UTILIZING THE PIGGYBAC TRANSPOSON IN TRANSGENIC CHICKEN STRATEGIES

by

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(Under the Direction of Robert Beckstead)

ABSTRACT

The chicken is a well-established model system for studying vertebrate embryogenesis, but creating transgenic birds has proven difficult. Viral infections have been predominantly used to insert transgenes and have been moderately successful, however, the rate of germ line infection is low and the virus is not easily manipulated in the laboratory. To increase efficiency and ease of production we are using *piggyBac*, a transposable element (TE) system, paired with an in-vivo transfection reagent, JetPEI, to generate transgenic chicks.

The *piggyBac* system utilizes a transposase enzyme, which recognizes a specific DNA sequence called a transposon. The enzyme excises the transposon from its original location in the DNA and inserts it into a new genomic location. The transposon used in our studies contains a constitutively expressed green fluorescent protein (GFP) gene for tracking insertion by fluorescent microscopy. The TE system is delivered to cells using JetPEI, an in-vivo transfection reagent. JetPEI forms a capsule around DNA, which is endocytosed by cells. Once inside, the capsules rupture and TE DNA is released into the cell to integrate into the genome. The current question with this system is two-fold. First, is JetPEI able to transfect developing chick cells, namely germ cells and, second, will *piggyBac* efficiently integrate into the chick genome.

To answer this question we prepared a mixture of JetPEI with the *piggyBac* system. This was injected into Stage X white leghorn embryos which were incubated to hatch. Hatched chicks of differing ages were euthanized to evaluate GFP expression. Imaging showed GFP expression in multiple tissue types from all three germ layers. 6/18 males expressed GFP in the testes, which co-localized with positive staining from germ cell antibodies showing that JetPEI will transfect germ cells. Stable expression of GFP in multiple tissue types including testes from different age chicks indicates that *piggyBac* has integrated into the genome and is actively expressing the transgene. The efficiency and ease of manipulation of *piggyBac* in combination with JetPEI make this a powerful system for creating transgenic chicks.

INDEX WORDS: Transgenic chicken, *piggyBac*, JetPEI, Transposable element, in-vivo transfection reagent, germ cell, chick

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PRODUCTION OF TRANSGENIC CHICKENS USING THE *PIGGYBAC* TRANSPOSABLE ELEMENT

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DEDICATION

I would like to dedicate this dissertation to my wonderful and loving wife, Claibourne Jordan. Without her support and motivation this surely would not have been possible.

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CHAPTER 1

INTRODUCTION

The chick has been a model organism for developmental study for over 2000 years. The accessibility of the chick embryo for visualization and tissue manipulation has made it an ideal model for studying developmental processes (Stern, 2005) and has yielded many major discoveries in areas such as cell fate, limb development and somitogenesis (Burt, 2007). The chicken is also one of the most important animal protein sources in the world, with the US broiler industry totaling over 21 billion dollars in farm receipts in 2010 (www.usda.gov). Georgia ranks first in broiler production among all U.S. states. Understanding the genetics of and having the ability to affect the genome of the chicken is scientifically and economically important.

As molecular tools have dominated the field of developmental biology, the chick has fallen behind other systems as a genetic model. Research into developing the chick as a modern model system has been limited, as transgenic chick technology has only been recently developed (McGrew, et al., 2004) (van de Lavoir, et al., 2006). The predominant mode of transgenesis is injecting virus particles containing the transgene of interest into the sub-germinal cavity of a Stage X (EG&K) embryo blastoderm. At this stage, the embryo is 24 hours developed and is comprised of 50-60,000 cells (Spratt, 1963), of which only 100-200 become germ line cells. This low percentage of germ line cells present in the early embryo has kept transgenic efficiencies low in the chick, as it is these cells that must have the transgene for transmission to progeny. Researchers using virus injections to infect these cells have produced transgenic chickens but

with widely varying efficiencies (<1%-45%) (Rapp, et al., 2003) (McGrew, et al., 2004). There are also several negative attributes associated with using viruses to introduce genetic material. Viral DNA constructs can only accommodate small transgene fragments, usually less than 5,000 base pairs. Viral promoters are used to express the transgene *in-vivo* but are silenced in the germ line and, over time, in somatic cells, resulting in the loss of transgene expression. Upon integration, the virus deposits some of its native DNA promoter sequence into the host genome along with the transgene. In combination, these issues must be considered when choosing a transgenic strategy in the chick.

To overcome the inefficiencies and other difficulties associated with viral constructs and develop an alternative strategy for chick transgenesis, we sought to adapt reagents used in other model systems for use in the chick. Transposons are native modular DNA elements that are found in the genome of all Kingdoms of life. While most are artifacts from evolution, some are still active and have been characterized for laboratory use. The transposon contains a transposase enzyme gene flanked by inverted repeat nucleotide sequence that is specific for that transposon. When expressed, the active enzyme recognizes and binds its own inverted repeats, excises itself from the genome, then integrates itself into a new genomic locus. It is in this "cut and paste" manner that transposons move around the genomes of organisms. Transposons have been used in a variety of organisms and have been shown highly active in many vertebrate systems (Ivics, et al., 2009). For use as a molecular transgenic tool, the transposase gene can be separated from the transposon to act *in-trans*. This allows for control of transposition and affords the ability to utilize the now empty transposon to integrate a transgene of choice to the genome of a host. This ability has made transposon systems a very popular method of transgensis.

Of these transposons, the *piggyBac* system has shown promise of being the most active in transposition in vertebrate cell types and has the best integration and excision profile (Wu, et al., 2006) (Fraser, et al., 1996). *PiggyBac* has the capacity to transpose very large DNA sequences (Li, et al., 2011) making it ideal for integrating multiple sequences or large genes in a single transposition. This system has also been codon-optimized for use in mammals (Cadinanos and Bradley, 2007), which is very compatible with avian species, but had not been previously used in an avian system.

The *piggyBac* system by itself is insufficient to produce transgenic chicks. It must be paired with a reagent designed to introduce genetic material to cells *in-vivo*. For this purpose we chose a commercial *in-vivo* transfection reagent, JetPEI, to pair with our *piggyBac* transposon system. JetPEI is a linear polyethylenimine molecule developed to pack DNA or RNA into a small, charged bundle that is endocytosed by cells. It is has proven efficient at cellular transfection in multiple cell types (Al-Dosari and Gao, 2009) and has been paired with *piggyBac* in mouse cells *in-vitro* (Kang, et al., 2009). While yet unproven in the chick, the effectiveness of linear PEI in other organisms make it an ideal choice for nucleotide delivery.

While circumstantial evidence exists to support the hypothesis that pairing JetPEI and *piggyBac* would be an efficient method for transgenic chick production, there is no concrete data to support this claim. This project was designed to investigate this theory. Specifically, the aims of this project were to:

1. Determine if JetPEI could transfect cells of the early chick embryo *in-vivo*, especially germ line stem cells and their precursors;
2. Demonstrate that *piggyBac* would effectively transpose into chick cells *in-vitro* and *in-vivo*;

3. Verify that combining JetPEI and *piggyBac* would produce chimeric chicks with stable expression of the transgene in somatic and germ line cells and;
4. Analyze the efficiency of transposition in somatic and germ line cells.

During the development of this project an alternative hypothesis was posed for producing transgenic chicks. Recent evidence has shown that sperm can take up exogenous DNA and RNA and transmit a cDNA copy to the developing embryo at fertilization (Giordano, et al., 2000). While this technique has met with some success in other systems, it has not been widely used in the chick due to instability of chicken sperm outside of the bird. A sperm mediated gene transfer technique has recently been reported with high success in fish (Collares, et al., 2010), whose eggs are similar to chickens with very high yolk content. A preliminary project was designed to recapitulate this technique using chicken sperm to test the effectiveness of sperm to deliver exogenous DNA to embryos.

Specifically, this project was designed to:

1. Reproduce the previous report of sperm mediated gene transfer in fish in the chick;
2. Measure sperm quality parameters in reaction to the procedure and;
3. Obtain the transmission rate of the transgene to progeny with this technique.

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CHAPTER 2

LITERATURE REVIEW

THE CHICKEN: HISTORY AND IMPORTANCE

Domestication of the Red Jungle Fowl in India and southeast Asia nearly 8000 years ago (Fumihito, et al., 1994) began the breeding process resulting in the chicken that we know today. Initially, cock fighting played a major role in the domestication and spread of chickens throughout the ancient world, without much regard for meat or egg production (Encyclopedia Brittanica, 2007). As time progressed, breeding and selection practices in different parts of the world created hundreds of different breeds with a variety of different feather colors and physical features. Soon people began to realize the importance of chickens as a food source. Ancient Egyptians called them "the bird that lays every day" (Carter, 1923), and chickens were valued because they were cheap to acquire and maintain and could be eaten in one meal with no risk of spoilage from storage.

As people began to rely more on the chicken for food, two industries began to emerge. Many people still kept the most colorful and vibrant chickens as exhibition show animals and, just like dogs, bred them to maintain pure-bred lines. These lines continue today almost unchanged for the last one hundred years and serve as a reservoir of a multitude of chicken breeds. A separate commercial production industry also emerged as demand for chicken meat and eggs began to rise. Early on, chicken was a luxury item as most chickens were grown only to feed the family and not produced on a commercial scale. President Herbert Hoover promised in his 1928 campaign "a chicken in every pot and a car in every garage" promoting the idea that

people would prosper under his presidency and would be able to afford chicken on a regular basis. Production of chicken increased during World War II when beef was rationed for the war effort. The eastern shore of Delaware, Maryland and Virginia, the southern states and parts of California prospered from poultry production and began to strive to breed the perfect chicken. As companies produced better birds through genetic selection for important traits, two very distinct lines of commercial chickens were developed. Meat type chickens were selected for their ability to grow to market weight in weeks instead of months with less feed than before. Egg type chickens were selected for their propensity to lay an egg of uniform size and weight every day. Selection along these lines has continued through the present to give us the breasts, wings, thighs, drumsticks and eggs we see in grocery stores today.

