken, turkey, brown kiwi, and zebra finch *LB-FABP* promoter regions revealed conserved binding sites within a 2.1 kb promoter region for transcription factors hepatocyte nuclear factor 1 (HNF1), hepatocyte nuclear factor 6 (HNF6), and CCAAT-enhancer-binding proteins (C/EBP). A lentiviral vector containing the 2.1kb *LB-FABP* promoter and the *eGFP* gene was constructed. A packaging cell line derived from human embryonal kidney cells (293FT cells), were used to package the lentiviral particles. The lentivirus was then tested to ensure its functionality using HepG2 cells (hepatoblastoma cell line) prior to microinjection into fertilized quail eggs. While over 270 birds were screened from seven G0 (generation zero) lines, no transgenic offspring were identified from the chimeric founders. In conclusion, microarray data and RT-PCR confirmed the liver specificity of *LB-FABP*. However, further studies are needed to confirm the functionality of the constructed vector *in vivo*.

3.2. Introduction

The identification and characterization of liver specific and developmental stage specific genes have and will continue to advance our understanding of liver physiology, function, and etiology. Such tissue specific gene promoters have the potential to drive the expression of target genes in a tissue specific manner. This method has been proven effective in multiple studies within our laboratory, including studies utilizing the *RBP7* promoter, which is specific to adipose tissue, and the *MUC2* promoter, which is specific to intestinal tissue (Ahn et al., 2015; Woodfint et al., 2017).

Previously, liver specific expression in transgenic animals has been accomplished using the albumin and transthyretin promoter in mice (Pinkert et al., 1987; Nicolas, et al., 2002). However, microarray data indicated that albumin is not liver specific within chicken. As such, a novel promoter needed to be identified to drive the expression of a transgene within transgenic birds. Analysis of chicken microarray data and RT-PCR identified 15 genes specific to the liver of chickens (Figure 2). One of the identified genes was the liver basic fatty acid binding protein (*LB-FABP*). LB-FABP is categorized in the family of FABPs, whose main function is regulating fatty acid transport and uptake. Typically, an *FABP* gene contains four exons and three introns, which will be spliced out prior to the RNA being translated into a protein (Chmurzyńska, 2006). Previous research indicated that *LB-FABP* was liver specific through Northern blot analysis. The same study found decreased expression during embryogenesis, but dramatically increased post-hatch. In terms of diurnal expression, LB-FABP expression was highest at the early period of the light cycles and then decreased towards the dark period. Furthermore, feeding was a vital factor regulating the expression of LB-FABP (Murai et al., 2009). Generation of a transgenic Japanese quail system

would not only confirm the liver specificity of LB-FABP, but also allow for further studies regarding liver function, physiology, and performance.

3.3. Materials and Methods

Animal use and ethics statement

Animal care and use procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University (IS00003354, January 1, 2018, IACUC). Japanese quail (*Coturnix japonica*) were housed in battery cages at The Ohio State University Poultry Facility in Columbus, Ohio. A standard starter or breeder diet and water was provided to the animals *ad libitum*. Chickens (*Gallus gallus*) were housed in individual cages at The Ohio State University Poultry Facility prior to necropsy and tissue collection. Animals were euthanized via CO_2 inhalation followed by cervical dislocation for tissue collection.

Total RNA isolation and microarray analysis

Fat, muscle, heart, lung, liver, kidney, brain, and spleen samples were collected from three birds and pooled by tissue type. Total RNA was extracted with Trizol (Life Technologies Inc., Grand Island, NY) and nanodrop (NanoDrop Technologies, Wilmington, DE) was used to determine the RNA quantity and gel electrophoresis was used to determine RNA quality. Frozen samples were then sent to The Ohio State University Microarray Core Facility for microarray analysis. RNA was reverse transcribed into cDNA and then labeled. cDNAs were hybridized to the Affymetrix GeneChip^{TM} Chicken Genome array chip (Affymetrix, Santa Clara, Ca). This particular gene chip covered 32,773 transcripts corresponding to over 28,000 chicken genes. Data was received as a relative expression of genes. Chicken microarray data was analyzed in order to find the most appropriate candidate gene for the study, as previously described (Ahn et al., 2015).

Total RNA extraction, cDNA synthesis, and RT-PCR

In an effort to further confirm the liver specificity of LB-FABP, fat, thigh muscle, pectoralis muscle, heart, lung, kidney, and liver were collected from 20 day old chickens. Total RNA was isolated using Trizol reagent (Life Technologies) according to the manufacturer's protocol. RNA quality was assessed by gel electrophoresis and quantity was measured using a NanoDrop spectrophotometer (NanoDrop Technologies). cDNA was generated by using 1 μg of total RNA and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with conditions of 65°C for 5 min, 37°C for 52 min, and 70°C for 15 min. cDNA samples were then used to conduct PCR for expression patterns of *LB-FABP* using the following primers: Forward: 5`- ACACTTGGAAAAGAGGCTGACAT-3', Reverse: 5'-TGTCTTTAAACTCTCTTGCTTCTTCTG-3'.

PCR was also performed for 13 other liver specific genes identified by microarray analysis. The conditions for RT-PCR was 95^{o}C for 1 min and 30 s, then 22 – 33 cycles of 94^{o}C for 30 s, 58^{o}C or 59^{o}C (the annealing temperature was dependent on the Tm of the primer set) for 30 s, 58^{o}C for 30 s and 72^{o}C for 30 s, and lastly 72^{o}C for 10 min. For four of the genes, PCR was conducted using 1 μl of cDNA, 3 ul of 10mM deoxynucleotide triphosphate mix (dNTP), 4 ul 10x PCR Rxn Buffer with MgCl2, 1 ul each of the forward and reverse primer, 38.75 ul of distilled water, and 0.25 Hot Start Takara Taq DNA Polymerase (Takara Bio USA, Mountain View, CA, USA). The remaining nine genes were analyzed using 1 ul cDNA, 5 ul 10x PCR Rxn Buffer, 3 ul 50 mM MgCl2, 1 ul 10mM deoxynucleotide triphosphate mix (dNTP), 1 ul each of the forward and reverse primer, 38.75 ul distilled water, and 0.25 ul Platinum DNA Polymerases (Invitrogen). Ribosomal protein S13 (*RPS13*) was used as a housekeeping gene to ensure the equal amount of

cDNA for each sample. Dilution of cDNA occurred in order to make the band brightness between tissues similar.

Analysis of transcription factor binding sites

The MatInspector program (Genomatix Software GmbH, Munich, Germany) was used to analyze potential transcription factor binding sites within the 2.1kb chicken *LB-FABP* promoter (Woodfint, 2017) (Figure 3). The liver-enriched binding sites include HNF1, HNF6, and C/EBP. The UCSC blat program (University of California Santa Cruz, Santa Cruz, Ca) was used to identify conserved sites within four avian LB-FABP sequences (chicken, turkey, brown kiwi, and zebra finch).

Vector construction and production of lentiviral particles

The 2.1 kb sequence of chicken *LB-FABP* gene was amplified from chicken genomic DNA by PCR with a forward primer containing the BamH1 site and seven over hanging nucleotides, 5′-TCAGACTGGATCCTGTATGGAAGAAAACAAGGCAGC -3′, and a reverse primer containing the BamH1 site and seven overhanging nucleotides, 5′-TCAGACTGGATCCTGGAGGATAAGGTGCAGTGTGA -3′, and then cloned into pCR2.1-TOPO vector (Invitrogen). BamH1 restriction enzyme was used to digest the pCR2.1 recombinant vector. The 2.1 kb *LB-FABP* promoter was inserted into the pLTReGW lentiviral vector containing *eGFP*. The resulting vector, pLTeGW-LB-FABP -eGFP, was designed to express eGFP specifically in the liver through the direction of the *LB-FABP* promoter. Co-precipitation of calcium phosphate and the pLT-LB-FABP-eGFP vector was used to produce lentiviral particles. One day prior to the transfection, 293FT cells were plated on 100 mm culture dishes in complete medium consisting of Dulbecco's Modified Eagle Medium (DMEM, Life Technologies Inc.) with

10% fetal bovine serum (FBS, Life Technologies Inc.), 1% penicillin/streptomycin (pen/strep, Life Technologies Inc.), 1 mM MEM sodium pyruvate (Life Technologies Inc.), and 0.1 mM MEM non-essential amino acids (Life Technologies Inc.). Nine μg of pLT-LB-FABP-eGFP, 9 μg of ViraPower Packaging Mix (Life Technologies Inc.), and 87 μL of 2 M calcium solution (Clontech Laboratories Inc., Mountain View, CA, USA) were added to a final volume of 700 μL of sterile H2O (Clontech Laboratories Inc.) to prepare the transfection solution. Then, 700 μL of 2× HEPES-Buffered Saline (HBS) (Clontech Laboratories Inc.) was added dropwise while slowly vortexing the solution. The transfection solution was incubated for 5 min at room temperature and added dropwise to the complete medium. Following 10 h of transfection, the medium was replaced with 5 mL of fresh complete medium. After 48 h, the supernatant was collected and filtered through a 0.22 μm pore-sized filter (Henke-Sass, Wolf GmbH, Tuttlingen, Germany). The supernatant was pelleted via centrifugation at 25,000 rpm for 2 h with an ultracentrifuge (L7-65R, Beckman Coulter, Fullerton, CA, USA). The pellet was then resuspended in Opti-MEM as a 100× concentrated lentiviral particle soup and stored as 40 μL aliquots at −80°C until further use.

Transfection of 293FT cells and infection of HepG2 cells with LB-FABP-GFP construct and viral particles

The vector concentration was measured and concentrated to 1μg/mL before lipofectamine transfection. Human embryonal kidney cells (293FT cells) were plated and grown to approximately 70% confluency. On the day of transfection, 293FT cells were transfected with the pLT-LB-FABP-eGFP vector using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

Prior to injection of lentiviral particles into quail eggs, the efficiency of the particles was tested with HepG2 cells, a human liver carcinoma cell line. HepG2 cells were cultured in three

wells of a 12 well culture plate (Greiner Bio-one, Kremsmünster, Austria). Varying concentrations of pLT-LB-FABP-eGFP virus were added to each plate (2µl, 5µl, and 10µl). Plates were visualized using a florescent microscope 48 hours later. The same protocol was performed within QM7 cells (quail muscle cell line). This was done to determine the ability of the lentiviral particles to drive the expression of GFP in a liver specific manner.

Production of the founder quail

Wild-type Japanese quail eggs were cleaned with 70% ethanol and placed upside down for two hours and then laterally on a tray for approximately 2 h at room temperature. Tweezers were used to create a small window on the side of the egg when the blastoderm had settled. Then, 2 to 3 µL of the concentrated lentivirus was injected into the subgerminal cavity of 103 stage X quail embryos using a microinjection system (Tritech Research, Inc., Los Angeles, CA, USA) under a stereomicroscope (Olympus America Inc., Center Valley, PA, USA) (Ahn et al., 2015; Woodfint et al., 2017). The window was sealed with paraffin film, and the eggs were incubated for 14 days at 37.5°C with 60% relative humidity before being placed in a hatching tray. Seven hatched founder chicks were grown to sexual maturity (approximately six weeks) and mated to wildtype quail.

Mating and selection of transgenic offspring

The seven chimeric founder quail were mated to wildtype quail at sexual maturity. Eggs were collected each week and stored in a cooler (13°C) until incubation (two weeks). Hatchlings were tagged and reared for 14 days to collect feather pulp for genomic DNA extraction. The pulp from one feather was incubated in 300 µL of cell lysis solution (200 mM NaCl, 50 mM Tris-Cl, 10 mM EDTA, 1% SDS, pH 8.0) containing Proteinase K (0.1 mg/mL, Invitrogen) at 55°C for at

least 2 h. Then, 300 μL of Phenol:Chloroform:Isoamyl Alcohol was added to the tube, vortexed, and centrifuged at 13,400 rpm for 2 min to remove the protein. The supernatant was transferred to a new tube and genomic DNA was precipitated by adding 240μl of isopropanol and 80μl of protein precipitate solution, inverting, and centrifuging at 13,400 rpm for 5 min. The pellet was washed with 70% ethanol and centrifuged at 13,400 rpm for 2 min. After drying, TE buffer containing RNase A (10 mg/mL, Qiagen, Valencia, CA, USA) was added to dissolve the pellet. The genomic DNA was utilized for genotyping PCR with two different primer sets in an effort to further confirm a positive or negative result. The forward of the first primer set was located in the rev response element (RRE) of the vector (5'-ACAGAGACAGATCCATTCGATTAGTG-3') and the reverse was located within the *LB-FABP* promoter (5'-AAAGCTCCAAGCCTTGGGA-3'). The forward of the second primer set was located in the *eGFP* (5'-AGAACGGCATCAAGGTGAACTT-3') and the reverse in the woodchuck hepatitis virus post-translational response element (WPRE) (5'-AATCCTGGTTGCTGTCTCTTTATG-3') (Figure 4). Following the identification of a positive transgenic offspring, that offspring would have be retained for breeding. The offspring of the transgenic bird, generation 2 (G2), will be used for the functional study. The amplification conditions for *LB-FABP* were 94°C for 2 min, 40 cycles of 94°C for 40 s, 56°C for 1 min, and 72°C for 1 min, and a final extension time of 10 min at 72°C. Ribosomal protein s13 (*RPS13*) was used as a control. *RPS13* was amplified from each tissue using the primers; RPS13-F, 5′-AAGAAGGCTGTTGCTGTTCG-3′ and RPS13-R, 5′-GGCAGAAGCTGTCGATGATT-3′.

3.4. Results

Microarray analysis

Chicken microarray data was analyzed in order to identify liver specific genes. This was done through comparing gene expression levels between liver and other chicken tissues (brain,

fat, heart, kidney, liver, lung, muscle, and spleen) (Figure 1). *LB-FABP* was identified as d a 35-fold higher expression in chicken liver compared to other tissues. Negligible amounts of expression were also present within brain, fat, heart, kidney, lung, muscle, and spleen tissues. Of the 15 candidate genes (*A2M, ABCB1, COCH, CYP2W2, GFRA3, A2ML2, PIT54, PRPS2, SERPINA5, SLCO1B3, AVBD8,* C8A, and *SULT)* analyzed for this study, *LB-FABP* had the highest level of liver expression.