Innovation and development in equipment, husbandry and disease prevention rose concordantly with the increase in poultry production. Research at universities led to innovations in housing, vaccines and feed rations along with new technologies in processing, packaging and storage. In 2010 the United States broiler industry had an economic impact of 45 billion dollars with farm receipts totaling over 21 billion dollars (data provided by USDA). The United States has become the number one producer of poultry in the world, making chicken a valuable commodity in the dynamic of the US economy.

While the chicken is most widely known as a food source, it has also been an excellent model for basic scientific study, especially the study of development. Over 2000 years ago Aristotle studied and taught "embryology" at the Lyceum, using incubated chicken embryos as his model. His famous work, *Historia Animalum,* is regarded as the first reference to the chick embryo as a model for embryology. From his observations of chick embryos, he concluded that preformation was an incorrect theory and that all animals, including humans, developed in a

step-wise manner into an adult form. Study of the chick embryo since that time has led to many fundamental discoveries in the realm of developmental biology. Studies during the 17th and 19th centuries led to the findings of blood circulation (Harvey, 1628) and the neural tube, somites and evidence of a beating heart before any blood formation (Malpighi, 1672, 1675) . Separate germ layers (Pander, 1817) (Von Baer, 1828) and the neural crest (His, 1868) were also discovered in the chick during this time. After the turn of the 20th century, developmental studies in the chick furthered our understanding of gastrulation cell movements (Gräper, 1929), embryo polarity regulation (Waddington, 1932), formation of limb buds through discovery of the Apical Ectodermal Ridge (AER) (Saunders, 1948) and grafting experiments (Tickle, et al., 1975), and migration and differentiation of cell populations through the use of quail-chick chimeras (Douarin, 1973). Even chicken mutants such as the talpid3 have been used to study limb development (Ede and Kelly, 1964) (Francis-West, et al., 1995). Work in the chick embryo has contributed many key concepts and developmental facts that prove to be true across multiple species and has been a predominant model in embryological studies.

While the chick embryo is known for advancements in the developmental biology world, some of the most momentous findings associated with it have come in the fields of virology, cancer and immunology. The discovery of Rous Sarcoma Virus in the chick established a link between cancer and viruses (Rous, 1911)(Nobel Prize 1966) and helped lead to the isolation and characterization of the first cellular oncogene (c-src) (Stehelin, et al., 1976) (Nobel Prize 1989). Reverse transcriptase and the "DNA provirus hypothesis", which exposed the mechanisms by which RNA viruses become incorporated into the host's genome (Temin, 1964) (Temin and Mizutami, 1970) (Nobel Prize 1975), were discovered and formulated in the chick embryo. The discovery of the function of the Bursa of Fabricious and its role in B-lymphocyte production in

chickens (Glick, et al., 1956) was of huge importance and opened the door to the discovery of T-lymphocyte origin in the thymus. Genomics was also being practiced over 100 years ago and terms such as alleles, genetic linkage and epistasis were coined from work on phenotypic traits in the chicken (Bateson and Saunders, 1902) (Sutton, 1903) (Bateson and Punnett, 1911). The first genetic maps created used sex-linked traits in the chicken (Spillman, 1909) and soon thereafter, a genetic linkage map of the chicken was created (Serebrovsky and Petrov, 1930) (Hutt, 1936). More recently, the discovery of the DT-40 vertebrate (chick) cell line that undergoes homologous recombination between transfected and genomic DNA as efficiently as budding yeast (Buerstedde and Takeda, 1991) has led to many profound discoveries. As molecular technologies have advanced, so too has research in the chicken, with transgenesis becoming a predominant mode of studying genetic function. Advances in chick transgenesis will be discussed in a later section.

As discussed above, the chicken and chick embryo are significant players in two distinct fields; agriculture and biological science. Since chicken meat and eggs are some of the cheapest and most nutritious animal protein sources in the world, it is understandable that applied researchers would want a thorough understanding of chick development. For the basic researcher however, the chick embryo also provides an advantageous model with a substantial history of scientific discovery. Being applicable to both areas of research makes the chick an invaluable system.

THE CHICKEN: ANATOMY OF REPRODUCTION

The structure of avian sperm is similar to many other species and is composed of a head containing the nucleus and acrosome, a mid-piece where the mitochondria are housed and a long flagellum (Bakst and Howarth, 1975) (Thurston and Hess, 1987). Avian spermatogonial cells

have been characterized into four types based on structural differences and staining patterns (Lin and Jones, 1992) and proceed through cell divisions to give 32 spermatids per immature spermatogonial cell. Spermiogenesis is the transformation of spermatids into mature sperm cells without further cell divisions and is carried out in a stepwise manner (de Reviers, 1971) (Reviers, 1971) (Gunawardana and Scott, 1977). Following spermiation, chicken sperm are suspended in seminal fluid secreted from cells lining the seminiferous tubules. Chicken sperm are immotile when stored in the excurrent ducts (Ashizawa and Sano, 1990) and only become motile upon ejaculation (Munro, 1938) (Howarth, 1983).

The hen reproductive tract consists of the ovary and the oviduct. During embryonic development, the distribution of primordial germ cells becomes asymmetrical, with a larger portion populating the left gonad. By incubation day 10 the right ovary and oviduct begin to regress (Hutson, et al., 1985), with regression complete by hatch. Most avian species develop only one ovary and oviduct, a process deemed to be a way of minimizing weight to enhance flight. At hatch, the female chick ovary contains all of the oocytes and oogenesis ceases. Once females reach sexual maturity (~20 weeks in chicken) the oocytes are arranged into follicles with an obvious hierarchy based on size determined by yolk deposition. The hierarchy is as follows: the F1 follicle is the largest and most mature, containing the most yolk. The F2 follicle is the next largest followed by F3, F4 and F5. There are also thousands of additional follicles visible in the ovary designated by size and color as large yellow, small yellow and small white follicles. Yolk deposition is ongoing in the large yellows, just begun in the small yellows and has not begun in the small whites. The F1 follicle is the first to be ovulated. After F1 ovulation, the follicle is considered post-ovulatory and is resorbed by the bird. The follicular hierarchy then shifts so that the F2 is now designated as the F1 and so forth down the cascade. A new large

yellow follicle will then join the hierarchy at the F5 position. During the time prior to the next ovulation, yolk is being deposited into each follicle of the hierarchy so that they may be proper size upon ovulation. Ovulation occurs every 24-26 hours, and is regulated by complex hormonal control and environmental factors (Etches, 1996).

Upon ovulation the oocyte is "captured" by the uppermost portion of the oviduct, the infundibulum. Here fertilization occurs and the first layer of albumin is produced. Fertilization proceeds when one sperm penetrates the vitelline membrane and fuses with the oocyte at the germinal disc. The ovum then moves down the oviduct into the magnum where most of the albumin is added. The ovum proceeds to the isthmus where shell membranes are added. From here, the ovum continues into the shell gland (uterus) where the hard, calcium rich shell is added. The newly formed egg spends the majority of its time in the shell gland (~20 hrs in chicken). Upon oviposition, the egg leaves the shell gland and passes through the uterovaginal sphincter into the vagina. The vagina plays no actual role in the development of the embryo or formation of the egg. The uterovaginal region does function in storing sperm in specialized sperm-storage tubules. As many as 100 sperm may be stored in these tubules (Bakst, et al., 1994) and they function to maintain fertilization of a clutch of eggs without the need for synchronized copulation. The egg spends no real time in the vagina and is immediately laid by being expelled through the cloaca. The time from ovulation to oviposition corresponds to the length of time between each egg laid which, in chicken, is ~24 hrs.

THE CHICKEN: EARLY EMBRYONIC DEVELOPMENT

In chickens, the female or hen, is the heterogametic sex, possessing a Z and a W sex chromosome while the male or rooster, is homogametic, possessing only Z chromosomes. In this scenario, the female determines the sex of the progeny (Hance, 1926). In nature, copulation

occurs when a rooster perches on a hen's back and drips semen from a rudimentary phallus onto the exposed cloaca of the hen. The hen then takes the sperm into the vagina through reverse peristalsis, where it is stored in sperm-storage tubules until needed for fertilization. Sperm leave the tubules and travel up the oviduct to the infundibulum, where fertilization occurs. When coming into contact with the ovum, sperm bind to receptors on the outer membrane (Robertson, et al., 2000) and dissolve the vitelline membrane with hydrolytic enzymes released from the acrosome (Wishart and Horrocks, 2000). Many sperm may penetrate the vitelline layer but only the sperm that penetrates at the germinal disc can fuse its nuclei with the ovum to form a zygote ~3 hours after ovulation (Waddington, et al., 1998). As the embryo passes down the subsequent portions of the oviduct, embryo development begins and continues until oviposition. During this 24-26 hour period the embryo undergoes cleavage and establishment of the axes of polarity (Waddington, 1932). At the time of lay, the embryo consists of several regions. The area opaca can be seen by the naked eye and encircles the blastodisc. Koller's sickle, marginal zones and the endophyll area are all found in the centermost area pellucida. Since the embryo has been undergoing cell divisions during the cleavage process it consists of ~40-60,000 cells at the time of lay (Stepinska and Olszanska, 1983) (Spratt, 1963).