Reverse transcriptase PCR

RT-PCR further confirmed the liver specificity of *LB-FABP* and other genes thought to be specific to the chicken liver (primer sequences in Table 1). Subcutaneous fat, thigh muscle, pectoralis muscle, heart, kidney, lung, and liver cDNA samples were tested to confirm tissue specificity. Ribosomal protein S13 (*RPS13*) was used as a housekeeping gene to ensure the same amount of cDNA was loaded for each sample. All analyzed genes were confirmed as liver specific, though band intensity varied between genes (Figure 2) indicating different amounts of liver expression.

Promoter analysis

LB-FABP promoter analysis of four distinct species (chicken, turkey, medium ground finch, and brown kiwi) through the MatInspector and University of California Santa Cruz (UCSC) Blat programs identified 2 major conserved sites and the presence of several transcription factor binding sites within a 2.1kb region upstream of the *LB-FABP* gene (Figure 3). Those binding sites include hepatocyte nuclear factor 1 (HNF1), hepatocyte nuclear factor 6 (HNF6), and CCAAT enhancer binding protein (C/EBP). HNF1, HNF6, and C/EBP are known to be liver enriched and crucial factors in the transcriptional regulation of liver specific genes (Schrem et al., 2002). Another gene (Myomesin 3) was found in the same orientation

upstream of the *LB-FABP* promoter, limiting the potential insert size for the lentiviral vector. As such, a 2.1kb *LB-FABP* promoter region was chosen for this study.

Transfection of LB-FABP-GFP construct into 293FT cells

Lipofectamine transfection of the *LB-FABP* construct into 293FT cells led to strong eGFP fluorescence of the cell cultures (Figure 5). This demonstrated the ability and efficiency with which the construct was able to be packaged within the 293FT cells, and also that the eGFP protein was properly fluorescing when transcribed. A lack of fluorescence would indicate an issue with the vector construct.

Infection of LB-FABP-GFP construct viral particles into HepG2 cells

HepG2 cells were infected with varying amounts (2μl, 5μl, and 10μl) of the lentiviral particles at approximately 50% confluency and incubated for 24 hours. The media was then removed and replaced with fresh media. Forty-eight hours later, observations via fluorescent microscopy indicated that the viral particles were able to strongly drive the liver specific expression of eGFP (Figure 6). eGFP signal strength increased with increasing concentration, indicating a dose dependent response to the viral particles.

The same procedure was performed on QM7 cells to confirm the liver specificity of the construct. No fluorescence was observed in the QM7 cultures (not pictured). This indicated that not only was the construct properly packaged in 293FT cells and functional, but also that it drove the expression of eGFP in a tissue specific manner, not ubiquitously.

Genotyping PCR of G0 founder birds

Of the 103 quail eggs injected with the viral particles, 14 hatched, giving a success rate of 13.6%. No positive birds were identified through any of the generated founder lines. Two hundred and seventy-nine birds, offspring of the seven founder quail, have been screened using genotyping

PCR. Birds with any form of non-specific banding or hint of a correct band were confirmed a second time.

3.5. Discussion

This study confirms the liver specificity of *LB-FABP* and 14 other chicken genes through microarray data. LB-FABP and 13 other genes were further confirmed through RT-PCR. Furthermore, cell culture tests confirmed that the lentiviral vector was being packaged and that the viral particles were able to drive the expression of eGFP in a liver specific manner. Despite these encouraging findings, no transgenic birds were identified.

The *LB-FABP* promoter was chosen for this study due to its high liver specificity, as demonstrated by microarray data and RT-PCR, as well as the promoter analysis which revealed several transcription factor binding sites, which are known to be integral in liver development and function. Those transcription factor binding sites included HNF1, HNF6, and C/EBP.

Liver-specific and liver-enriched genes, aside from *LB-FABP*, have also been shown to contain binding sites for HNF1, including: albumin, alpha-fetoprotein, alpha-fibrinogen, beta-fibrinogen, alpha1-antitrypsin, transthyretin, and aldolase B. Both HNF1α and HNF1β are expressed during embryotic development (Hayashi et al., 1999). HNF-6 is liver enriched as well but expressed within endoderm derived tissues. Similar to HNF1, binding sites for HNF6 are located within the transthyretin gene as well as another liver-specific gene, α-FP (Hayashi et al., 1999). Furthermore, HNF-6 is present within the liver during embryogenesis and plays a vital role in liver development (Wang et al., 2016). C/EBPα has been determined to be necessary for liver development and the lack of C/EBPα leads to lethal outcomes as evidenced by a 2001

knock-out study (Linhart et at., 2001). The liver specific aspect of C/EBP has been well documented (Hayashi et al., 1999, Szpirer et al., 1992, Lee et al., 1997, and Drewes et al., 1991).

In addition to these transcription factor binding sites, two conserved areas were identified when the *LB-FABP* promoter was compared between four avian species. Typically, conserved regions are involved in the functionality of the gene and subsequent protein (Stojanovic et al., 1999). As such, it was necessary to identify conserved regions between multiple species and retain those sequences within the promoter sequence used in this study. The LB-FABP vector was also chosen for this study based on the results from the cell culture tests.

The lack of transgenic offspring could be due to a number of reasons. The simplest possibility being that the issue arose due to improper or poor microinjection technique. Microinjection of the virus must occur in the subgerminal cavity of the blastoderm. If the microinjection needle does not fully pierce the blastoderm, the virus will have an opportunity to integrate into the quail genome. Similarly, if the microinjection needle passes completely through the subgerminal cavity the virus will not be deposited in the correct location. Furthermore, an excess amount of virus injected into the subgerminal cavity will cause a lethal disruption of the embryo. Other physical issues that could have arisen include the potential decrease in viral titer due to thawing and refreezing. Previous studies have noted a minimum of 5% decrease to a maximum of 50% decrease in viral titer simply due to thawing and refreezing (Krajden et al.; 1999 and Ugai et al., 2002).

The lack of transgenic birds could also be due to inappropriate integration of the viral particles, or lack thereof. Even if the virus was injected correctly, chimerism is random and does not guarantee integration into the germline, which is needed to pass the gene on to future progeny.

If the virus did integrate into the germline of one of the founder quails, it is likely that low germline transmission efficiency hindered the generation of transgenic quail. At approximately 40 birds screened per line (279 birds screened between seven transgenic lines), there is a possibility that not enough birds were screened per line and that, if screening had continued, a transgenic bird would have been positively identified.

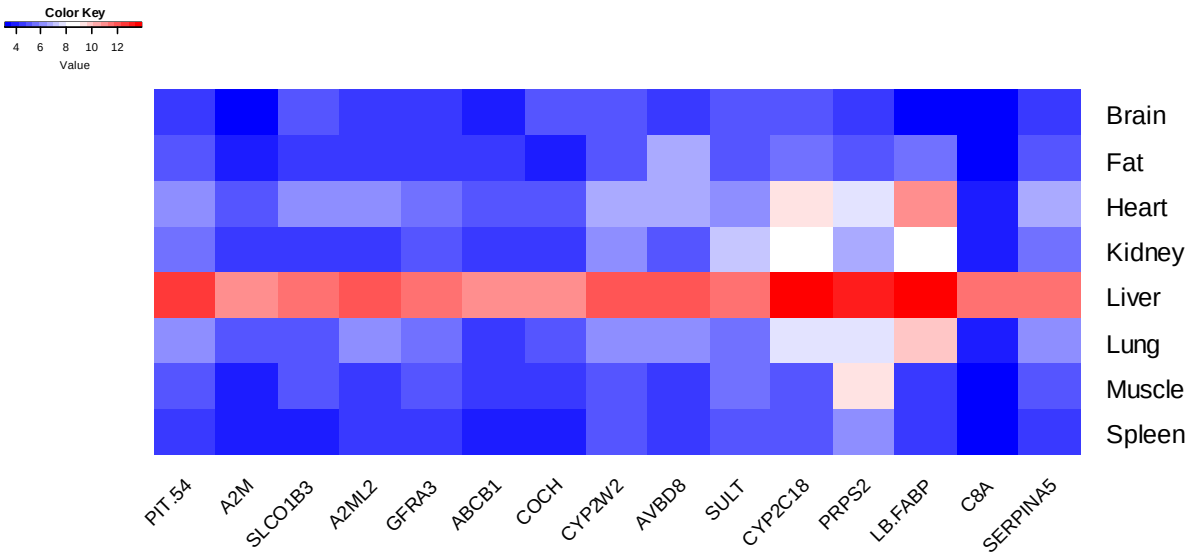
While no transgenic birds were obtained in this study, this model, could be a powerful tool to drive the expression of target genes to study the function and development of the liver, as well as gain a better understanding of the liver under normal and diseased conditions. Future measures to ensure a transgenic line could include injecting a higher number of eggs. This would, potentially, increase the number of birds screened. Also, the sperm of founder males could be tested to determine if they carry the transgene. Blood could also be tested from founder birds to determine if non-germline integration occurred. This would allow us to focus our attention on lines that are most likely to produce positive transgenic birds.

Once transgenic birds are obtained, a functional study should be performed on the G2 generation to further confirm the specificity of LB-FABP. Western blot analysis should be performed on both transgenic and wild type birds' livers to confirm the presence of the eGFP solely within the liver of transgenic birds. Furthermore, western blot analysis on a tissue distribution from a transgenic bird will confirm that the eGFP is not present in tissues other than the liver. Gross analysis of the transgenic liver under ultraviolet light will demonstrate the strength of the eGFP signal. Histology and immunohistochemistry can also be used to confirm liver specificity and the presence of eGFP within the tissue. Fasting and refeeding trials can be done to determine the effects of feeding on the activation of the *LB-FABP* promoter. Finally, *eGFP* can be replaced with a protein that is either agriculturally or medically relevant. This will allow for greater

production from an agricultural standpoint or could be a new model for the study of the liver. In conclusion, the identification of tissue specific genes, as well as their promoters, is integral in gaining a better understanding of tissue and organ physiology, function, and etiology as well as improving food animal production.

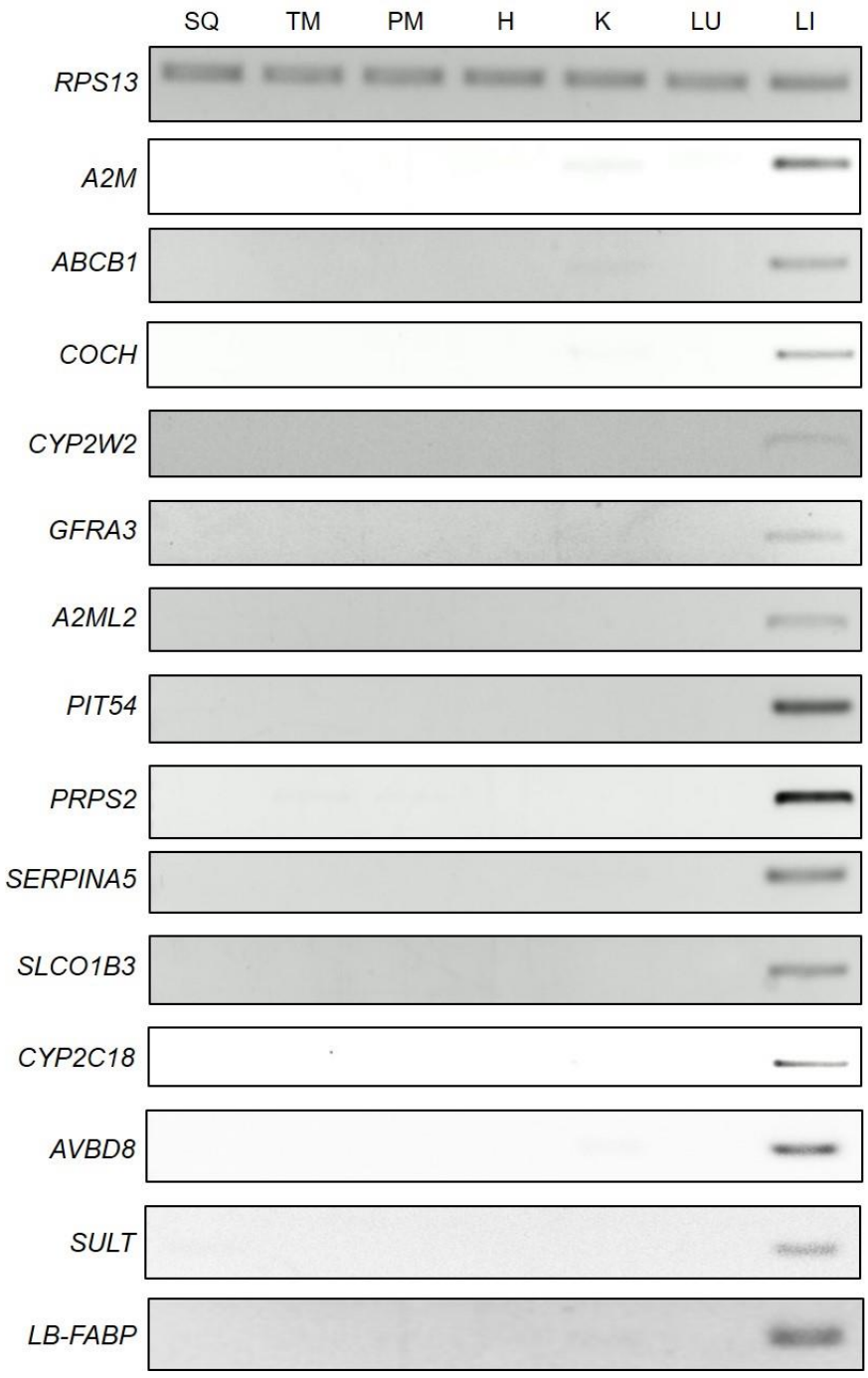
3.6. Figures

Figure 1: Heatmap of liver specific genes



Using the R statistical program, chicken microarray data was transformed into a heatmap representing the relative expression levels of 15 different genes (*PIT54, A2M, SLC01B3, A2ML2, GFRA3, ABCB1, COCH, CYP2W2, AVBD8, SULT, CYP2C18, PRPS2, LB-FABP, C8A,* and *SERPINA5*) in eight different tissues (brain, fat, heart, kidney, liver, lung, muscle, and spleen). Blue indicates a low amount of expression while red indicates a high amount of expression. Microarray data confirmed that all 15 genes were liver specific with *LB-FABP* having the highest amount of expression within the liver.

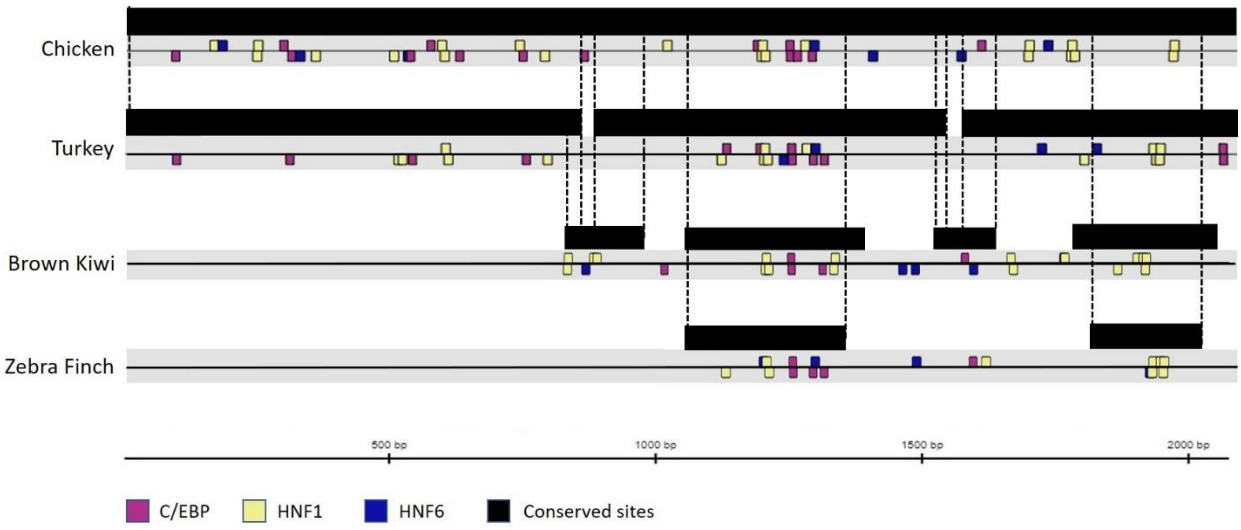
Figure 2: RT-PCR Analysis of Chicken liver specific genes



A tissue distribution of 20 day of chicks was used to confirm the liver specificity of 14 different genes thought to be liver specific, via microarray data results. Tissues include: **Lane1:** Subcutaneous fat (SQ), **Lane 2:** Thigh muscle (TM), **Lane 3:** Pectoralis muscle (PM), **Lane 4:**

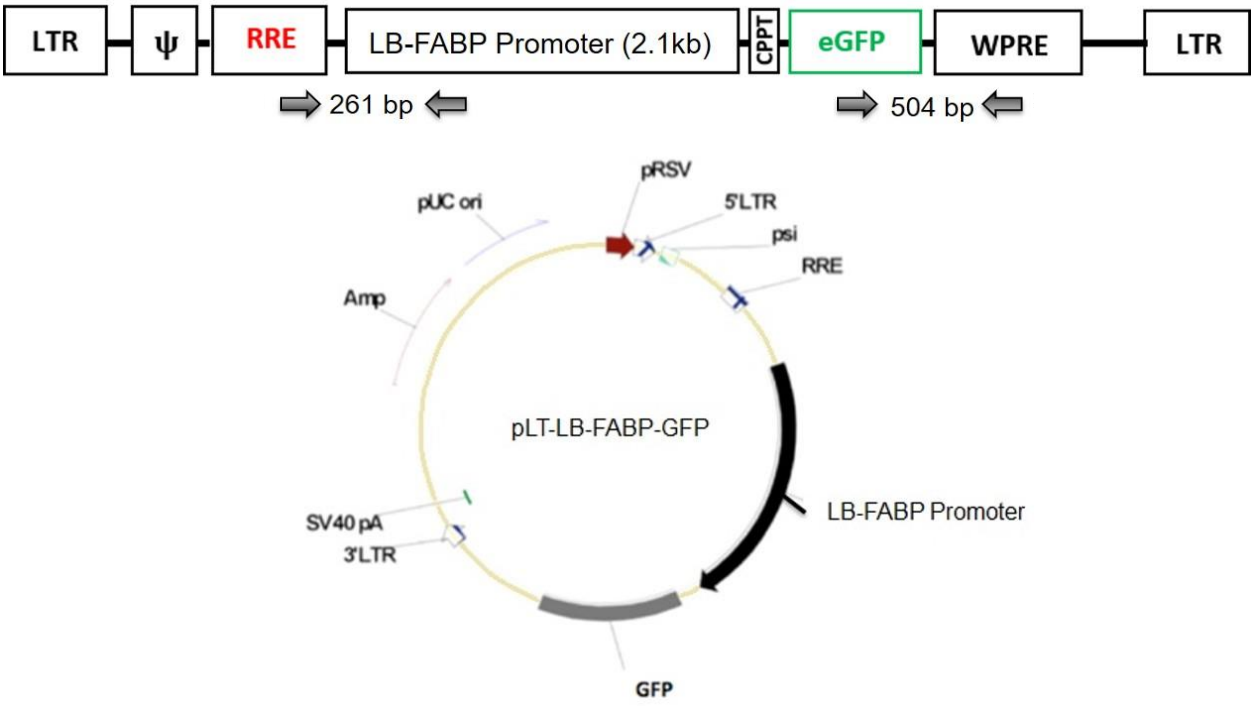
Heart (H), **Lane 5:** Kidney (K), **Lane 6:** Lung (LU), and **Lane 7:** Liver (Li). *RPS13* was used as a housekeeping gene to ensure the same amount of cDNA was loaded for each sample.

Figure 3: *LB-FABP* Promoter analysis



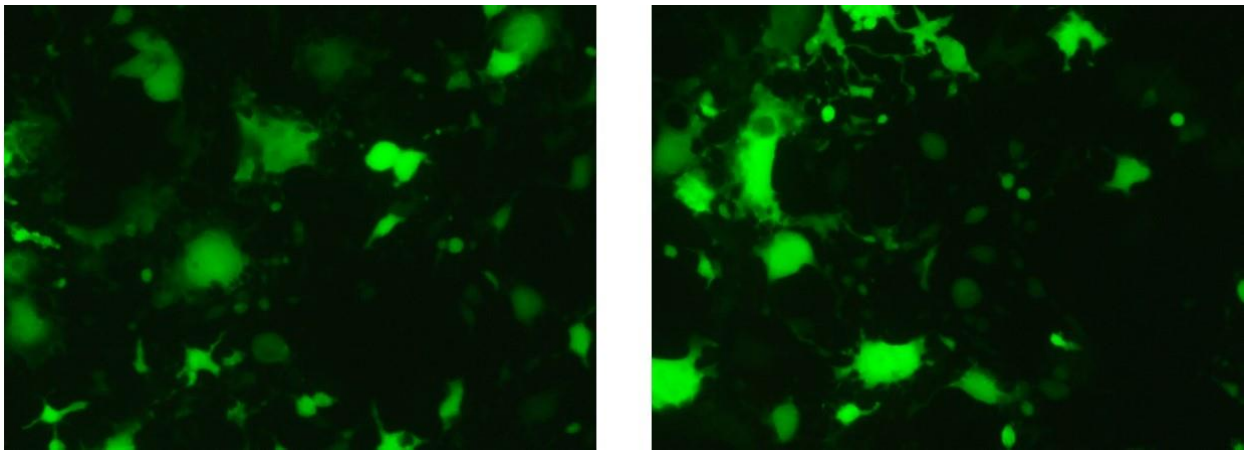
Promoter analysis was performed using the Mat Inspector program. The *LB-FABP* promoter of multiple bird species were analyzed for liver specific transcription factors including C/EBP, HNF1, and HNF6. Black boxes indicate conserved sites within the sequences of all the analyzed species.

Figure 4: *LB-FABP* Construct



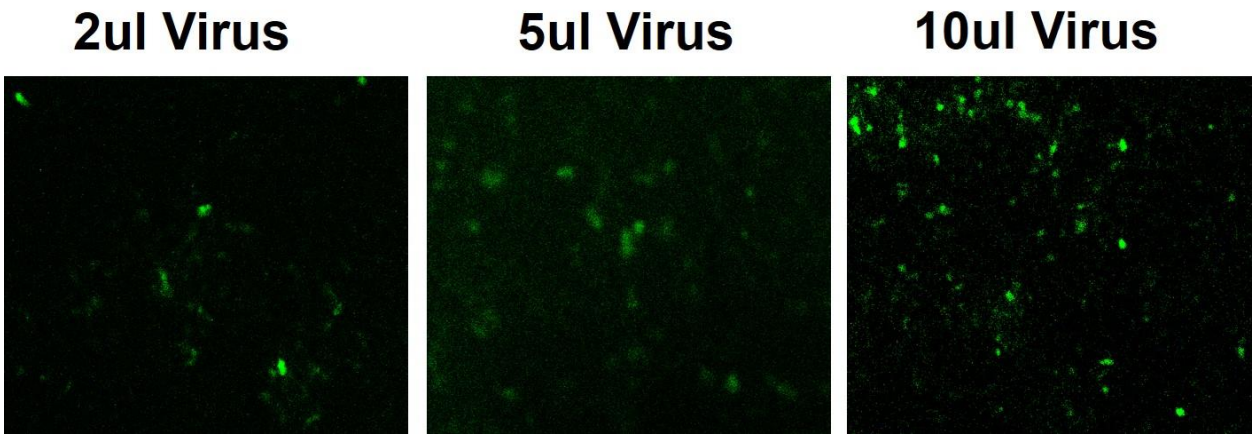
Top: Linearize version of a portion of the lentiviral vector containing the *LB-FABP* Promoter. Arrows indicate the two primer sets used to screen transgenic birds via genotyping PCR. The first set is between the RRE and WPRE with a product size of 494bp and the second set is between the *eGFP* and the WPRE with a product size of 282bp. **Bottom:** Full lentiviral vector containing the *LB-FABP* promoter and *eGFP*.

Figure 5: eGFP expression in 293FT cells



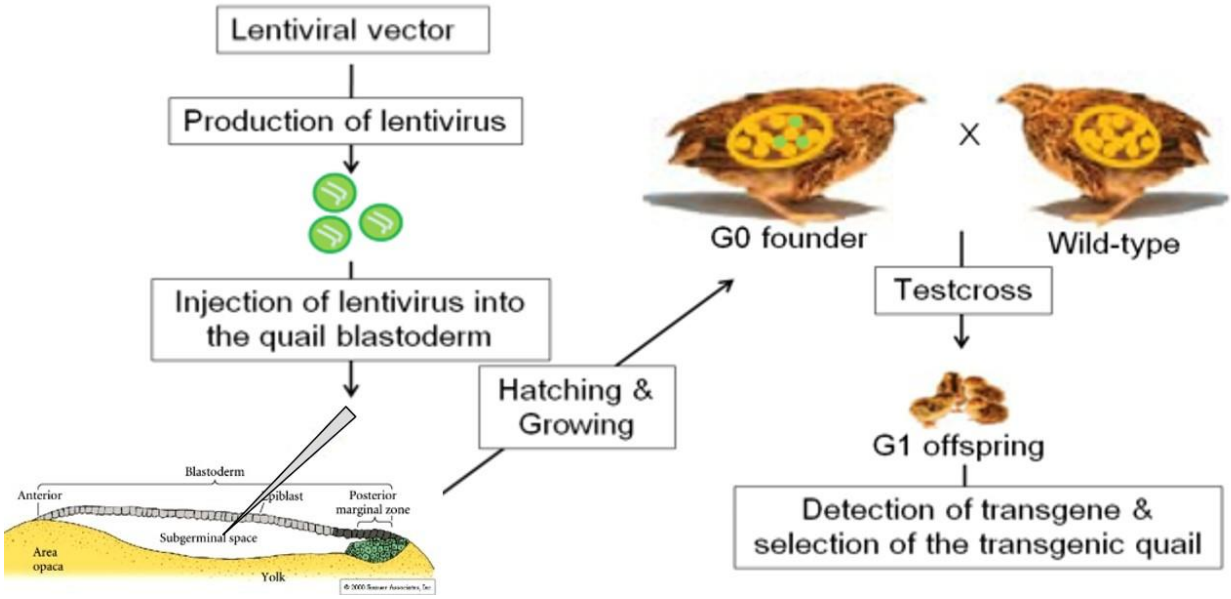
(**Left and Right)** Fluorescent microscopy of 293FT cells transfected, via lipofectamine transfection, with the *LB-FABP* construct. A strong Florescent signal was seen within 293FT cells following transfection.

Figure 6: eGFP expression in HepG2 cells



Fluorescent microscopy of HepG2 cells infected with increasing amounts of virus (2µl, 5µl, and 10µl). The results indicated that the virus was dose dependent.

Figure 7: Generation of Transgenic quail



The above image is a schematic briefly depicting the process for the generation of transgenic quail.