When development proceeds after lay, embryos are staged based on developmental cues. There are two pre-dominant staging systems, that of Eyal-Giladi and Kochav (EGK) (Eyal-Giladi and Kochav, 1976) and Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951). Freshly laid, unincubated eggs are referred to as Stage X (EGK) and will remain at this stage in developmental diapause until incubated. Once warmed to proper incubation temperature (~99.5°F for chicken), the embryo will resume development and begin to form immature structures. As gastrulation begins, the primitive streak shows first, at Stage 2 (HH) and reaches

its full length by Stage 3+ (HH). By Stage 6 (HH) the head process has begun to extend anteriorly, but there is no somite formation yet. Stages 7-14 (HH) are based primarily on the number of visible pairs of somites, with the first pair being visible at Stage 7 (HH) and ~3 additional pairs being visible at each subsequent stage. Stage 7 (HH) also corresponds to ~24 hours development. At Stage 10 (HH) the heart tube begins to form and fold out of the embryo. The eye has also begun to form at this time. Stages 13 (~48 hrs development)-15 (HH) see the embryo turn onto its left side, the heart clearly beating and pumping blood through early atria and ventricles and the clear definition of the eye and lens. By Stage 20 (HH) (~72 hrs development), the limb buds are defined and distinguishable, the tail bud has formed, the pharyngeal arches are present and the allantois is becoming enlarged.

Of particular interest from a transgenic perspective is the development and maturation of primordial germ cells (PGC's). PGC's are a morphologically distinct cell type and are easily distinguishable through staining techniques (Sang, 2004). They are present but dispersed in the sub-germinal cavity of Stage X (EGK) embryos (Petitte, et al., 1997) in the form of endophyll cells. As mentioned above, at oviposition the blastoderm is composed of ~40-60,000 cells. Of these, 150-200 are endophyll cells. Endophyll cells differentiate into germ cells in the early area pellucida and begin migration during the early stages of development. By Stage 5 (HH) they have moved out of the embryo proper and into the germinal crescent at the anterior end of the area pellucida. Between stages 10 and 13 the germ cells migrate into the blood stream where they are circulated around the embryo. Germ cells leave the blood stream at ~Stage 16-17 (HH) and move through the gut to the forming gonadal ridges. Several theories exist as to the mechanism of this migration, but none are considered complete. By three days incubation (Stage

20, HH) germ cell population of the gonads is complete. From here the germ cells will proliferate and eventually differentiate into spermatozoa or ovum in mature birds.

TRANSGENIC TECHNOLOGY IN THE CHICK

It seems only natural to think that with the host of seminal discoveries made in the chick, it would maintain itself as a leading model system in the modern research era. This has not been entirely the case, however. The chicken remains at the forefront of research in the applied and industrial area, but has not kept pace with other model systems in developmental biology. In the last 30 years, molecular genomics has taken the developmental biology field by storm, with new tools becoming available at an incredible pace. One such tool is transgenesis, first developed in mice in the early 1980's (Gordon and Ruddle, 1981). Since that time transgenic technology has become the predominant standard for genetic analysis. Transgenesis is the process of introducing new traits into animals by incorporating the genes that encode those traits into the host genome. A second caveat of transgenesis is that the novel trait must then be passed on to the progeny of that animal thereby demonstrating inheritability. In order to transmit a trait to progeny, that gene must be integrated to the germ cells of the parent line.

With the chick being an historical leader in developmental research, one would assume it would also be a model system in transgenic research. Indeed it would be of great interest to pair molecular genetic data from the chick with past phenotypic observations from tissue manipulation and disruption studies. In many ways, the chick is an outstanding model for transgenic research. First and foremost is the availability and accessibility to chick embryos and the ease of which they can be manipulated (Stern, 2005). With an almost constant production of 1 egg per hen per day and the ability of a rooster to fertilize up to 5000 eggs per month (personal communication), the chick system provides a screening potential rivaling that of *Drosophila*.

Another attribute of the chicken is a short gestational period and short time to sexual maturity. Fertilization of an embryo to day of hatch is only 22 days (including the 24 hrs in the oviduct) and from hatch to sexual maturity is 4-5 months, depending on the breed of chick. Chickens are also relatively easy and inexpensive to maintain and propagate so greater numbers can be kept for research. And since germ cells are of the utmost importance in the realm of transgenesis, one would think using the chick model would be beneficial since the location of germ cells at lay is known, as is the germ cell migration pattern. But in this lies the major obstacle to chick transgenesis: the embryo has already developed for 24 hours before researchers are able to access the embryo.

As mentioned earlier, the embryo consists of 40-60,000 cells at lay, of which only 150-200 are germ cell precursors. This low percentage makes it increasingly difficult to target germ cells in a Stage X (EGK) embryo. In other model systems such as mouse, *Drosophila*, *Xenopus*, or *C. elegans*, one can target embryos at the 1 or very few cell stage. This greatly increases the percentage of transgenic germ cells as all of the cells derived from those original cells should carry the transgene. While transgenesis may be easier in other systems, that is not to say it has not been accomplished in the chick. Repetition of the original pro-nuclear injection procedure from the mouse was carried out in chick, with a very low level of success (Love, et al., 1994), as oocytes are not conducive to culture through to hatch. Petitte et al., (1990) were the first to show that blastodermal cells from one embryo could be transferred through injection to another Stage X blastoderm and produce chimeric birds. This method was improved upon by damaging the recipient embryo through radiation, which increased chimerism greatly (Carsience, et al., 1993). These early experiments showed the potential for chick transgenesis and that blastodermal cells of chick embryos held the potential for efficient production of transgenic offspring.

Efforts to isolate and culture embryonic stem cell (ESC) like blastodermal cells were also attempted in parallel with direct injection experiments. Brazolot et al., (1991) were able to maintain blastodermal cells for a short time in culture and transfect them with *lacZ* before injection to recipient embryos. Their results showed that these cells would integrate into the somatic tissue of the developing embryo. Conditions were eventually established to maintain cells in culture long term (Pain, et al., 1996) and germ-line chimeric chicks were created with these cells from short-term cultures (Speksnijder, et al., 1999). Although a promising technology, these ESC like cells have not proven a full pluripotency potential and still remain difficult to culture. For these reasons, researchers turned to primordial germ cell isolation and culture methods for the production of transgenic chickens. PGC's have been isolated from multiple places in the embryo at varying stages of development including the germinal crescent (Vick, et al., 1993a), the circulating blood (Tajima, et al., 1993) and the partially developed gonad (Jae Yong, et al., 2002). In all of these cases, introduced PGC's of differing breeds were shown to contribute to the germ line with varying efficiency. Just as with blastodermal cells, removal of native PGC's either through chemical ablation or physical removal increased germ line efficiency of these PGC's (Vick, et al., 1993b) (Naito, et al., 1994) (Kagami, et al., 1997) and more recently (Nakamura, et al., 2010) (Park, et al., 2010). Initial studies described production of germ line chimeras from genetically altered PGC's (Vick, et al., 1993b) (Wentworth, et al., 1996) and Naito et al. (1998) described the transfection of PGC's with liposomes and the ability to detect the transgene in germ cells of gonads.

While transgenesis with PGC's from different breeds of chicken had proven successful, the inability to produce transgenic chicks at a useful frequency from genetically altered PGC's still limited this technology. Breakthroughs in PGC technology came with the standardization of

long-term culture conditions, first for gonadal derived PGC's (Jae Yong, et al., 2002) and then migrating PGC's (van de Lavoir, et al., 2006). In the same set of experiments, van de Lavoir et al. showed the ability to genetically alter the cultured PGC's and produce second generation transgenic chicks. Since that time the culture procedure has been reproduced in another breed of chickens (Macdonald, et al., 2010) and PGC's have been modified in culture by multiple methods (Leighton, et al., 2008) (Motono, et al., 2010). Isolation methods have also improved (Mozdziak, et al., 2005; Yamamoto, et al., 2007) which is crucial to the efficient production of transgenic chicks from germ cells due to the small percentage of germ cells to total cells at any time of chick development. Most recently Lu et al. (2012) have reported the production and integration of induced pluripotent stem cells from quail injected into chicken which bypasses the need for isolation of PGC's.

Although germ cell culture and modification has improved greatly in the last 15 years, the predominant method of producing transgenic chicks has been through the use of viral injections into the Stage X (EGK) sub-germinal cavity of developing chick embryos. Viral vectors work by cloning the transgene of interest into virus DNA constructs and then making a viral particle in cell culture that will package the transgene into an active virus. Viruses are then injected into tissues and infect as they normally would. Native viral machinery then integrates the transgene into the host cell genome. Avian leucosis retroviruses (ALV) were used early on in the development of avian transgenic technology (Salter and Crittenden, 1989) (Thoraval, et al., 1995) along with reticuloendotheliosis virus (REV) (Bosselman, et al., 1989). Following initial discoveries, this technology was shelved for some time due to lack of available vector systems and fear of using vectors from viruses that are widespread in the poultry industry (Sang, 2004). Avian retroviruses were revisited later by Harvey et al. (2002) and Rapp et al. (2003), but these

experiments were met with very low transmission rates (<1%) not suitable for studies using multiple gene expression vectors. Retroviral vectors were soon replaced with lenti-viral vectors that showed a much higher propensity for transgenic production and germline transmission (McGrew, et al., 2004) and have been the predominant vector system used since (Chapman, et al., 2005) (Scott and Lois, 2006) (Lillico, et al., 2007) (Lyall, et al., 2011). There are several types of vectors based on varying viral backbones, with transgenic efficiency being different for each.

There are, however, many problems associated with using virus to introduce genetic material. Viral DNA constructs can only accommodate small transgene fragments (Scott, et al., 2010). This limits the size of integratable sequence to ~5,000 base pairs, which is a relatively small amount of sequence. Viral promoters are used to express the transgene in-vivo but are silenced in the germ line and, over time, in somatic cells resulting in loss of expression of the transgene. Upon the low frequency of successful integration, the virus deposits some of its native DNA promoter sequence into the host genome along with the transgene. In combination, these negative attributes must be considered when choosing a transgenic strategy in the chick.

TRANSPOSONS

When considering transgenic chick production methods, one cannot argue that the lenti-viral systems available today are the most efficient. But with the drawbacks listed above, other methods of stable and efficient chick transgenesis are being sought out. DNA transposons are a group of DNA elements that naturally occur in all kingdoms of life and were first discovered and characterized in Maize (McClintock, 1950). In nature, they are a single fragment of DNA that contains the gene for a transposase enzyme flanked by transposon inverted repeats (Figure 3.1). When the transposase gene is transcribed and the mRNA translated into a functional protein, the

enzyme recognizes its own inverted repeats and cleaves itself from the DNA. The transposase then searches the DNA for a new site for transposition, thereby carrying itself around the genome with a "cut and paste" mechanism. Transposon integrations throughout evolution have played a direct role in genome size, with larger genomes carrying a higher percentage of stable transpositions (Ågren and Wright, 2011). Many transposon systems have been identified from multiple Kingdoms (fungi, plants, animals) and species (nematodes, arthropods, fish, frogs and humans) (Plasterk, et al., 1999), but most are remnants of long inactivated transposons. Most characterized transposons belong to the Tc1/*mariner* superfamily that consists of three smaller families: Tc1, *pogo*, and *mariner* (Robertson, 1995). The most common of these family members are Tc1, Minos and *Sleeping Beauty* (SB). Transposons were first used successfully in plant and invertebrate models, including *C. elegans* (Rushworth, et al., 1993) (Bessereau, et al., 2001) and *Drosophila* (Spradling, 1986) (Cooley, et al., 1988) (Bellen, et al., 2004). Initially there were no transposon systems that would efficiently integrate and be active in vertebrate models. That changed in 1997 when Ivics et al. molecularly reconstructed a dead Tc1/*mariner* type transposon named *Sleeping Beauty* (SB) found in several fish genomes (Ivics, et al., 1997).

Since the emergence of SB, vertebrate transposon transgenesis has increased, with several different transposon systems showing efficient transposition in vertebrate models. *PiggyBac* and *Minos* from drosophila, *Frog Prince* from amphibians, *Hsmar1* from humans and *Harbinger3_DR*, *Tol1* and *Tol2* from fish have all shown activity in vertebrate cell lines or *in-vivo* (Ding, et al., 2005) (Wilson, et al., 2007) (Pavlopoulos, et al., 2007) (Miskey, et al., 2003) (Miskey, et al., 2007) (Sinzelle, et al., 2008) (Koga, et al., 2008) (Kawakami, et al., 2000). Indeed even the *Ac/Ds* element originally discovered by McClintock in maize will efficiently transpose in zebrafish embryos (Emelyanov, et al., 2006), indicating that some of these

transposons have the ability to remain active across not just species, but Kingdoms as well. Each transposon has its own unique characteristics that make it more or less suitable for genetic modification processes. All transposons show some type of integration site specificity, ranging from a simple AT dinucleotide in Tc1/*mariner* family members (Thomas, et al., 2002) to 15 nucleotide palindromic sequences for *Harbinger_D3* (Sinzelle, et al., 2008). They are also influenced by DNA structure at the chromatin level for integration (Geurts, et al., 2006) and some have a tendency for "local hopping," i.e transposition very close to the original insertion site. With the variety of transposons available however, a system to fit the experimental needs can be found.

Transposons have several features that make them an ideal choice for genetic modification. A major advantage over viruses is their ability to be separated from their transposase gene. In fact, the transposase gene can be removed from within the inverted repeats and replaced with almost any sequence. The transposase gene can then be expressed *in-trans* from a helper plasmid or introduced as mRNA directly, thereby promoting earlier transposon integration. Separating the transposase from the transposon makes controlling integration rates easier and supports stable integration events and long-term expression of the transgene of interest. Transposition can be controlled by titrating the amount of transposase encoding plasmid or mRNA (Wang, et al., 2008), thereby limiting the amount of enzyme available for integration. Injecting transposase mRNA instead of plasmid DNA also gives the advantage of earlier transposition since the cell does not have to produce the mRNA itself and more stable integration since the mRNA will be degraded by the cell. Another distinct advantage of transposons, in general, over viruses is their ability to mobilize larger cargo sets. Viral constructs can only support 5-8 kilobases of sequence (Scott, et al., 2010), whereas transposons have been shown to

move cargo from 10 up to 100 kilobases (Koga, et al., 2007) (Balciunas, et al., 2006) (Urasaki, et al., 2006) (Li, et al., 2011), depending on which system is used. The increased cargo can be almost any type of DNA element as well. A third advantage to using transposons is no silencing effects. Viral integration mechanisms often form sequences and the viruses themselves contain sequences that are targeted for gene silencing (Ivics and Izsvák, 2010). In contrast, transposon integrations are rarely silenced, with less than 4% of all integrations from SB, *Tol2*, and PB being silenced in HeLa cells (Grabundzija, et al., 2010). Even with these qualities, transposon mediated transgenesis has been lacking in the chicken model, with only a few instances reported (Sherman, et al., 1998) (Sato, et al., 2007) (Lu, et al., 2009). For all the reasons listed above, we chose to use a transposon system instead of viruses in our transgenic chick strategy.

PIGGYBAC

PiggyBac (PB) is a DNA transposon system that was originally isolated from the Cabbage Looper moth, *Trichoplusia ni* (Cary, et al., 1989) and has been shown to be active in several invertebrate systems (Lobo, et al., 1999) (Thibault, et al., 2004). More recently, *piggyBac* has been used in mammalian cells including mouse (Ding, et al., 2005) and human (Wilson, et al., 2007) to achieve efficient integration. The transposase gene has also been optimized for mammalian codon usage (Cadinanos and Bradley, 2007), making it much more efficient in vertebrate systems. PB has only recently become a major player in transposon mediated transgenesis as *Sleeping Beauty* was the main system for many years. There are several reasons why PB has recently been utilized, all of which are important to consider when deciding on a transposon system.

First, analysis of *piggyBac* insertions and transpositions revealed that removal of the transposon through natural transposition restored the native TTAA genomic integration site

(Fraser, et al., 1996) (Elick, et al., 1996). This is not the case for other transposons, which may leave behind or take additional sequence upon transposition. This fact has been exploited by researchers to create transgene free induced pluripotent stem cells (Woltjen, et al., 2011) by removing the pluripotent transgenes once a pluripotent state was achieved and native genes were reactivated. Second, native PB transposase activity is higher than that of several other transposon systems including *Tol2*, *Mos1*, and two forms of a hyperactive *Sleeping Beauty* transposase, SB11 and SB12 (Wu, et al., 2006) (Wilson, et al., 2007). Subsequent to those findings, a hyperactive form of PB has been generated through mutant screening in yeast (Yusa, et al., 2011) and characterized in mouse ES cells. In addition to being more active in mouse ES cell culture, this hyperactive form of PB maintains the traceless excision property of native PB. A third beneficial property of the *piggyBac* system in its propensity to integrate into transcription units, with a preference for start sites (Wilson, et al., 2007) (Wang, et al., 2008) (Liang, et al., 2009) (Galvan, et al., 2009). This attribute makes PB ideal for genome-wide mutagenesis screens and directly relevant to the developmental biologist. The final trait of PB that makes it stand out is the ability to transpose large DNA sequences. Ding et al. (2005) reported the capability to transpose >9 kilobases without any reduced activity. More recently, Li et al. (2011) showed that PB has the capacity to transpose 100 kilobases of sequence and retain precise integration and excision profiles with stable expression of the large transgene. Taken together, the characteristics of *piggyBac* make it an excellent system for the efficient integration of transgenes and pursuit of transgenic animals for developmental or mutagenic study.

IN-VIVO TRANSFECTION

While transposon systems provide a very viable solution for integrating transgenes, they do not provide a system in which to introduce the DNA to cells *in-vitro* or *in-vivo*. This must be

overcome to develop a transgenic strategy for any animal. Viral infection has been a popular strategy for gene delivery but has many drawbacks including host immune response, DNA cargo capacity limitations and high cost. Alternatively, there are many brands of non-viral gene delivery reagents available that have shown promise for cellular transfection with relatively no adverse cellular reactions (Dinçer, et al., 2005). These non-viral systems can be divide into two categories; lipid or polymer based cationic complexes (Felgner, et al., 1987) (Wu and Wu, 1987). Both systems act by complexing negatively charged DNA into positively charged bundles (Yamano, et al., 2010). These positively charged bundles then bind with the negatively charged cell membrane through an electrostatic interaction (Boussif, et al., 1995) (Pires, et al., 1999) (Simões, et al., 1999). The bundles are endocytosed by cells wherein the DNA is released with differing mechanisms, depending on the type of reagent used (Tros de Ilarduya, et al., 2010). Once inside the cell, DNA is released by the transfection reagent and enters the nucleus either through passive entry during the cell cycle when the nuclear membrane is disintegrated (Brunner, et al., 2000) or by active entry through nuclear pores. Understanding the mechanism involved in transport of DNA into the nucleus is critical as nuclear uptake is a significant barrier to gene delivery (Zabner, et al., 1995).