3.7. Tables

Table 1: Primers for liver specific gene RT-PCR

GENE NAME	GENBANK NO.	FORWARD PRIMER SEQUENCES (5′-3′)	REVERSE PRIMER SEQUENCES (5′-3′)
A2M	XM_015292681.1	GAAAATAATTCTGAATGGGATCACCT	TGGAATTTGTGAAAACCTTTAAACC
ABCB1	NM_204894.1	CGTATTGCTATTGCTCGAGCTCT	AGTACTGCCTTTAATTTACATGTTGCA
AVBD8	NM_001001781.1	CTGAATTTGGACATGAAGATCCTTT	CTGAGGTCCTGGCGAACATTA
COCH	NM_204937.1	TTCTTTCACAACCAGAAACGTATTTG	GGTATGAGATTCCCTTGGTTCAGA
CYP2C18	NM_001001757.1	TCCCTTTGCTGTCTCCCATC	TCTCCTGCACAGATGCGTTT
CYP2W2	XM_004945126.2	GCAGAGATTTGTCACCCTTCTG	CAAGGCTTTCTCCAATGCAGT
GFRA3	XM_015294043.1	AGAAGGAGAAGTGTGACCAGATACTTG	CTCCAGAATATACAGAGGAGGCCATA
LB-FABP	NM_204634.1	ACACTTGGAAAAGAGGCTGACAT	TGTCTTTAAACTCTCTTGCTTCTTCTG
LOC427942	XM_004938106.2	GTGGAGGTGGATATGCTGTCAG	TGAACTGCAAGGGGAATTGTATT
PIT54	NM_207180.1	TGGGGTGAACACAACTGCTATC	ACATCTTTGCCATTCATGCAAC
PRPS2	NM_001006264.1	CAGAGTAGCCATTCTTGTCGATGATAT	ATGGCCTCAGCCAGGATCAT
SERPINA5	XM_015287768.1	CAGGAAAGTCCGATCTGAAGGTT	CAGCAAAAATGGACGGTTGAA
SLCO1B3	NM_001318449.1	AGGCTGTATGACTCCAATGCATT	TCAGATGTTCACCATTTTTGCAG
SULT	NM_205060.1	GAGCGTGATAGAGAGGAGCAGTTT	CAGATTGAAGAGCACAGTGGAAA

Chapter 4: Avians as bioreactors

4.1. Introduction

Bioreactors are genetically modified animal systems that have the potential to reduce the production cost and improve production efficiency of pharmaceutically relevant recombinant proteins. This has been documented in a wide variety of animal species including goats, cattle, and chickens (Huang et al., 2007; Yang et al., 2008; Table 2). These transgenic animals secrete a target protein into milk, egg whites, blood, or other bodily fluids, which is then purified and ready for human use (Wang et al., 2013). Over the past few decades, avians have become an increasingly desirable animal model for bioreactor production systems. This is because recombinant proteins can be laid down in the egg whites and collected with the egg (Ivarie, 2006). Egg whites contain approximately 3.6 grams of protein (Petitte and Mozdziak, 2007). Ovalbumin contributed to over 50% of the egg white protein (Ivarie, 2006). Identification of the magnum specific ovalbumin promoter has led to these tissue specific avian bioreactors. Chickens and quail also have a short generation time which allows for a transgenic line to be established quickly. This rapidly expanding area of research has a relatively limited amount of published work. To our knowledge, all relevant publications have been thoroughly reviewed for this article.

4.2. Transgenic animal bioreactors

Between 2015 and 2017, the U.S. Food and Drug Administration (FDA) approved over 100 novel drugs for commercial use. Many these drugs are protein-based, leading to an increased need for protein production within the pharmaceutical industry. This can lead to an increased financial burden for individuals requiring these medications. According to a 2016 industry report,

the total spending in 2015 on prescription drugs in the United States was $310 billion dollars. This number is expected to increase by 2020 to a spending amount between $370-$400 billion.

This increased need for pharmaceutically relevant proteins has led researchers to investigate potential alternatives to the cell-based production system. Currently, approximately 30% of approved therapeutic proteins are produced within E. coli systems. However, this recombinant system does not allow for the natural glycosylation found in certain proteins (Baeshen et al., 2015). Chinese hamster ovary (CHO) mammalian cell systems allow for appropriate post-translational modification and have quickly become the leading method for the commercial production of pharmaceutical proteins (Wurm, 2004). Transgenic animals as bioreactors may be the next step in improving the efficiency of pharmaceutical protein production.

Multiple species have previously been used to generate proteins for potential use in humans. The FDA approved the first bioreactor system in 2009. A biotherapeutics company engineered transgenic goats, whose milk contained human antithrombin, a 432-amino acid glycoprotein naturally produced in the liver (Sullivan et al., 2014). In 2014, the FDA approved a pharmaceutical protein, produced from transgenic rabbits, that could treat hereditary angioedema (Editorial staff, 2014). Recently, chickens have become increasingly popular as a model for these transgenic studies due to their egg laying ability, short generation time, and capability of producing glycoproteins.

4.3. Avian Bioreactors

The chicken egg white consists of approximately 3.5 grams of protein (Harvey et al., 2002). While there are many different egg white proteins, approximately 80% of egg white is comprised of just four proteins: Ovalbumin (54%), Ovotransferrin (12%), Ovomucoid (11%), and Lysozyme (3.4%) (Stevens, 1991; Mann, 2007). The aspect of the reproductive tract responsible for laying

down egg white proteins is the tubular glands of the magnum (Whenham et al., 2014). As a result, researchers have been investigating magnum-specific promoters as a method for overexpressing a target protein within chicken egg whites. Multiple patents have been filed concerning the production of exogenous proteins in the egg whites of transgenic chickens. Papers have also been published on this topic in recent years, a majority of which utilize the Ovalbumin promoter as shown in Table 1.

4.4. Early avian bioreactor systems

A 2002 study first demonstrated the potential of chickens as a bioreactor (Harvey et al., 2002). At the time, the elements required for oviduct specific expression were not known and a ubiquitously expressed promoter, cytomegalovirus (CMV), was used to express β-lactamase within the egg whites of transgenic birds. Six of the hens had significant levels of β-lactamase in their egg whites (56.3-250 ng/ml). The hemizygous progeny of the transgenic birds had concentrations of egg white β-lactamase ranging from 0.47-1.34 μg/ml. This expression level was shown to be constant through four generations (Harvey et al., 2002). This group published a paper the following year where they reported the first successful production of a biologically active human protein in the egg whites of transgenic hens, human glycoprotein interferon α-2b (hIFN) (Harvey et al., 2002). The average hIFN expression of the eggs was determined to be 2.7 ug/ml. Following this ground-breaking work, other studies were done with avians as bioreactors using a wide variety of methods (Table 2).

Two separate studies introduced the viral particles via injection into the embryonic heart at 55 hours of incubation. In a 2005 study, transgenic chickens were generated from chimeric chickens and expressed an antiprion single chain Fv-Fc fusion antibody. However, the progeny had a lower expression level than their chimeric predecessors (Kamihira et al., 2005). A 2008

publication was able to produce human erythropoietin (hEpo) within the eggs and serum of chimeric founder chickens. Unfortunately, no transgenic offspring were obtained from the original chimeric birds (Kodama et al., 2008). The low expression level and lack of progeny could be due to leakage of the target protein into the blood stream or no insertion of the transgene into germline cells.

Though these studies approached the idea of eggs as bioreactors somewhat differently, they contained several similarities. In the studies that obtained progeny, the progeny consistently had lower protein expression compared to the chimeric founders. One explanation for the lowered expression in subsequent generations is that high concentrations within the bodies of transgenic embryos were lethal. There were also variations in expression levels between chimeric hens. Though the exact reason for this was unknown, the variation could be due to different ratios of chimerism, or potentially epigenetic factors, such as methylation (Rapp et al., 2003, Jang et al., 2011). While these studies are an important start for the use of avians as bioreactors, it is necessary to devise a tissue-specific method of exogenous protein production to ensure high yields of protein products within the eggs and also to maximize the health and production of transgenic birds.

4.5. Ovalbumin as a magnum specific promoter

In 2005, the ovalbumin promoter was first used as a tissue specific promoter to generate chimeric chickens with estrogen-induced expression of monoclonal antibodies within their egg whites (Zhu et al., 2005). The first protein produced was a chimeric human-mouse monoclonal antibody specific for the dansyl hapten and the second was a fully human antibody specific for a prostate specific membrane antigen (Zhu et al., 2005). Two different constructs were used and transfected into two types of chicken embryonic stem cells (male and female) to potentially avoid conflict of sexes between donor cells and recipient eggs. One vector contained 7.5kb and the other

contained 15kb. Some of the chimeras generated from both lines expressed low levels of the monoclonal antibodies with concentrations ranging from 1.4-10.8 ng/ml when using the 7.5kb construct, and 0.5-3.4 ng/ml when using the 15kb construct (Zhu et al., 2005). The results from this study indicated that the promoter size did not largely affect the expression level. This is likely due to the fact that both constructs contained the steroid dependent regulatory element (SDRE) and negative regulatory element (NRE) and were estrogen induced.

A majority of studies use between a 2.0kb and 3.0kb promoter which contains both the SDRE and NRE (Lillico et al., 2006; Byun et al., 2010; Cao et al., 2015; Lui et al., 2015; Park et al., 2015). These regulatory elements are thought to confer magnum specificity. These two regulatory elements work in complex to allow for the activation of the Ovalbumin promoter (Schweers et al., 1990). Three of the studies documented the utilization of additional estrogen response elements within the constructs, making the transgene more estrogen inducible.

Over the past decade, several studies have focused on avians as bioreactors using the Ovalbumin promoter in order to induce magnum-specific production of proteins. This is due to the high quantity of Ovalbumin within the egg and expression of the Ovalbumin gene is exclusively found in the magnum. A wide variety of proteins have been produced within the avian egg. These proteins include: human epidermal growth factor (hEGF), human lysozyme (hLy), human neutrophil defensin 4 (HNP4), single chain Fv-Fc fusion protein (ScFv-Fc), human interferon B1a (hIFNB1A), and green fluorescent protein (GFP) within chickens. Within quail, recombinant human interleukin 1 receptor antagonist (rhIL1RN) was produced in the egg (Table 1).

Variation in expression among transgenic lines is a concern of avian transgenics. One element that may contribute to the variation in transgene expression among different transgenic lines is the site of transgene integration. Insertion of the transgene is random, and its location

within the chromosome can have a direct effect on expression level and tissue specificity (Lee et al., 2017). If integration occurs near the centromere, it is likely to have a lowered expression due to silencing and regulation occurring near the centromere (Dobie et al., 1996). Furthermore, if integration occurs near a strong tissue specific gene and promoter, that promoter can affect the expression of the transgene. Expression can occur in the target tissue by the transgene promoter, and in the off-target tissues by the promoter near the integration site. Multiple insertion sites also often occur with transgene delivery systems. Therefore, it is important to identify the location and number of insertion sites. This information will lead to the identification of a transgenic line containing a high expressing transgene integration site. That line can then be bred to maximize transgene expression. Methods to determine the integration of the transgene have been established in recent studies (Bryda et al., 2006, Shin et al., 2014). Furthermore, variations between the expression levels in the different studies could be attributed to the use, or lack, of an estrogen response element (ERE), or to the translation efficiencies and rates of degradation of target proteins. The ERE allows for the construct to be estrogen inducible and could potentially increase the protein expression within the egg.

4.6. Future Studies

While many advancements have been made within this area of research over the past few decades, improvements must still be made to solidify the use of avians as bioreactor systems. These improvements include tissue specificity, minimal leakage into serum, appropriate amount of protein expression, inducibility of the promoter, and maintenance of the transgenic lines.

Identifying a new magnum specific gene, aside from Ovalbumin, could be beneficial to improving this system. A proteomics study recently identified several different egg proteins that could potentially be ideal candidate genes for future studies (Rath et al., 2017). Prior to utilizing

any of these novel promoters, further studies should be performed to confirm tissue specificity and expression capacity. The very tight tissue specificity of the promoter would minimize production from other tissues and leakage into the serum, exclusively concentrating the produced protein within the egg whites of transgenic birds.

Leakage is also an issue of concern as excessive leakage of exogenous protein into the blood could be lethal and result in the loss of transgenic birds and ultimately the transgenic lines. For example, a recent study failed to generate transgenic quail because expressing hEPO within the blood stream produced lethal results (Koo et al., 2017). The use of tissue-specific promoters, as opposed to ubiquitous promoters could reduce this issue. However, there is a high amount of Ovalbumin within the egg. This high production could potentially lead to a considerable amount of leakage even though it is produced in magnum. A slightly lower protein concentration within chicken eggs could still be purified and useful within the pharmaceutical industry, and potentially lead to a more stable production of transgenic lines. Additionally, a lower concentration would be less likely to interfere with embryo development, allowing for continued transgenic generations. The inducibility of the system is also important. Several of the discussed papers used hormone response elements in order to induce the production of the pharmaceutically relevant proteins; this ensured that the protein was only produced when hens began laying.

It is also important to maintain transgenic lines for continued production. Multiple articles stated that the generated chimeric chickens produced target proteins but not progeny. Without the viable progeny, continued injections would be needed to maintain the production of transgenic birds. This is a very inefficient model for long term use. It would be beneficial for the transgenic line to be maintained naturally through the male lineage. There are multiple benefits in using the male bird to continue the lines. Firstly, males could be breed to multiple females, increasing the

hemizygous egg output and potential transgenic progeny. Also, transgenic males could be kept for breeding as they would contain the transgene, but not the gonadal tissue needed to produce the target protein.

Genome specific editing via the clustered regularly interspaced palindromic repeats/ CRISPR associated protein 9 (CRISPR/CAS9) system could also improve the generation of transgenic birds. Sequence specific double stranded breaks and homologous recombination would lead to a specific, and known, integration site as opposed to random integration. A gene similar to the reverse orientation splice acceptor (ROSA)26 gene, commonly used in mammals, could be targeted (Li et al., 2014). The interruption of this gene does not affect the viability of the offspring. Such a system would alleviate some issues involving transgene integration and offspring production (Figure 8). While this is a very promising field that has made many advancements, there are still some issues that are currently associated with using chickens as bioreactors.