As mentioned above, these differing types of transfection reagents operate in the same manner (Elouahabi and Ruysschaert, 2005), by bundling DNA for cell delivery. Of the two types, lipid based transfection, or lipofection, has been the most studied and used nonviral gene delivery method (Al-Dosari and Gao, 2009). Indeed, lipoplexes have been used in a variety of forms for gene delivery in multiple tissues (Morille, et al., 2008) including 110 clinical trials (6.2% of total) for gene therapy (http://www.wiley.com/legacy/wileychi/genmed/clinical/). Although popular, lipid-based transfection strategies have limitations. When designing an

experiment where gene delivery is necessary, transfection efficiency is critical. Lipid-based systems have been shown to vary greatly in efficiency based on the structure of the lipid, the charge ratio used to form DNA-lipid complexes and properties of any co-lipids used (Wasungu and Hoekstra, 2006). While there have been many formulations to try and overcome these issues, transfection efficiency is still lower with true lipoplex complexes than other systems. Another hindrance of lipoplex systems is that cationic lipids do not circulate for very long in the bloodstream. Cationic lipids are eliminated upon interaction with serum molecules or blood components and therefore initially accumulate in the pulmonary vasculature (Liu, et al., 1997) and, later, in the liver. This makes transfection of pulmonary vasculature more efficient (Barron, et al., 1999) but hinders delivery of DNA to other tissues through commonly used intravenous injection. Coating the lipolexes with substances such as polyethyleneglycol will increase duration of circulation in the blood but at the expense of transfection efficiency (Pedroso de Lima, et al., 2001). The biggest drawback to using lipid based transfection is cell toxicity and short-term gene expression (Al-Dosari and Gao, 2009). Most lipoplexes cause induction of pro-inflammatory cytokines (Tousignant, et al., 2000) (Li, et al., 1999) (Sakurai, et al., 2002) as well as increased leukocyte and thrombocyte counts and liver enzymes (Tousignant, et al., 2000) which leads to cell death and limited gene expression. Gene expression in furthermore limited by lipoplexes cell cycle dependent mechanism of DNA release and entry into the nucleus of transfected cells (Brunner, et al., 2000), limiting expression to only actively dividing cells with accessible nuclei. It is for these reasons that other gene delivery systems have been widely used as of late.

Cationic polymers are a growing class of nonviral gene delivery reagents that include histones, synthetic polypeptides, polyethylenimine (PEI) and chitosan to name a few. Polyplexes

are usually favored to lipoplexes because they are typically more stable, even after endosomal escape inside the cell (Al-Dosari and Gao, 2009). Of these, PEI has been widely used since its inception in 1995 (Boussif, et al., 1995) and is considered the most effective polymer based transfection reagent (Al-Dosari and Gao, 2009). Polyethylenimine comes in several forms but the linear, low molecular weight formulation is the most effective at cellular transfection with little cytotoxic response (Gosselin, et al., 2001) (Wightman, et al., 2001). PEI has been used to deliver genes to many tissue types in-vivo including lung (Wiseman, et al., 2003), brain (Hassani, et al., 2007), pancreas (Vernejoul, et al., 2002), retina (Liao and Yau, 2007), bladder (Ohana, et al., 2005), and tumor cancers (Scaiewicz, et al., 2010). It has also been used to deliver siRNA molecules in-vivo (Aigner, 2006) (Urban-Klein, et al., 2005). More importantly, PEI has been shown to transfect primary cells such as mouse stem cells (Yamano, et al., 2010) which can be difficult to transfect.

Polyethylinimine has proven to be a very flexible gene delivery reagent in the types of cells and tissues it can transfect, but it also has other advantages over polyplex and lipoplex systems as well. The most direct benefit of PEI is no immune response from either single or systemic delivery of PEI/DNA complexes (Bonnet, et al., 2008) (Hwang and Davis, 2001). This allows for repeated administration in gene therapy protocols or lab trials. The polyehtylenimine polymer itself also seems to aid in DNA protection from degradation inside the cell. Complexes of PEI exert a “proton sponge effect” inside endosomes, essentially neutralizing the pH by absorbing incoming protons onto non-protonated amines in the polymer (Akinc, et al., 2005). This absorption leads to an influx of chloride ions and causes increased osmotic pressure inside the endosome, eventually causing it to rupture (Sonawane, et al., 2003). Through this mechanism the PEI polymer protects the DNA by slowing the acidification process that is necessary for

endosome to lysosome transition and facilitates escape from the endosome inside the cell. Even after release from the endosomal compartment, there is evidence that PEI polymers remain stable until the complex is transported to the nucleus, wherein the complex disintegrates and DNA is released (Chen, et al., 2008). Intact complexes inside the cytoplasmic component of the cell would protect the DNA from degradative enzyme activity and ensure intact DNA delivery to the cell nucleus and host genome. This mechanism could also explain the reported cell cycle independence of linear PEI transfections (Brunner, et al., 2002). All the advantages listed above in concert with multiple routes of acute or systemic administration (http://www.polyplus-transfection.com) give credence to PEI as an efficient and multi-faceted system for gene delivery.

SPERM MEDIATED GENE TRANSFER

All methods of transgenesis, especially chick transgenesis, involve manipulating early embryos in some form. This leads to inconsistent transgenesis and mosaicism as well as embryonic death. For this reason, an alternative procedure to early embryo injections or manipulations was first introduced in 1989 (Lavitrano, et al., 1989) utilizing sperm as a carrier for transgenes. The benefits of using sperm as transgene delivery systems are many. In most animal models, sperm is readily available and can be manipulated or maintained in the laboratory. Artificial insemination techniques are developed for most model systems. The transgene of interest would be present at the single cell stage of embryonic development and should, therefore, remain in every cell of the animal if it is integrated into the genome, and no embryos or adult animals would need to be sacrificed to create the transgenic progeny. It is understandable then that development of this technique could greatly increase transgenic technologies in all model systems and possibly new ones.

The first report of using a sperm mediated gene transfer technique was by Lavitrano et al., (1989). They incubated naked plasmid DNA with mature mouse spermatids and then artificially inseminated with this sperm to achieve progeny. They initially reported detection of plasmid sequence in 30% of the progeny from this insemination, which is a robust figure. Shortly after this report, however, multiple laboratories reported an inability to reproduce the findings (Brinster, et al., 1989), indicating a lack of understanding of the inherent mechanisms involved in gene uptake and transfer or the possibility that the data was not reproducible. Since these first reports, much study has gone into elucidating the mechanisms of DNA uptake and maintenance by sperm. The ability of sperm to take in exogenous DNA was first reported by Brackett, et al., (1971) when they showed that infectious virus was produced in ovum fertilized with sperm incubated with SV40 DNA. Since that initial experiment, the actual mechanism of DNA uptake has been characterized and involves the interaction of exogenous DNA with DNA-binding proteins of 30-35 kDa which are present on the sperm cell surface (Lavitrano, et al., 1992) (Zani, et al., 1995). It has also been shown that major histocompatibility complex (MHC) II and CD4 molecules play a part in the binding and internalization of exogenous DNA (Mori, et al., 1990) (Wu, et al., 1990) (Lavitrano, et al., 1997). The ability and propensity of sperm to take up foreign DNA is, however, very counterintuitive considering that sperm are carriers of the genetic information being passed to progeny and would therefore need to be protected from random DNA intrusion. Indeed there are many self-defense mechanisms employed by sperm to prevent the uptake of exogenous DNA including inhibitory factors in the seminal plasma of mammals that prevents binding of exogenous DNA to sperm cells (Zani, et al., 1995). Internalization of exogenous DNA also triggers expression of native endonucleases in a dose dependent manner

that extensively degrades the foreign DNA molecules (Maione, et al., 1997). It is these layers of defense mechanisms that keep the genetic code carried in sperm cells intact during transmission.

A separate question in the process of sperm mediated gene transfer is what happens to DNA that is taken up by the sperm? To answer this question, we must first analyze the structure of the DNA inside the nucleus of the sperm. Studies of sperm chromatin revealed that it is nuclease-sensitive, organized into nucleosomes and has a very low level of methylation (Uschewa, et al., 1982) (Banerjee, et al., 1995; Gatewood, et al., 1990) (Gatewood, et al., 1990) (Pittoggi, et al., 1999) resembling active chromatin in somatic cells (Spadafora, 2008). Upon nucleotide sequence analysis of clones from fractions of sperm genomic DNA, enrichment in retrotransposon DNA encoding the LINE-1 open reading frame 2 (ORF 2) reverse transcriptase (RT) gene was discovered (Pittoggi, et al., 1999, 2000). This surprising finding led to experiments to determine if there was active reverse-transcription taking place inside the sperm cell nucleus. Researchers incubated sperm with only RNA molecules and were subsequently able to PCR DNA fragments from the original RNA sequence, proving that there was RT activity. They also showed that RT molecules were associated with the sperm nuclear scaffold through immunoelectron-microscopy using anti-RT antibodies (Giordano, et al., 2000). Later, it was shown that RT activity is triggered by exogenous DNA molecules as well (Pittoggi, et al., 2006). Through the process of internalization, transcription and splicing and reverse transcription a cDNA copy of the original DNA molecule is made which is stably maintained inside the sperm.