4.7. Current Issues

One of the key issues facing these studies is the efficiency of the system. Many of the discussed publications faced extremely low hatch rates of chimeric birds and generation of subsequent transgenic lines. The typical hatch rate of chimeric Japanese quail generated through microinjection is approximately 10%, as evidenced through transgenic projects performed within our lab (Woodfint et al., 2017, Ahn et al., 2015). Of those hatched birds, it is not guaranteed that the viral particles are integrated within the gametic tissue. Assuming appropriate integration into the gametes takes place, and depending on the integrations sites, approximately one in one-hundred birds will be transgenic. Also, the stability of the amount of protein within the transgenic lines is an issue. Some studies that were able to produce a transgenic line noticed a decrease in expression in following generations. High expression within chicks may have been lethal, leading to the

survival of only low expressing transgenic birds. Once these issues are addressed, the use of avians as a bioreactor will be more efficient and desirable as a commercialized system.

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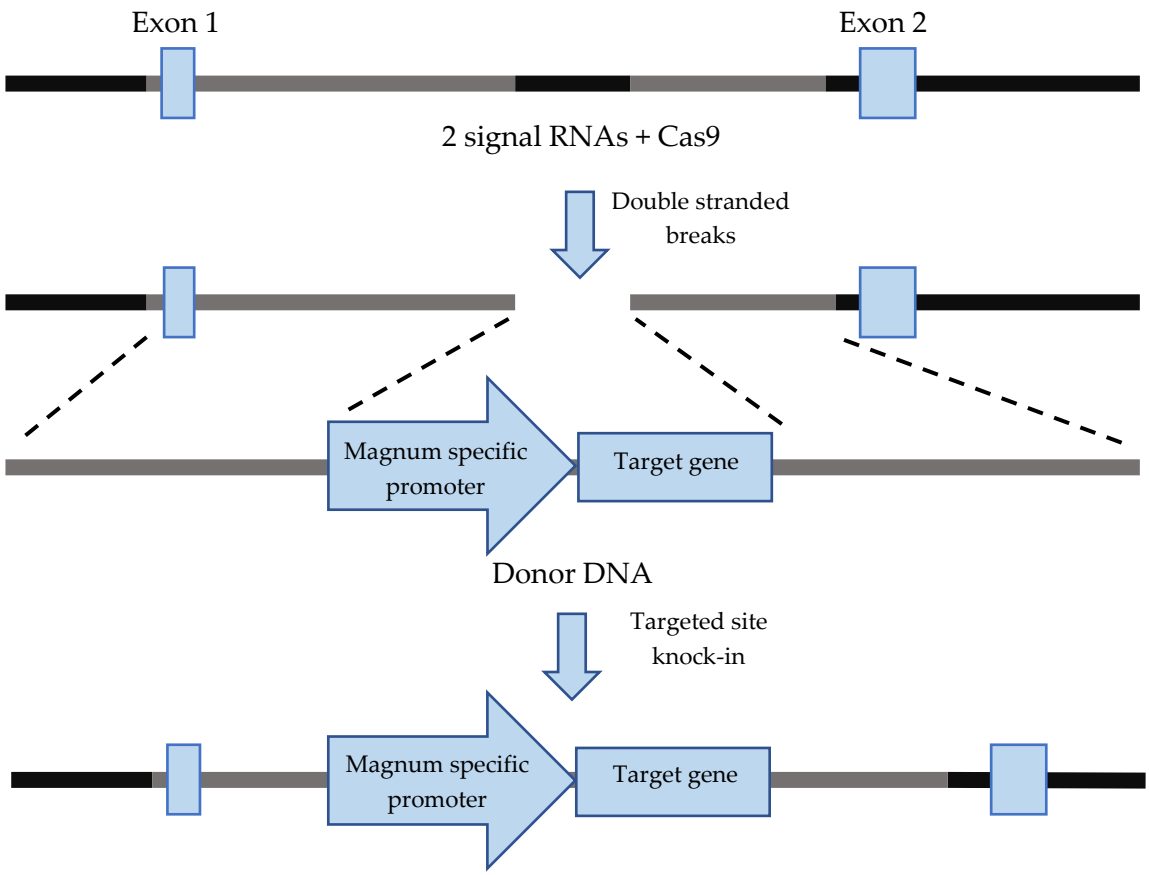
4.9. Figures

Figure 8: Targeted knock-in into specific chicken locus



Targeted knock in of magnum specific promoter and target gene construct into a specific chicken gene locus.

4.10. Tables

Table 2: Publications with Avians as Bioreactor

Promoter	Target Gene	Method	Year
CMV[1]	β-lactamase	Avian Leukosis Virus	Harvey et al., 2002
CMV	hIFN[3]	Avian Leukosis Virus	Rapp et al., 2003
RSV[2]	ScFv-Fc[4]	Retroviral Vector	Kamihira et al, 2005
Ovalbumin[5]	mAbdns[6] & mAbF1[7]	Injection of transformed cES cells	Zhu et al., 2005
Ovalbumin (+ERE)	ScFv-Fc & hIFNB1a[8]	Lentiviral Vector	Lillico et al., 2007
β-actin	hEpo[9]	Retroviral Vector	Kodama et al., 2008
Ovalbumin	rhIL1RN[10]	Lentivirus	Kwon et al., 2010
Ovalbumin	GFP[11]	Recombinant Lentivirus	Byun et al., 2011
Ovalbumin	HNP4[12]	Lentiviral Vector	Liu et al., 2015
Ovalbumin (+ERE)	hLy[13]	Lentiviral Vector	Cao et al., 2015
Ovalbumin (+2 ERE)	hEGF[14]	Injection of transformed PGCs	Park et al., 2015

[1]Cytomegalovirus
[2]Rous sarcoma virus
[3]Human interferon
[4]Single chain Fv-Fc fusion protein
[5]7.5kb & 15kb promoter contains natural ERE
[6]Human-mouse antibody specific for the dansyl hapten
[7]Prostate specific membrane antigen antibody
[8]Human interferon B1a
[9]Human erythropoietin
[10]Recombinant human interleukin 1 receptor antagonist
[11]Green fluorescent protein
[12]Human neutrophil defensin 4
[13]Human Lysozyme
[14]Human epidermal growth factor

Chapter 5: Identification of the *MUC2* Promoter as a Strong Promoter for Intestinal Gene Expression through Generation of Transgenic Quail Expressing GFP in Gut Epithelial Cells

Abstract

Identification of tissue- and stage-specific gene promoters is valuable for delineating the functional roles of specific genes in genetically engineered animals. Here, through the comparison of gene expression in different tissues by analysis of a microarray database, the intestinal specificity of mucin 2 (MUC2) expression was identified in mice and humans, and further confirmed in chickens by RT-PCR (reverse transcription-PCR) analysis. An analysis of cis-acting elements in avian *MUC2* gene promoters revealed conservation of binding sites, within a 2.9 kb proximal promoter region, for transcription factors such as caudal type homeobox 2 (CDX2), GATA binding protein 4 (GATA4), hepatocyte nuclear factor 4 α (HNF4A), and transcription factor 4 (TCF4) that are important for maintaining intestinal homeostasis and functional integrity. By generating transgenic quail, we demonstrated that the 2.9 kb chicken *MUC2* promoter could drive green fluorescent protein (GFP) reporter expression exclusively in the small intestine, large intestine, and ceca. Fluorescence image analysis further revealed GFP expression in intestine epithelial cells. The GFP expression was barely detectable in the embryonic intestine but increased during post-hatch development. The spatiotemporal expression pattern of the reporter gene confirmed that the 2.9 kb *MUC2* promoter could retain the regulatory element to drive expression of target genes in intestinal tissues after hatching. This new transgene expression system, using the *MUC2* promoter, will provide a new method of overexpressing target genes to study gene function in the avian intestine.

5.2. Introduction

The specificity of gene expression for tissue- or cell-types and developmental stages is often regulated by promoters and enhancers. Therefore, identification of tissue- and stage-specific genes and characterization of their promoters is important in order to promote transgene expression in a tissue and stage-specific manner in genetically engineered animals for the elucidation of transgene function. To identify numerous tissue-specific genes in both mice and humans, comparative microarray database analysis based on the Gene Expression Omnibus (GEO) repository was previously used in our studies, Int. J. Mol. Sci. 2017, 18, 196; doi:10.3390/ijms18010196 www.mdpi.com/journal/ijms Int. J. Mol. Sci. 2017, 18, 196 2 of 13 and several novel tissue-specific genes were discovered [1,2]. In addition, our comparative analysis of the promoter region of tissue-specific genes has identified conserved cis-acting elements as a major aspect of tissue-specific regulation and expression [3–5]. The effectiveness of promoter sequences in directing the expression of genes of interest in specific target tissues or cells was widely proven in transgenic studies that used promoters of adipocyte fatty acid binding protein (aFABP, also known as aP2 and FABP4) [6], albumin [6], and insulin [7] genes, to name a few, for targeting adipocytes, liver, and pancreatic beta cells, respectively. In terms of intestine-specific transgene expression, the regulatory regions of several genes were previously used to direct gene expression. For example, a 12.4 kb promoter-enhancer complex or a 9 kb regulatory region of the mouse Villin gene was used to direct expression of transgenes such as reporter genes, the oncogenic K-ras gene, and the Cre recombinase gene in mice to label the intestine, induce intestinal tumorigenesis, and induce gene knockout within the epithelium [8–12]. The promoter of intestinal fatty acid binding protein (I-FABP) has also been used to direct the intestinal expression of transgenes such as growth hormone (GH) and cystic fibrosis transmembrane conductance regulator (CFTR) genes [13,14].

Although results from studies using the Villin promoter indicated high expression of downstream genes in the embryonic stage, which is useful in studying the effects of transgene integration on embryonic development, the entire vertical (crypt-villus axis) and horizontal (duodenum-colon axis) expression requires a relatively large 12.4 kb or 9 kb sequence of the regulatory region. In addition, studies using I-FABP promoter reported localization of the transgene mimicking its endogenous localization, but they also reported variability issues regarding the degree of transgene expression and phenotypes. In contrast to the distinct expression patterns of Villin and I-FABP, mucin 2 (MUC2), which is a member of the mucin family encoding gel-forming glycoproteins that aids in the protection of the gastrointestinal tract [15,16], showed a constitutive expression pattern throughout the chicken intestinal tissues in this study. This led us to investigate chicken *MUC2* promoter for a reporter transgene expression in an intestine-specific manner. Transgenic technology will be a useful method in studying intestinal biology. For example, it will allow for a better understanding of direct and specific in vivo roles of potential genes. This technology can also be used to understand encoding factors involved in intestinal digestion and absorption processes as well as intestinal secretory and endocrine factors. However, intestine-specific promoters for modulating gene expression in the intestinal tissues of avian species have yet to be identified. The objectives of this study were to identify intestine-specific genes in avian species through comparative analysis, generate transgenic Japanese quail that contain the enhanced green florescent protein (eGFP) gene under the control of a promoter region of an intestine-specific gene (the 2.9 kb chicken *MUC2* promoter) via a gene transfer using lentiviral particles, and investigate distinct spatiotemporal expression of the GFP reporter gene. The identification of promoters that can effectively drive expression of a gene of interest in a space and time-dependent manner will

pave the way for the investigation of gene function in the intestine for both agricultural and biomedical purposes

5.3. Results

Microarray Analysis of Intestine-Specific Mucin Genes in Mouse and Human

Through the analysis of expression of mucin (MUC) genes in mouse and human tissues based on the aforementioned microarray GEO DataSets (GDS3142 for mice and GDS596 for humans), *MUC13* and *MUC2* were identified as genes that are predominantly expressed in the small intestine of both mice and humans. The MUC13 mRNA showed 61.2- and 50.3-fold higher expression in the small intestine of mice and humans, respectively. The *MUC2* mRNA showed 26.9- and 31.7-fold greater expression in the same tissue (Table 1). As a result, these two mucin genes were selected for further comparative examination of small intestine enrichment among chicken tissues.

Confirmation of Intestine-Specific Expression of Mucin Genes in Chickens

The expressions of *MUC13* and *MUC2* were confirmed in various tissues of chicken using reverse transcription-PCR (RT-PCR) with primers in exons (Figure 9). The expression of chicken *MUC13* was detected in various parts of the small intestine; however, *MUC13* was also expressed in the thymus, muscle, liver, lung, and kidney of the chicken. In contrast, *MUC2* was exclusively expressed in different parts of the small intestine, ceca, and large intestine. This suggests that MUC2 is an intestine-specific gene across species, including the avian species, whereas MUC13 is intestine-specific in mammals, such as mice and humans. Therefore, our comparative analysis using GEO DataSets and RT-PCR has led to the discovery of MUC2 as a common intestine-specific gene, whose promoter may regulate intestine-specific expression.

Analysis of the MUC2 Promoter

To investigate regulatory regions in the promoter sequence of *MUC2*, the 5 kb upstream regions in birds were compared (Figure 11). Using the BLAST (Basic Local Alignment Search Tool)-like Alignment Tool (BLAT) search (available online: http://genome.ucsc.edu), highly conserved regions across bird species were identified. As shown by the black boxes in Figure 11 chicken, turkey, and quail shared multiple regions with other birds, such as the zebra finch and medium ground finch, within the 2.9 kb region that appear to be evolutionarily important. Moreover, predicted binding sites for intestine-specific transcription factors such as caudal type homeobox 2 (CDX2), GATA binding protein 4 (GATA4), hepatocyte nuclear factor 4 α (HNF4A), and transcription factor 4 (TCF4) were significantly distributed in the 2.9 kb sequence, especially in the case of the quail. Thus, this 2.9 kb *MUC2* promoter was chosen for the generation of transgenic quail with intestine-specific expression of enhanced green fluorescent protein (eGFP).