The final concern with sperm mediated gene transfer is the ability of the sperm to pass the newly synthesized cDNA “transgene” on to progeny as a functional active molecule. It is not suggested that transgenes are integrated into the genome of the sperm carrier. In actuality, most conclude that the cDNA copy maintains itself as a non-integrated episomal structure (Khoo, et

al., 1992; Kuznetsov, et al., 2000) (Kuznetsov, et al., 2000) (Collares, et al., 2010) (Collares, et al., 2011). There have been reports of genome integration when using protocols to avoid interaction with the sperm cell membrane, i.e. using liposomes, (Shemesh, et al., 2000) or a combination of liposomes and restriction endonucleases (Harel-Markowitz, et al., 2009) (Churchil, et al., 2011). Although non-integration is the predominant mechanism in sperm cells, it is accepted that the cDNA sequences are stably maintained at low-copy number (<1 copy per genome) episomally, mosaically distributed and show non-Mendelian inheritance patterns in progeny (Spadafora, 2008). It is conceded that over time the presence of these episomal cDNA copies are lost, underlying the importance of stable integration for the maintenance of transgenic animals. While still relatively underutilized as a transgenic production protocol, sperm mediated gene transfer show promise of being a universal method of transgenic animal production, with simple and efficient procedures.

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CHAPTER 3

NOVEL USE OF JETPEI AND PIGGYBAC TO TRANSFECT GERM CELLS AND STABLY EXPRESS GREEN FLUORESCENT PROTEIN IN CHICKEN[1]

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ABSTRACT

The chicken is a well-established model system for studying developmental biology and is recognized as one of the top food production animals in the world. For this reason the chicken is an excellent candidate for transgenic applications, as the technology can be applied to both areas of research. Transgenic technology has not, however, been broadly applied in the chicken model, primarily due to difficulties in establishing germ-line transmission. Transgenic technologies are available in the chick, namely using non-replicating viral particles to infect germ cell pre-cursors, but are unsuitable for many applications because of size and sequence restraints and low efficiency. To create a more versatile method of chick transgenesis, we utilized the transposable element system *piggyBac* (PB) paired with an in-vivo transfection reagent, JetPEI. *PiggyBac* has been previously shown to be highly active in mammalian cells and will transpose into the chicken genome. Here, we show that JetPEI can transfect multiple chick cell types, most notably germ-line stem cells. We also show that pairing these two reagents is a viable and reproducible method for integration of transgenes into the chicken genome. Efficient and stable expression of the Green Fluorescent Protein (GFP) transgene was seen in multiple tissue types including heart, brain, liver, intestine, kidney and gonad. Combining an in-vivo transfection strategy with the PB system provides a simple and flexible method for efficiently producing stable chimeric birds and could be used for production of transgenics.

Keywords: piggyBac, JetPEI, transgenic, chicken, germ-line cell

INTRODUCTION

The domestication of the Red Jungle Fowl, which occurred nearly 8,000 years ago (Fumihito, et al., 1994), was an historical moment in the evolution of society. From an agricultural standpoint, chicken has become one of the most important protein sources in the world so understanding the genetics and development are financially important. The chicken, or more specifically the chick embryo, has also been a leading model system in developmental biology research for the past 100 years. This system has led to many important discoveries such as B cells and tumor viruses (Brown, et al., 2003), cell fate and tissue origins through quail-chick chimeras (Fontaine-Perus, et al., 1982), core knowledge of limb development through discoveries such as the apical ectodermal ridge (Saunders, 1998), and a detailed knowledge of somite segmentation (Olivier, 2004), to name a few. The chick has been so well studied because of the availability of embryos, short development time and ease of experimental embryology to elucidate developmental processes (Stern, 2005). More recently, however, research in the chick has diminished due to a lack of efficient molecular tools and the advancement of other model organisms.

Transgenic animals have become the dominant model in molecular and developmental biology since the creation of transgenic mice in the 1980's (Gordon and Ruddle, 1981). Advancements in chicken transgenic technology soon followed with the use of avian retroviral injections (Salter and Crittenden, 1989) which target primordial germ cells (PGC's) that are present but dispersed in the sub-germinal cavity of embryos at Stage X of development (Petitte, et al., 1997). These retro-virus particles would produce transgenic animals but the efficiency was very low (<1%) (Rapp, et al., 2003), which made them ineffective. New Lenti-viral vectors increased efficiency from <1% to up to 45% transgenic offspring (McGrew, et al., 2004), but the

highest efficiency of these vectors are proprietary. Viral vector integration systems also have the disadvantages of sequence and size restraints (Scott, et al., 2010) and viral promoter silencing resulting in no expression of the transgene, especially in the germ line. To overcome these limitations, we aimed to develop a simplified and efficient method of transfection and integration of transgenes into developing chick cells, most importantly PGC's.

PiggyBac (PB) is an extremely efficient transposable element system originally isolated from Trichoplusia ni which has been shown to be active in mammalian (Ding, et al., 2005) and human (Wilson, et al., 2007) cells. It has also been shown to be more active than other transposon systems, including Sleeping Beauty and Tol2 (Wu, et al., 2006). PB uses a precise "cut and paste" mechanism to excise and integrate a transposon and its cargo into novel locations in the host genome (Fraser, et al., 1996). The PB transposon has the capacity to transpose large DNA sequences (Ding, et al., 2005), reportedly up to 100 kilobases, and is designed to transpose all types of DNA and RNA elements. For these reasons PB has the activity and versatility advantage over viral and other transposon systems. Recently, PB has been shown to integrate into the developing chick spinal cord and stably express GFP (Lu, et al., 2009). Because of these advantages, we decided to utilize PB in our transgenic strategy.

Using PB affords greater flexibility for integration of transgenes into host DNA but is not useful on its own for lack of a cellular delivery method. An in-vivo transfection reagent could overcome the delivery issue, but the success of transgenesis would depend greatly on the transfection efficiency of the reagent. JetPEI (Polyplus Transfection, Illkirk France) is a cationic polymer that bundles and transfects DNA through electrostatic interactions with DNA and cell membranes. It has been shown highly versatile and efficient at cellular transfection (Akinc, et al., 2005; Boussif, et al., 1995) and does not induce an inflammatory response (Bonnet, et al., 2008).

JetPEI has been used to deliver DNA and RNA including siRNA in vivo to multiple tissue types (Hassani, et al., 2007; Liao and Yau, 2007; Vernejoul, et al., 2002; Wiseman, et al., 2003). The polymer will transfect mouse stem cells (Yamano, et al., 2010) and has been combined with PB to transfect mouse cancer cells (Kang, et al., 2009) in vitro, but has not been previously shown to transfect germ cells in vivo.

Here we show that utilizing JetPEI in combination with PB is an effective method for developing chimeric chicks that stably express the green fluorescent protein gene both in-ovo and after hatch. JetPEI is efficient at transfecting multiple cells types through intravenous injection and will transfect germ cell precursors using Stage X injections. Pairing PB with JetPEI yields efficient and stable expression of transgene throughout the development of the chick embryo and adult bird.

MATERIAL AND METHODS

Plasmid Construction

PB plasmid vectors used in this study were previously described (Cadinanos and Bradley, 2007) but have been modified by cloning restriction fragment digests into the PB vector backbone. Plasmid mPB, the PB transposase, was restricted with SalI, EcoRI (Fermentas) to remove the 906bp CMV promoter. A 1724bp fragment containing the constitutive CMV/Chick B-actin fusion CAG promoter from pCAG-Cre-ERT2 (Clontech) was cloned into the SalI, EcoRI sites, giving 7546bp plasmid CAG-mPB (Figure 3.1a). Subsequently, the 775bp enhanced green fluorescent protein gene from plasmid pEGFP-N3 (Clontech) was cloned into EcoRI, NotI sites of plasmid CAG-mPB to give plasmid CAG-GFP. Plasmid 5'-PTK-3', the PB transposon, was restricted with XhoI and SpeI to remove the 2892bp SA-IRES-PURO sequence. Plasmid ends were blunted with blunting enzyme (Fermentas). A 2476 bp SpeI and NotI restriction fragment

of CAG-GFP containing the CAG promoter and EGFP gene was also blunted using blunting enzyme (Fermentas) and subcloned into 5'-PTK-3'. The resulting 5959bp plasmid became 5'-CAG-GFP-3' (Figure 3.1b). All cloning was verified by restriction digest and sequence analysis.

Preparation of Plasmids and JetPEI Solutions

Plasmids CAG-mPB and 5'-CAG-GFP-3' were ethanol precipitated to achieve concentrations between 4 and 5 µg/µl with A260/A280 ratios being greater than 1.9. JetPEI reagent was prepared according to manufacturer's protocol. In short, plasmid DNA and glucose solution were mixed in tube 1 so that final concentrations were .5 µg/µl and 5% with respect to total reaction volume. Glucose solution at 5% final concentration and JetPEI at a nitrogen/phosphate (N/P) ratio of 8 were added to tube 2. Nuclease free water was added to each tube to reach total reaction volume. Tube 2 was added to tube 1 and gently mixed. The reaction was allowed to proceed for at least 15 minutes prior to injection.

Embryo Injection

Injection of embryos was performed at two different developmental time points. First, fertile eggs were incubated for 4 days on their side in a standard desktop incubator (C.Q.F. Manufacturing, Savannah, GA). Shells were taped, albumin was removed and eggs were windowed as previously described (Van Raay, et al., 2008) to reveal embryos. 1-2 µl of JetPEI/5'-CAG-GFP-3' solution was injected intravenously into the omphalo-mesenteric (vitelline) artery. One ml of phosphate buffered saline (PBS) was added drop-wise on top of the embryo to prevent desiccation and windows were re-taped before placing eggs back into the incubator. Eggs were incubated for 24 hours post-injection and analyzed. Second, fertile, freshly laid Stage X eggs were windowed using a Dremel multitool (Dremel, Mt. Prospect, IL) to expose the inner shell membrane. Membranes were removed using a scalpel blade to expose the Stage X

blastodisc. 1-2 µl of JetPEI/5'-CAG-GFP-3' or JetPEI/CAG-mPB/5'-CAG-GFP-3' solution was injected under the epiblast layer into the sub-germinal cavity (Figure 3.2) of the chick embryo. Injections were performed using a Picospritzer III (Parker Hannifin Corp., Cleveland, OH) and a pulled glass needle. Hot glue was used to seal the window and eggs were placed back in the incubator for 3 days or until hatch.