Generation of Transgenic Birds

Genotyping PCR was used to positively identify transgenic birds. The offspring of chimeric founders were screened in order to identify Generation 1 (G1). Of the 105 injected eggs seventeen chimeric founders hatched, a success rate of 16.2%. A single chimeric founder produced one offspring that was positively identified as transgenic. The identification of this animal indicated that the vector construct was able to successfully integrate into at least one chimeric founder. Genotyping PCR was also used to identify transgenic Generation 2 (G2) birds, the progeny of the transgenic G1 bird. G2 birds were hatched normally in a Mendelian ratio (19 wild type and 20 transgenic birds (51.3%), indicating one integration site), with transgenic offspring expressing the eGFP in their intestinal tissue.

Western Blot Analysis of Transgenic Birds for Tissue Distribution

Western blot analysis revealed that the eGFP protein was specific to the intestinal tissues of the transgenic birds. The small intestine and large intestine of day 42 (D42) birds, one wild type and two transgenic birds from the G2 generation were used to perform Western blot (Figure 15a). The results indicated that the eGFP protein was present in the transgenic birds, but not in the wild-type birds. Western blot was performed using a tissue distribution from a transgenic bird to further determine the expression pattern of eGFP (Figure 15b). The tissues included fat, skeletal muscle, heart, liver, lung, proventriculus, small intestine, and large intestine. The target eGFP protein was detected within the small intestine and large intestine. Therefore, the 2.9 kb *MUC2* promoter was able to successfully direct expression of eGFP in only intestinal tissues.

Protein and Fluorescence Detection of eGFP in Epithelial Layer of Intestinal Tissues

Western blot analysis was also performed to confirm the intestinal specificity of MUC2 protein (Figure 16). Gastrointestinal tissues, esophagus, crop, proventriculus, ventriculus, small intestine, ceca, and large intestine were collected from a D21 transgenic G2 bird (Figure 14). Bands appeared at the expected size of 25 kDa for the small intestine, ceca, and large intestine. Coomassie blue staining was used to ensure the same amount of protein was loaded for each sample. Scrapings parallel to the length of the tissue were taken from the intestine of D42 transgenic G2 birds. Under a fluorescent microscope, villi were visible in the intestine of the transgenic animals. The epithelial cells in these villi expressed the eGFP protein (Figure 18).

Time Point Expression of eGFP

Embryonic day 13 (E13), E16, D0, D3, and D21 transgenic G2 offspring were collected to determine variation within the eGFP expression. Western blot analysis was used to detect any

differences. The Western blot indicated that there is an increase in eGFP expression from the embryo to post-hatch D21 (Figure 17).

5.4. Discussion

In this study, the intestinal specificity of mucin 2 (MUC2) in avian species was confirmed through microarray data and RT-PCR analysis. Consequently, the 2.9 kb promoter region of chicken *MUC2* was used to generate transgenic quail with an intestine-specific reporter gene expression. Among several mucin genes, intestine-specific expression of the MUC2 gene was confirmed through our comparative analysis. Also, the avian stomach, or ventriculus, did not express *MUC2* mRNA. These findings concur with previous works indicating that MUC2 is expressed on the mucosal surface in both the large and small intestine of humans and mice, but absent or barely detectable in other gastrointestinal tissues, including the stomach [17,18]. Due to the tissue-specific expression of MUC2, it was hypothesized that the regulatory promoter region of *MUC2* gene could drive intestine-specific expression of a transgene.

The specific pattern of gene expression in certain types of cells, particular developmental stages, and nutritional conditions has been shown to be regulated by conserved regulatory elements, including a promoter region found in previous studies along with our reports [4,5,19,20,21]. In the current study, conserved promoter regions were identified in the chicken *MUC2* gene that are specifically expressed in intestinal tissues of 7-week-old broiler chickens. Importantly, conservation of cis-regulatory regions in *MUC2*, which include binding sites for major transcription factors for intestinal homeostasis and functional integrity such as caudal type homeobox 2 (CDX2), GATA binding protein 4 (GATA4), hepatocyte nuclear factor 4 α (HNF4A), and transcription factor 4 (TCF4), were revealed. The homeodomain protein CDX2 is thought to be an intestine-specific master transcription factor because CDX2 is expressed in the hindgut, and

its expression is critical to sustain expression of downstream intestinal transcription factors such as HNF4A. CDX2 deficiency leads to severe hindgut abnormalities and colon dysgenesis [22] and reduced chromatin access that is required for transcription [23]. As a result, binding of other transcription factors including GATA4 and HNF4A is disrupted [24]. Another study reported that CDX2 directs co-occupancy of cis-regulatory regions with TCF4 which is essential for intestinal-specific gene expression [25]. In this regard, the regulatory region of *MUC2*, a heavily glycosylated, gel-forming mucin, was selected as the candidate promoter due to high intestine expression indicated in the gene expression profile and conservation of the regulatory region among several avian species. As a result, the chicken *MUC2* promoter containing nine CDX2 (the intestinal master transcription factor; [23]), two HNF4A, one GATA4, and one TCF4B binding sites could successfully promote expression of GFP in the large and small intestine of quail.

Japanese quail, Coturnix c. japonica, were used in this study because they reach sexual maturity quickly, are small in size, have a short incubation period, and have a high egg laying capacity. Also, the quail intestine appears to be permissive for the chicken promoter due to a high sequence homology between chicken and quail *MUC2* promoters. Our lab has been able to successfully generate transgenic quail for a number of different studies [4,19,20]. This includes other studies where tissue-specific genes were identified [4]. These studies were attributed to high efficiency of stable integration of recombinant lentiviral particles into the host genome, capacity of infecting both dividing and non-dividing cells, and self-inactivating properties [21,26]. This allowed lentiviral vectors to become reliable and safe gene delivery vehicles for either ubiquitous or tissue-specific expression of transgenes in multiple studies, including our current study [4,19,21,27,28].

The results of this study also indicated that the stimulation of transgene expression through the chicken *MUC2* promoter increases with age post-hatch. In a study by Jiang et al. [29], it was indicated that endogenous chicken *MUC2* mRNA expressions increased throughout embryonic development and continued to increase post-hatch. It was noted that following post-hatch day 7, the MUC2 expression remained high. Similarly, in this study, post-hatch day 3 and day 21 both exhibited high expression of eGFP protein which was regulated by the chicken *MUC2* promoter. Thus, these results suggest that the *MUC2* promoter can be used to promote target gene expression in the intestines at post-hatch ages.

In summary, by generating transgenic quail, we have demonstrated that the promoter of chicken *MUC2* contains regulatory elements that direct expression to the small intestine, ceca, and large intestine of quail in a developmental stage-dependent manner. These results show that the basic mechanisms that mediate intestine-specific expression are conserved between avian species. The relatively short promoter of the chicken *MUC2* isolated in this study offers a powerful tool for labeling intestinal cells and targeting expression in the intestines of quail as well as other avian species. In this regard, the epithelial specificity of MUC2 could be a valuable mechanism used to drive the expression of other advantageous molecules. This could provide beneficial information for the poultry industry and potentially improve production by modulating expression of genes that increase food intake, digestibility, gut mobility, gut development, and nutrient uptake. Regarding increasing food safety issues related to pathogenic bacteria in poultry, genes encoding innate anti-bacterial peptides can be delivered in vivo through this current expression system.

5.5. Materials and Methods

Animal Use and Ethics Statement

Animal care and use procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University (Protocol: 2015A00000135, 12 January 2016, IACUC). Japanese quail (Coturnix coturnix japonica) were housed at The Ohio State University Poultry Facility in Columbus, Ohio. A standard starter or breeder diet and water was provided to the animals ad libitum. Sacrificed animals were euthanized via CO2 inhalation followed by cervical dislocation for tissue collection.

Data Mining Using GEO DataSets

Microarray database in the Gene Expression Omnibus (GEO), a public genomics data repository, was examined to determine intestine-specific genes among mucin (MUC) genes, as described in our previous reports [1,2]. In particular, GEO DataSet (GDS) 3142 for the mouse MUC genes and GDS3113 for the human MUC genes were obtained from the NCBI website, and gene expression profiles for eight tissues (small intestine, spleen, muscle, liver, brain, lung, kidney, and heart) were sorted out based on the normalized expression value. The fold change assigned to gene expression in the small intestine (S. intestine) was estimated by dividing the MUC expression value in the small intestine by an average value of the other tissues (Table 1).

Analysis of Transcription Factor Binding Sites on the Promoter Region

The avian DNA sequences in the promoter region of *MUC2*, which include 5 kb upstream region from the start codon (ATG) of *MUC2*, were obtained from the NCBI website (available online: http://www.ncbi.nlm.nih.gov/gene). With these sequences, transcription factor binding sites were predicted using the MatInspector software (Genomatix Software GmbH, Munich, Germany). Among various transcription factors, previously reported intestine-specific

transcription factors (CDX2, GATA4, HNF4A, and TCF4) were selected for their binding sites on the *MUC2* promoter sequence [23,24,25].

Total RNA Extraction, cDNA Synthesis, and PCR

To determine tissue specificity of MUC2, fat, thigh muscle, pectoralis muscle, heart, liver, lung, kidney, spleen, duodenum, jejunum, ileum, ceca, and large intestine were collected from 7-week-old broiler chickens (n = 4). Total RNA from the broiler chicken tissues was isolated using Trizol reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. RNA quality was assessed by gel electrophoresis, and quantity was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was generated by using 1 μg of total RNA and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with conditions of 65 °C for 5 min, 37 °C for 52 min, and 70 °C for 15 min. cDNA samples were used to conduct PCR for expression patterns of *MUC2* using the following primers; MUC2-F, 5′-TGACTGAATGTGAAGGAACATGTG-3′ and MUC2-R, 5′-TTCATTTTGATGTTAAGCTGATGG-3′. The amplification conditions for *MUC2* were 95 °C for 1 min, 32 cycles of 95 °C for 25 s, 58 °C for 45 s, and 72 °C for 45 s, and a final extension time of 5 min at 72 °C. As a loading control, ribosomal protein s13 (*RPS13*) was amplified from each tissue using the primers; RPS13-F, 5′-AAGAAGGCTGTTGCTGTTCG-3′ and RPS13-R, 5′-GGCAGAAGCTGTCGATGATT-3′, with amplification conditions of 94 °C for 1 min, 27 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 20 s, and a final extension time of 5 min at 72 °C.

Vector Construction and Production of Lentiviral Particles

The 2.9 kb sequence of chicken mucin 2 (c-*MUC2*) gene was amplified from chicken genomic DNA by PCR with a forward primer containing the ClaI site (underlined), 5′-AATCGATTTTAGCAGCAGAG AATCCCCA-3′, and a reverse primer containing the PacI site (underlined), 5′-AGTTAATTAAGGCTAAGG TGGGTGAACTGTGA-3′, and was then cloned into pCR2.1-TOPO vector (Invitrogen). Two restriction enzymes, ClaI and PacI, were used to digest the pCR2.1 recombinant vector. The 2.9 kb *MUC2* promoter replaced a RSV promoter of the pLTReGW lentiviral vector containing eGFP that had been constructed previously [23]. The resulting vector, pLT-cMuc2-eGFP, was designed to express the eGFP gene specifically in the intestinal tissues through the direction of the *MUC2* promoter. The vector was first transfected into a human intestinal epithelial cell line (Caco-2 cells), and the expression of eGFP was confirmed in these intestinal cells.

Co-precipitation of calcium phosphate and the pLT-cMuc2-eGFP vector was used to produce lentiviral particles. One day prior to the transfection, 293FT cells were plated on 100 mm culture dishes in complete medium. This medium consisted of Dulbecco's Modified Eagle Medium (DMEM; Life Technologies Inc.) with 10% fetal bovine serum (FBS; Life Technologies Inc.), 1% penicillin/streptomycin (pen/strep; Life Technologies Inc.), 1 mM MEM sodium pyruvate (Life Technologies Inc.), and 0.1 mM MEM non-essential amino acids (Life Technologies Inc.). 9 μg of pLT-cMuc2-eGFP, 9 μg of ViraPower Packaging Mix (Life Technologies Inc.), and 87 μL of 2 M calcium solution (Clontech Laboratories Inc., Mountain View, CA, USA) were added to a final volume of 700 μL of sterile H2O (Clontech Laboratories Inc.) to prepare the transfection solution. Then, 700 μL of 2× HEPES-Buffered Saline (HBS) (Clontech Laboratories Inc.) was added dropwise while slowly vortexing the solution. The transfection solution was incubated for 5 min at room temperature and added dropwise to the

complete medium. Following 10 h of transfection, the medium was replaced with 5 mL of fresh complete medium. After 48 h, the supernatant was collected and filtered through a 0.22 μm pore-sized filter. The titer of lentiviral supernatant was measured by a standard ELISA method using the Lenti-X p24 Rapid Titer Kit (Clontech Laboratories Inc.) after the non-concentrated viral supernatants were serially diluted. The supernatant was pelleted via centrifugation at 25,000 rpm for 2 h with an ultracentrifuge (L7-65R, Beckman Coulter, Fullerton, CA, USA), resuspended in Opti-MEM as a 100× concentrated lentiviral particle soup, and stored as 40 μL aliquots at −80 °C until further use.

Production of the Founder Quail

Wild-type Japanese quail eggs were cleaned with 70% ethanol and placed laterally on a tray for approximately 4 h at room temperature. Fine-tipped tweezers were used to create a small window of about 4 mm in diameter on the lateral apex of the egg. Then, 2 to 3 μL of the concentrated lentivirus was injected into the subgerminal cavity of 102 stage X embryos using a microinjection system (Tritech Research, Inc., Los Angeles, CA, USA) under a stereomicroscope (Olympus America Inc., Center Valley, PA, USA). The window was sealed with paraffin film, and the eggs were incubated for 14 days at 37.5 °C with 60% relative humidity before being placed in a hatching tray. Seventeen hatched founder chicks were grown to sexual maturity.

Mating and Selection of Transgenic Offspring

The mature founders were mated with one wild-type quail of the opposite sex. Eggs were collected each week and stored in a cooler (13 °C) until incubating. Hatchlings were tagged and reared for 14 days to collect feather pulp for genomic DNA extraction. The pulp from one feather was incubated in 300 μL of cell lysis solution (200 mM NaCl, 50 mM Tris-Cl, 10 mM EDTA, 1%

SDS, pH 8.0) containing Proteinase K (0.1 mg/mL, Invitrogen) at 55 °C for at least 2 h. Then, 300 µL of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v/v) was added to the tube, mixed, and centrifuged at 13,000× g for 10 min to remove the protein. The supernatant was transferred to a new tube, and genomic DNA was precipitated by adding 300 µL of isopropanol, inverting, and centrifuging at 13,000× g for 5 min. The pellet was washed with 70% ethanol and centrifuged at 13,000× g for 2 min. After drying, TE buffer containing RNase A (10 mg/mL, Qiagen, Valencia, CA, USA) was added to dissolve the pellet. The genomic DNA was utilized for genotyping PCR with the primer set, RRE-F, 5′-AATCGCAAAACCAGCAAGAAA-3′ as the forward primer and MUC2-R, 5′-TGTCAAGCAATTTACAGTGAAATATG-3′ as the reverse primer to amplify a 494 bp fragment. Positive offspring were reconfirmed with the primer set, eGFP-F, 5′-GCATGGACGAGCTGTACAAGTA-3′ as the forward primer and WPRE-R, 5′-AATCCTGGTTGCTGTCTCTTTATG-3′ as the reverse primer to amplify a 282 bp fragment. Offspring of the positive G1 progeny from the founders were used as parents to produce transgenic quail for the functional study.

Tissue Collection and Microscopic Examination of GFP Expression

At 3 weeks post-hatch, two wild-type and two transgenic quail were collected to determine expression of eGFP. Fat, skeletal muscle, heart, liver, lung, proventriculus, small intestine, and large intestine were collected from each bird. From the two transgenic birds, the esophagus, crop, ventriculus, and ceca were also collected to examine eGFP expression along the entire gastrointestinal tract. All tissue samples were snap frozen in liquid nitrogen and stored at −80 °C until further analysis. Small intestine samples were collected from transgenic embryos or quail at day 13 of incubation (E13), day 16 of incubation (E16), day 0 post-hatch, day 3 post-hatch (D3), and day 21 post-hatch (D21). Part of the small intestine between the duodenum and jejunum was

snap frozen in liquid nitrogen and stored at −80 °C. The intestinal mucosa from the jejunum was scraped parallel to the length of the tissue from D42 transgenic quail and mounted on a glass slide to examine eGFP expression and fluorescence using an AXIO-Vert.A1 optical microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) equipped with an AxioCam MRc5 camera (Carl Zeiss Microscopy).

Western Blot Analysis

Frozen tissue samples were homogenized in ice-cold 1× lysis buffer (62.5 mM Tris, pH 6.8, and 5% SDS) with a Tissuemiser (Thermo Fisher Scientific, Waltham, MA, USA) and mixed with 2× Laemmli buffer (62.5 mM Tris, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 12.5% glycerol, 0.05% bromophenol blue). Gels stained with Coomassie brilliant blue were used to determine protein loading. Separation of proteins was performed on 12% SDS-PAGE using a mini-Protein system (Bio-Rad Laboratories, Hercules, CA, USA). Following SDS-PAGE and transfer to polyvinylidene fluoride (PVDF) membranes, the membranes were blocked in 4% nonfat dry milk dissolved in Tris-buffered saline-Tween (TBST; 20 mM Tris, 150 mM NaCl, pH 7.4, plus 0.1% Tween 20) for 30 min at room temperature. Then, membranes were incubated overnight at 4 °C with an eGFP primary antibody (1:5000 dilution; Clontech, Mountain View, CA, USA). After washing 6 times for 10 min in TBST, the membranes were incubated in horseradish peroxidase-conjugated secondary anti-mouse IgG (1:5000 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at room temperature for 1 h. The membranes were washed with TBST 6 times for 10 min each before detection with Amersham ECL plus Western Blotting Detection Reagents (GE Healthcare Biosciences, Pittsburgh, PA, USA). The blots were exposed to Hyperfilm (GE Healthcare Biosciences) to visualize the target proteins.

Statistical Analysis

A comparison of means of gene expression in multiple tissues was conducted by using one-way ANOVA followed by a Fisher's protected least significant difference test included in SAS 9.4 software (SAS Institute, Inc., Cary, NC, USA).

5.6. Conclusions

In this study, intestine-specific expression of mucin 2 (MUC2) in avian species was identified through comparative analysis of microarray data and RT-PCR analysis. Consequently, the 2.9 kb promoter region of chicken *MUC2* was used to generate transgenic quail for an intestine-specific expression of the GFP reporter gene. It was found that the promoter of chicken *MUC2* contains regulatory elements that direct expression to the small intestine, ceca, and large intestine of quail in a developmental stage-dependent manner. The relatively short promoter of the chicken *MUC2* isolated in this study offers a powerful tool to drive expression of target genes to study the function of genes in avian intestine.

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Author Contributions

The experiment was designed by Kichoon Lee from Ohio State University. Rachel M. Woodfint, Paula R. Chen, Jinsoo Ahn, and Yeunsu Suh performed experiments. Seongsoo Hwang and Sang Suk Lee contributed reagents and materials. Rachel M. Woodfint, Paula R. Chen, Jinsoo Ahn, and Kichoon Lee analyzed the results and wrote the paper.

Conflicts of Interest

The authors of this paper declare no conflict of interest.

5.7. References

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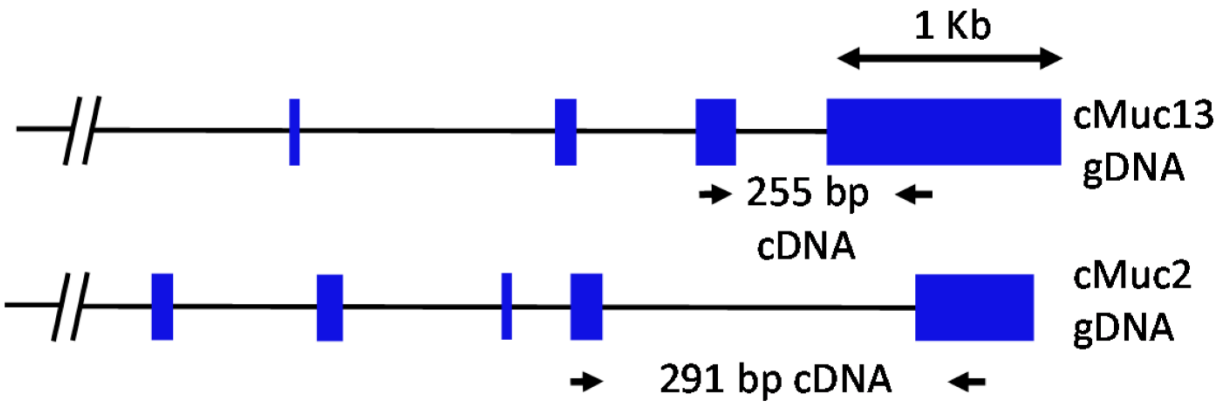
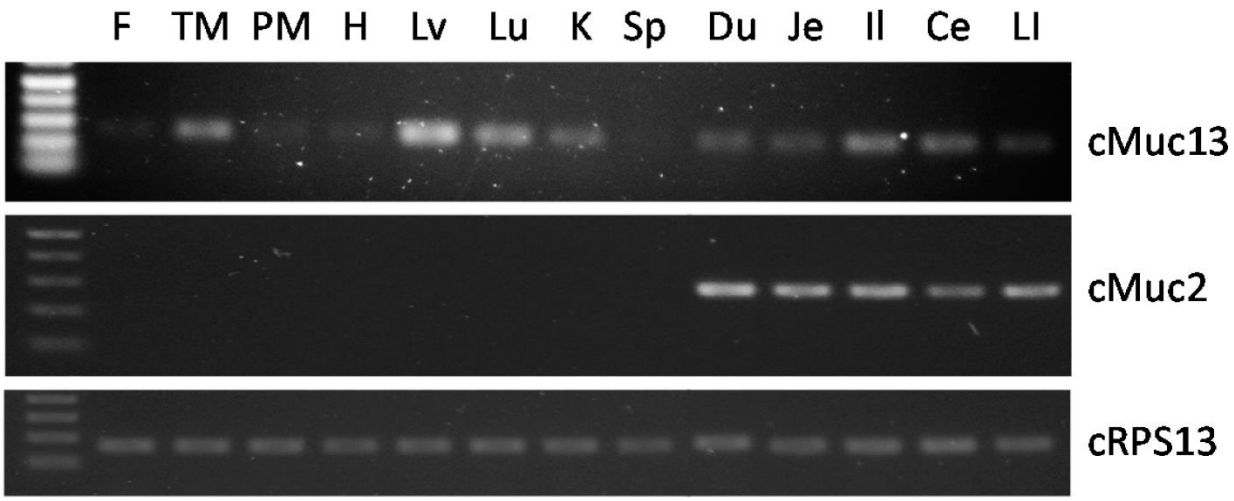
5.8. Figures

Figure 9: Exon-Intron diagram



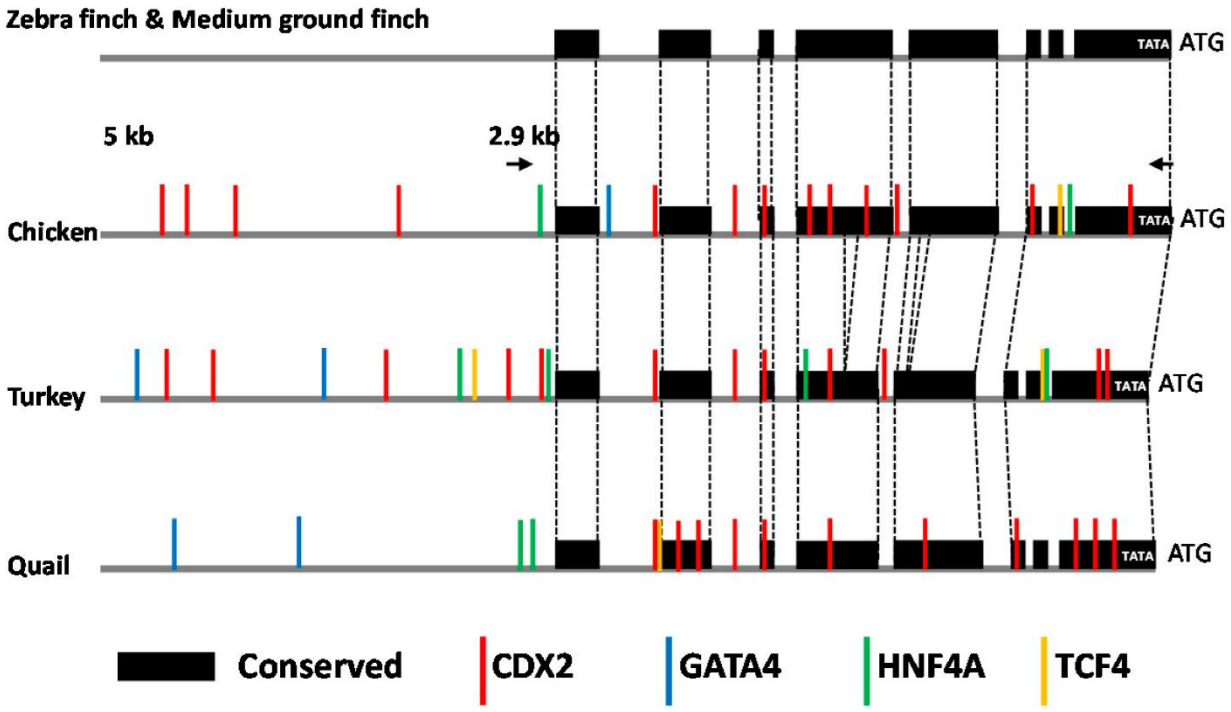
Schematic exon-intron diagram of chicken MUC13 and MUC2 genes. Locations of inter-exon primers for RT-PCR are marked with arrows and expected sizes of amplified PCR products are indicated. Blue boxes represent exons

Figure 10: Mucin 2 RT-PCR



RT-PCR tissue distribution including fat (F), thigh muscle (TM), pectoralis muscle (PM), heart (H), liver (Lv), lung (Lu), kidney (K), spleen (Sp), duodenum (Du), jejunum (Je), ileum (Il), cecum (Ce), and large intestine (LI). MUC13 was expressed in numerous tissues outside of the intestinal tissue. MUC2 was indicated to be intestine-specific, and chicken ribosomal protein s13 (*cRPS13*) was used as a control.