Transfection and Expression Analysis

Embryos injected intravenously were analyzed 24 hours post-injection. Stage X embryo injections were analyzed 3 days post-injection or were euthanized after hatch. Embryos were removed from the shell and yolk and viewed using a dissecting microscope with a GFP filter (Leica Microsystems GmbH, Wetzlar, Germany). Images were captured using Q-imaging software and equipment (QImaging, Surrey, BC, Canada). Chicks were euthanized by CO2 asphyxiation per Animal Care and Use Guidelines and were dissected to reveal internal tissues and organs. All animal care and use procedures were approved by The University of Georgia Animal Care and Use Committee. Chicks were viewed under the same dissecting microscope and imaged with the same software as embryos.

Sectioning and Immunohistochemistry

Tissues were collected from 3- and 5-day embryos and euthanized chicks by dissection and fixed in 4% formaldehyde. Embryos and tissues were washed in PBS and prepared for cryosectioning by an equilibration series of 5% sucrose and 15% sucrose before embedding in gelatin. Gelatin-embedded embryos and tissues were frozen and cryosectioned to generate 12-20 µm sections which were mounted on Superfrost® Plus glass slides. Immunohistochemistry was then performed to detect germline cells which are immunoreactive for the MC-480 (SSEA 1) and the EMA-1 antibodies. These antibodies were purchased as supernatant from the Developmental

Studies Hybridoma Bank at the University of Iowa and diluted 1:300 in PBS with 0.1% BSA and 0.1% Triton-X100. Appropriately matched Alexafluor secondary antibodies (Life Technologies) were used for fluorescent labeling. Stained slides were then analyzed by fluorescence microscopy to detect transgene expression (GFP) and germline cell markers.

RESULTS

JetPEI Will Transfect Multiple Tissue Types through Intravenous Injection

To test the efficiency of JetPEI at transfecting developing chick cells in vivo, we injected a JetPEI/5'-CAG-GFP-3' solution into the right omphalo-mesenteric (vitelline) artery of 4-day incubated chick embryos. Injecting transposon alone with no transposase gene will not cause integration into the chick genome, but the constitutive CAG promoter will express the GFP gene from plasmid 5'-CAG-GFP-3' and give an accurate readout of transfection efficiency. Embryos were incubated for 24 hours post-injection and then viewed under a dissecting microscope with a green fluorescent protein filter. Strong GFP expression was seen over the entirety of the chick embryo, indicating that JetPEI will transfect developing chick tissues (Figure 3.3a,b). No embryonic death was seen due to reagent toxicity. To determine specific tissue types that were transfected and expressing GFP, the embryos were dissected from the shell and yolk, fixed in 4% formaldehyde and sectioned for detailed tissue analysis. JetPEI effectively transfected multiple tissue types including vessel endothelium, neurons, and gonadal ridge (Figure 3.3e-g). Embryo sections corresponding to the gonad were stained with EMA-1 and SSEA-1 antibody. No co-labeling of germline cell markers with cells expressing GFP was observed (Figure 3.3g), indicating that JetPEI did not transfect germ cells within the gonadal ridge at this stage.

To test whether JetPEI could transfect a non-dividing cell type like PGC's, we injected freshly laid Stage X embryos with the same solution of JetPEI/5'-CAG-GFP-3' as before and

incubated the embryos to the same 5 day incubation time point. Embryos were dissected from the shell and yolk and viewed under the dissecting microscope with a GFP filter to examine fluorescence. Little fluorescence was seen in these embryos due to lack of integration of the GFP transgene into the embryo genome and dilution of the GFP signal from cell division. Very localized GFP expression was seen along the main axis of the lower portion of the chick embryo, corresponding to the location of the developing gonadal ridge (Figure 3.3c,d). Embryos were again fixed, sectioned and stained with EMA-1/SSEA-1 antibody to determine localization of germ cells in the area. Figure 3.3h-j shows a germ cell along the wall of the gut tube expressing GFP as well as staining positive for SSEA-1. At this time point, germ cells have not started rapid division and would therefore retain the transient GFP signal. This evidence proves that JetPEI could transfect germ cells as well as multiple other tissue types in the developing chick embryo.

Combining JetPEI with PB Delivers Stable Expression of Transgenes

Being able to transfect germ cells with a passive GFP construct is beneficial for studying short-term phenomena in the chick embryo but does not lend itself toward long-term analysis of development and transgenesis. To achieve stable integration of the 5'-CAG-GFP-3' PB transposon into the chick genome (and therefore long term expression) we co-injected the CAG-mPB helper plasmid that will constitutively express PB transposase in trans but not be integrated into the genome. These plasmids in solution with JetPEI were injected into Stage X embryos and incubated until hatch. Cull chicks were euthanized immediately, and samples of chicks and juvenile birds were euthanized at different time points to determine expression stability of the GFP transgene by visualization in tissue samples. The oldest birds sacrificed were 7 weeks old and all males. Figure 3.4a-f shows representative samples of GFP expression in varying tissue types including intestine, gizzard, heart, brain, pelvic bone and testes from cull chicks. Similar

expression was seen in all chicks examined, regardless of age. Positive testes were sectioned and stained with EMA-1/SSEA-1 antibodies to evaluate GFP expression in germ cells. Figure 3.4g-I shows positive GFP expressing cells co-staining positive with EMA-1/SSEA-1 antibodies indicating that the GFP transgene was stably integrated into those cells. Of 18 total male chicks and juvenile roosters sampled, 6 had positive GFP expression in the testes. The ability to pair JetPEI with the PB system to achieve stable expression of transgene cargo and the ease with which this system may be manipulated and adapted make this method a powerful tool in studying many aspects of chick development and may provide a novel method for introducing commercially beneficial genes into the chick genome.

DISCUSSION

Here we show that JetPEI will transfect multiple chick tissue types including PGC's. Our experiments also show that pairing the piggyBac transposon system with JetPEI is a very efficient method to produce stably expressing chimeric chicks. The ease with which this system can be modified lends itself to multiple types of studies in early and late chick embryos and in juvenile or adult chickens. The JetPEI/PB transposon system has several advantages over other methods of transgenesis in the early chick embryo. The first and perhaps most important advantage is integration by the PB transposon which provides stable expression of the transgene of interest rather than passive expression through plasmid electroporation or transfection. Second, PB has the capacity to carry large amounts of sequence cargo in its transposon, reportedly up to 100kb (Li, et al., 2011), making it possible to integrate very large gene sequences or multiple transcripts from one vector. Third, PB is very versatile in the types of cargo it can deliver. Inducible systems (Cadinanos and Bradley, 2007), Cre/loxP technology (Lu, et al., 2009), and siRNA have all been introduced using the PB transposon in other model

organisms. Fourth, JetPEI functions to package DNA into very small nanoparticles which do not cause inflammatory responses or cell death when introduced into host tissues as opposed to viral particles or liposome mediated transfections which often produce such results (Kawakami, et al., 2006). The properties of the linear polyethylenimine molecule also aid in escape from the endosome through a proton sponge mechanism as well as favor non-cell cycle mediated transfer into the nucleus of cells (Brunner, et al., 2002), promoting DNA uptake into the nucleus regardless of cell state.

When using a JetPEI/PB mediated transgenesis approach, one may be concerned that success is based on random transfection and integration into germ cells that cannot be directly targeted at the time of injection. While this is a valid concern, using JetPEI/PB does not differ from the predominant method of viral integration in that they are both random events, giving rise to lower efficiency in the chick model than other systems. That being said, the JetPEI/PB system appears to be relatively efficient when compared to other methods. We detected GFP expression in 6/18 (33%) immature chick testes. This does not directly imply germline transmission, but immunohistochemistry shows positive expression of GFP in germ cells in these testes. Exact evaluations of efficiency and transmission cannot be determined without sexually mature roosters for sperm samples and breeding.

It has been posited that using transposon technology to create transgenic animals can have adverse effects such as deleterious disruption of the host genome from numerous integrations of the transposon. Although possible, this is not necessarily a detrimental attribute of transposons. Often, multiple integrations will be beneficial and increase the chance of transgene transmission to the progeny. If the transposon does show a propensity to cause cell or embryo death through hyper-integration, however, the ratio of transposon to transposase can be titrated

for injections. By making the amount of transposase plasmid the rate limiting step to integration, you can control the number of integrations and promote stability in the host genome. And while not explicitly shown in this work, stable integration can be inferred from comparison of injections with transposon but no transposase plasmids to injections with both transposon and transposase plasmids that have been titrated to proper amounts. You can see in Figure 3.3c,d that there is little GFP expression present after only 3 days of incubation and the majority of expression seen is in non-dividing cells. In Figure 3.4, you can see robust GFP expression in all tissues shown, no matter what age the chicks were when sampled.

The combined effectiveness of JetPEI and PB in the chick opens many avenues to optimize the system for specific experiments. With new dual reporter PB transposon constructs now available (Transposagen), promoter profiling will become much easier in the chick model. Internal expression controls in this dual vector will allow you to quickly identify useful embryos. The flexibility of JetPEI administration can also lead to studies in juvenile or adult birds, as JetPEI is active through many different modes of administration (Polyplus Transfection). Separately JetPEI and piggyBac are powerful reagents in the genome modification toolbox; but when combined, they become an even more formidable presence in the transgenic chick landscape.