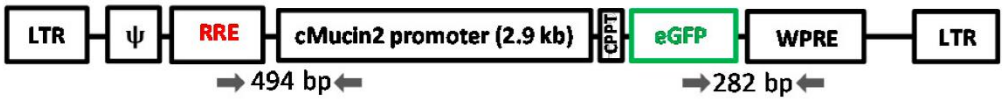
Figure 11: Mucin 2 promoter analysis



Prediction of the distribution of binding sites for an intestine-specific transcription factor. The binding sites of intestine-specific transcription factors (e.g., CDX2 (red), GATA4 (blue), HNF4A (green), and TCF4 (yellow)) are highlighted on the promoter region of the MUC2 gene from chicken, turkey and quail. Highly conserved DNA sequences between avian species are marked with a black box;

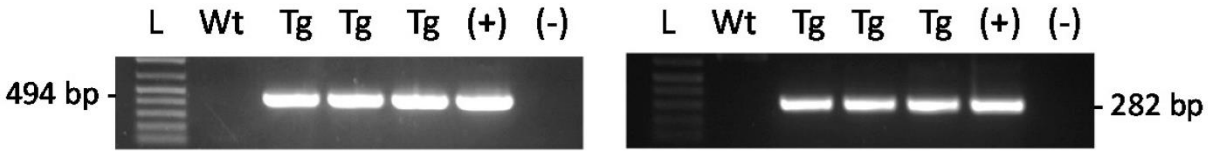
Figure 12: Mucin 2 construct

Figure 12: Viral vector construct. Construct contains 2.9 kb chicken MUC2 promoter. Located



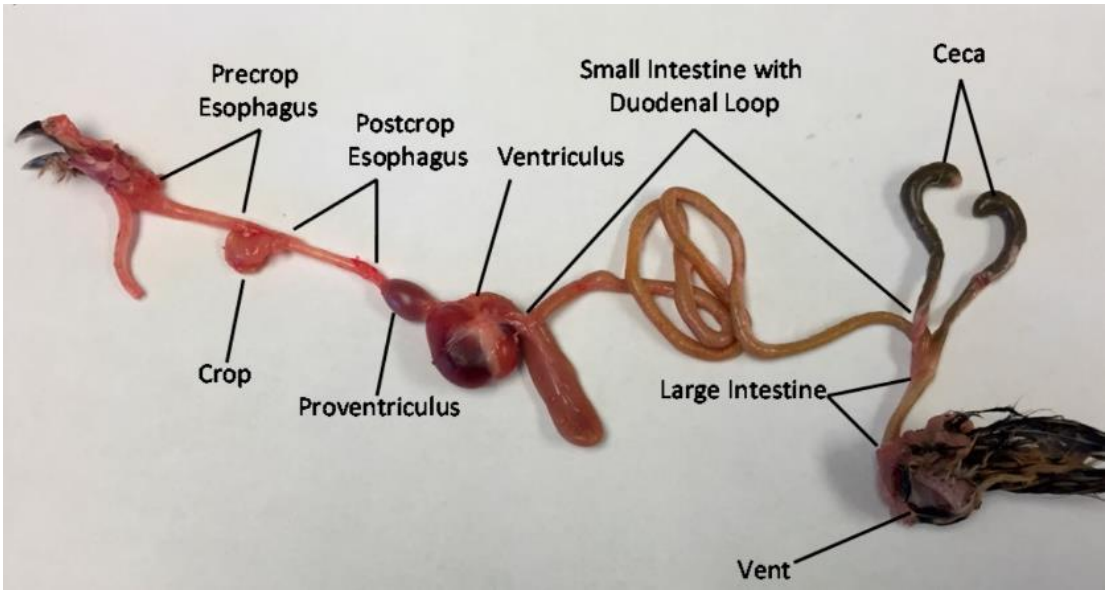
downstream of the promoter is eGFP.

Figure 13: Genotyping PC R



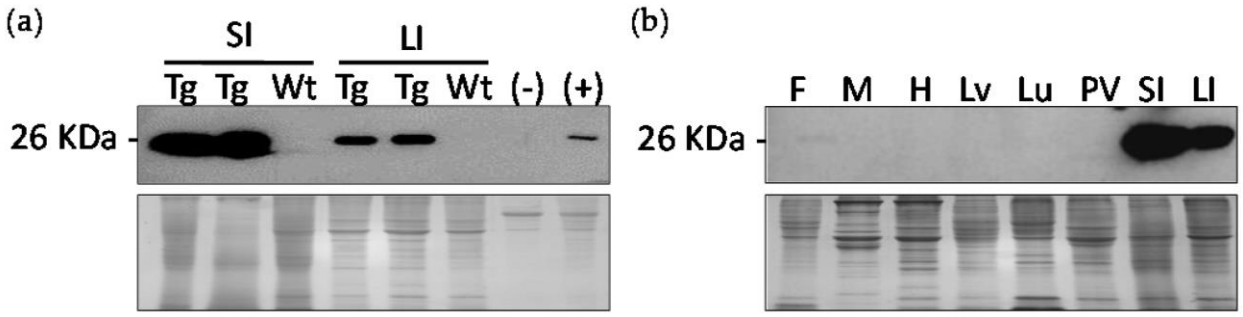
Confirmation of two primer sets. Two primer sets were utilized to confirm the presence of the transgene in animals. L (DNA ladder), Wt (wild-type), Tg (transgenic quail), + (positive control), − (negative control).

Figure 14: Avian Intestine



Digestive tract of adult Japanese quail.

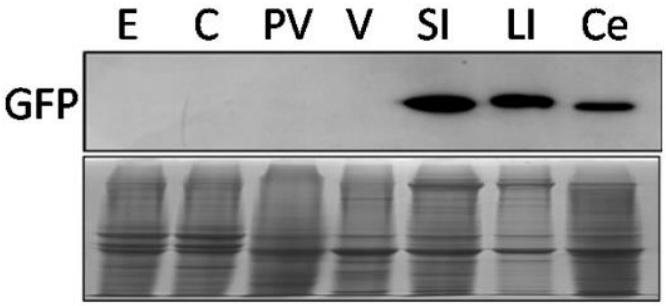
Figure 15: Western blot analysis (1)



(a) Western blot analysis of transgenic Japanese quail intestines compared to wild-type quail intestines. At 26 kDa, eGFP was expressed in the small and large intestine of transgenic quail but not in the wild-type. Coomassie blue staining was utilized to ensure an equal amount of protein

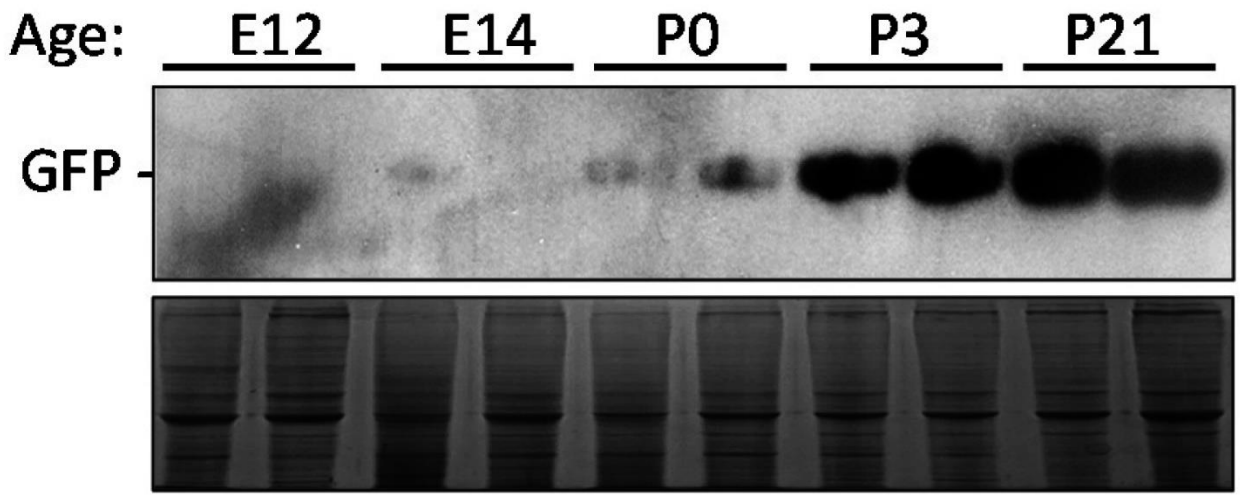
was loaded for each sample; (b) Western blot analysis of transgenic quail tissue distribution including fat (F), muscle (M), heart (H), liver (Lv), lung (Lu), proventriculus (PV), small intestine (SI), and large intestine (LI). Coomassie blue staining was used to ensure an equal amount of protein was loaded for each sample.

Figure 16: Western blot analysis (2)



Western blot analysis of transgenic quail intestinal tissue distribution including esophagus (E), crop (C), proventriculus (PV), ventriculus (V), small intestine (SI), large intestine (LI) and ceca (Ce). Coomassie blue staining was used to ensure an equal amount of protein was loaded for each sample.

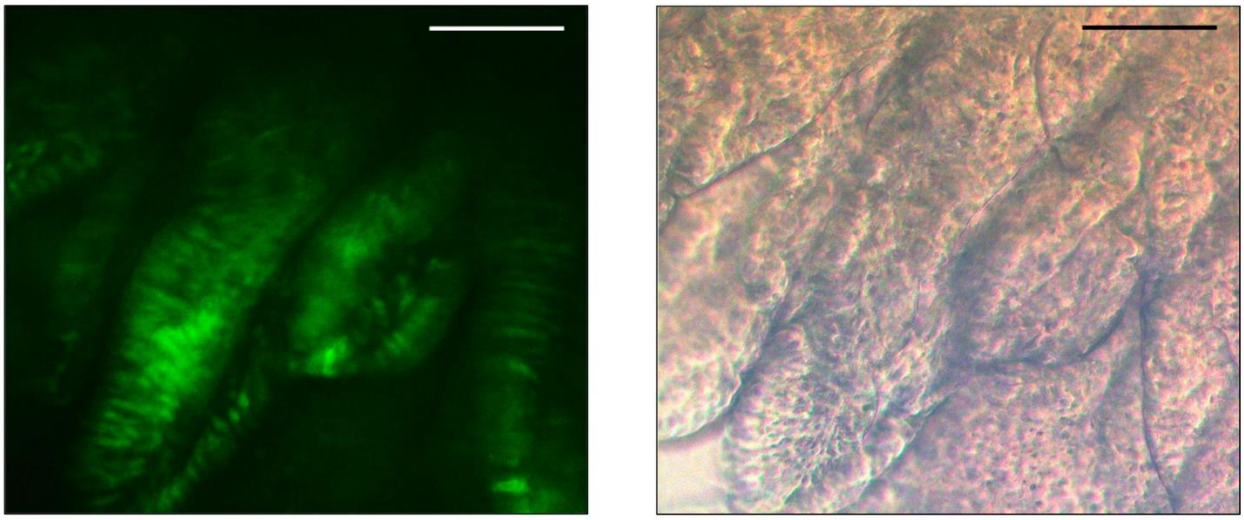
Figure 17: Western blot analysis (3)



Western blot analysis of different developmental time points of transgenic Japanese quail. These time points include embryonic day (E) 13, E16, post-hatch day (D) 0, D3, and D21. The eGFP expression increases with age. Coomassie blue staining was used to ensure an equal amount of protein was loaded for each sample.

.

Figure 18: Mucosal scrapings



Representative fluorescence and bright-field microscopic images of villi in the transgenic quail small intestine. The scale bar represents 100 µm.

5.9. Tables:

Table 3. Microarray data analysis for *mucin* gene expression patterns in various tissues based on GDS3142 for the mouse and GDS3113 for the human.

Gene	Fold [a]	S. Intestine	Spleen	Muscle	Liver	Brain	Lung	Kidney	Heart	*p* Value
Mouse										
Muc13	61.2	8463 ± 448	131 ± 2	145 ± 5	144 ± 7	124 ± 9	130 ± 8	137 ± 3	157 ± 6	<0.0001
Muc3	41.5	3752 ± 1093	82 ± 8	91 ± 4	98 ± 1	77 ± 3	82 ± 2	86 ± 3	117 ± 6	<0.0001
Muc2	26.9	2570 ± 195	90 ± 3	93 ± 2	100 ± 6	85 ± 1	86 ± 3	95 ± 5	120 ± 6	<0.0001
Muc4	5.9	472 ± 40	70 ± 3	69 ± 4	73 ± 5	71 ± 1	139 ± 11	68 ± 1	71 ± 3	<0.0001
Muc5ac	1.2	157 ± 9	114 ± 4	130 ± 3	142 ± 5	113 ± 2	117 ± 5	111 ± 3	165 ± 3	<0.0001
Mucl1	1.1	77 ± 7	60 ± 3	78 ± 2	66 ± 2	63 ± 1	66 ± 5	66 ± 1	73 ± 4	<0.05
Muc5b	1.1	158 ± 3	147 ± 2	119 ± 3	153 ± 13	104 ± 2	243 ± 21	132 ± 1	141 ± 7	>0.05
Muc16	1.0	95 ± 2	74 ± 6	77 ± 1	96 ± 6	73 ± 4	124 ± 7	80 ± 2	114 ± 2	>0.05
Muc20	1.0	109 ± 6	108 ± 6	106 ± 7	107 ± 7	105 ± 7	95 ± 3	104 ± 8	125 ± 4	>0.05
Muc15	1.0	71 ± 5	63 ± 1	69 ± 3	75 ± 6	71 ± 3	67 ± 2	68 ± 2	76 ± 3	>0.05
Muc1	0.6	144 ± 4	98 ± 6	102 ± 7	102 ± 4	85 ± 2	916 ± 33	198 ± 23	127 ± 4	NA
Human										
Muc13	50.3	100,638 ± 785	204 ± 10	250 ± 12	663 ± 172	277 ± 38	927 ± 659	11,295 ± 945	402 ± 128	<0.0001
Muc2	31.7	26,029 ± 501	339 ± 35	1168 ± 318	1498 ± 852	891 ± 324	694 ± 186	613 ± 24	550 ± 45	<0.0001
Muc20	2.0	50,305 ± 651	7068 ± 83	9927 ± 289	24,134 ± 413	2257 ± 260	27,753 ± 1550	103,500 ± 1705	5216 ± 354	<0.0001
Muc7	0.8	463 ± 78	400 ± 106	687 ± 163	1219 ± 740	494 ± 213	443 ± 143	370 ± 114	484 ± 115	NA
Muc21	0.5	429 ± 113	559 ± 320	431 ± 5	1744 ± 315	1048 ± 303	598 ± 14	999 ± 208	450 ± 118	NA
Mucl1	0.5	345 ± 96	1267 ± 780	613 ± 87	1032 ± 237	633 ± 83	549 ± 327	415 ± 116	511 ± 142	NA
Muc4	0.4	1310 ± 339	470 ± 79	730 ± 372	358 ± 79	314 ± 59	18,487 ± 1343	496 ± 279	357 ± 110	NA
Muc15	0.1	374 ± 130	929 ± 152	820 ± 224	540 ± 125	598 ± 121	4075 ± 536	19709 ± 895	611 ± 156	NA

[a] The fold was calculated by dividing the value of the small intestine by an average value of the other tissues. NA: not available.

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Chapter 5 (Previously published work)

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