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FIGURES AND LEGENDS

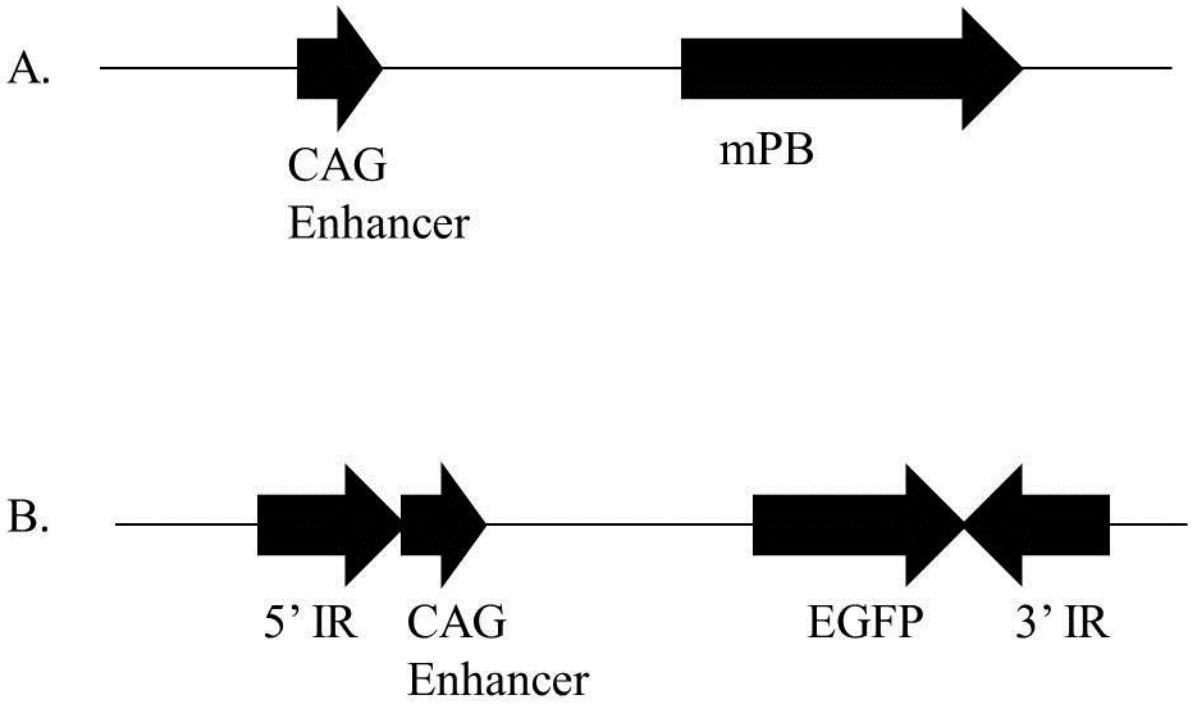

Figure 3.1. *PiggyBac* plasmids. (A) *piggyBac* helper plasmid encoding the *piggyBac* transposase (mPB) gene driven by a constitutive chick β-actin/CMV fusion promoter (CAG). (B) *piggyBac* transposon modified to contain the enhanced green fluorescent protein gene (EGFP) driven by the CAG promoter between the *piggyBac* inverted repeats (IR).

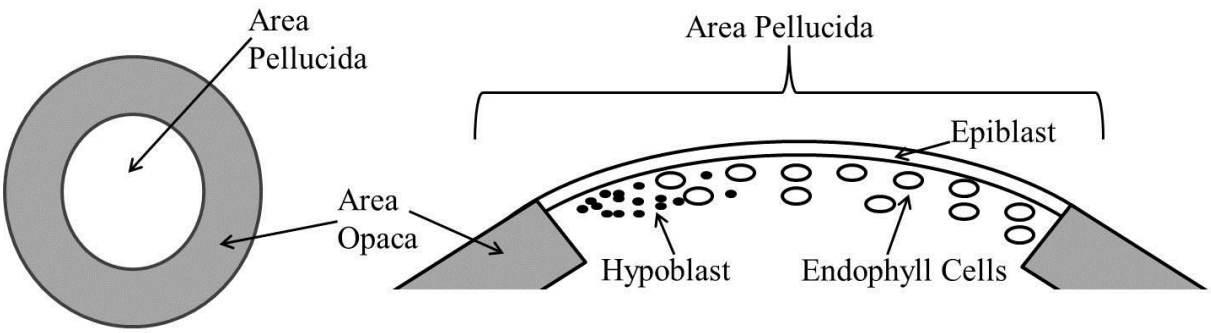

Figure 3.2. Anatomy of the chicken Stage X (HH) blastoderm. Overview and cross-sectional view of a freshly laid, Stage X blastoderm showing the relative position of germ cell precursors (Endophyll cells).

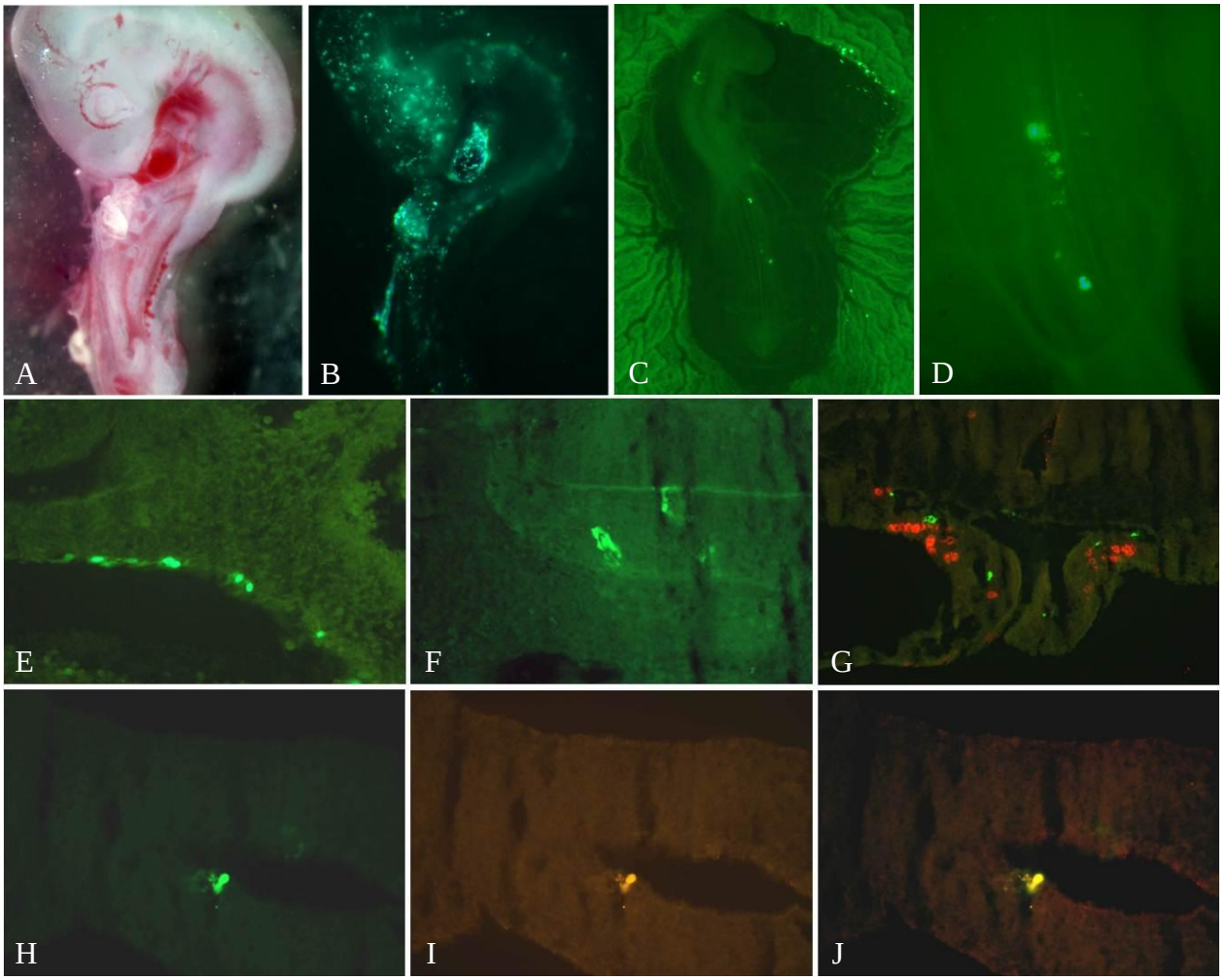

Figure 3.3. Tissue transfection with JetPEI. (A,B) Bright field and fluorescent images of 5-day incubated chick embryos injected with 5'-CAG-GFP-3'/JetPEI. After 24 hours incubation, widespread GFP is seen throughout the chick embryo. (E-G) Representative sections of the embryo shown in (B) reveal transfection of multiple cell types including vessel endothelial cells (E), neurons (F), and mesonephros (G). Staining with germ cell-specific antibodies (SSEA-1/EMA-1) show no germ cell transfection in this sample (G). (C) Whole-mount fluorescent image of a 3-day incubated chick embryo after Stage X injection of 5'-CAG-GFP-3'/JetPEI. Much less GFP expression was seen overall, with the majority corresponding to the developing gonadal ridge. (D) Enhanced image of gonadal ridge area from (C). (H-J) Expression of GFP and

labeling with SSEA-1/EMA-1in germ cells. Sectioning of the area in (D) shows cells expressing GFP (H) and labeling positive with germ cell specific SSEA-1/EMA-1antibodies (I). Overlaying these images confirms GFP expression in germ cells (J).

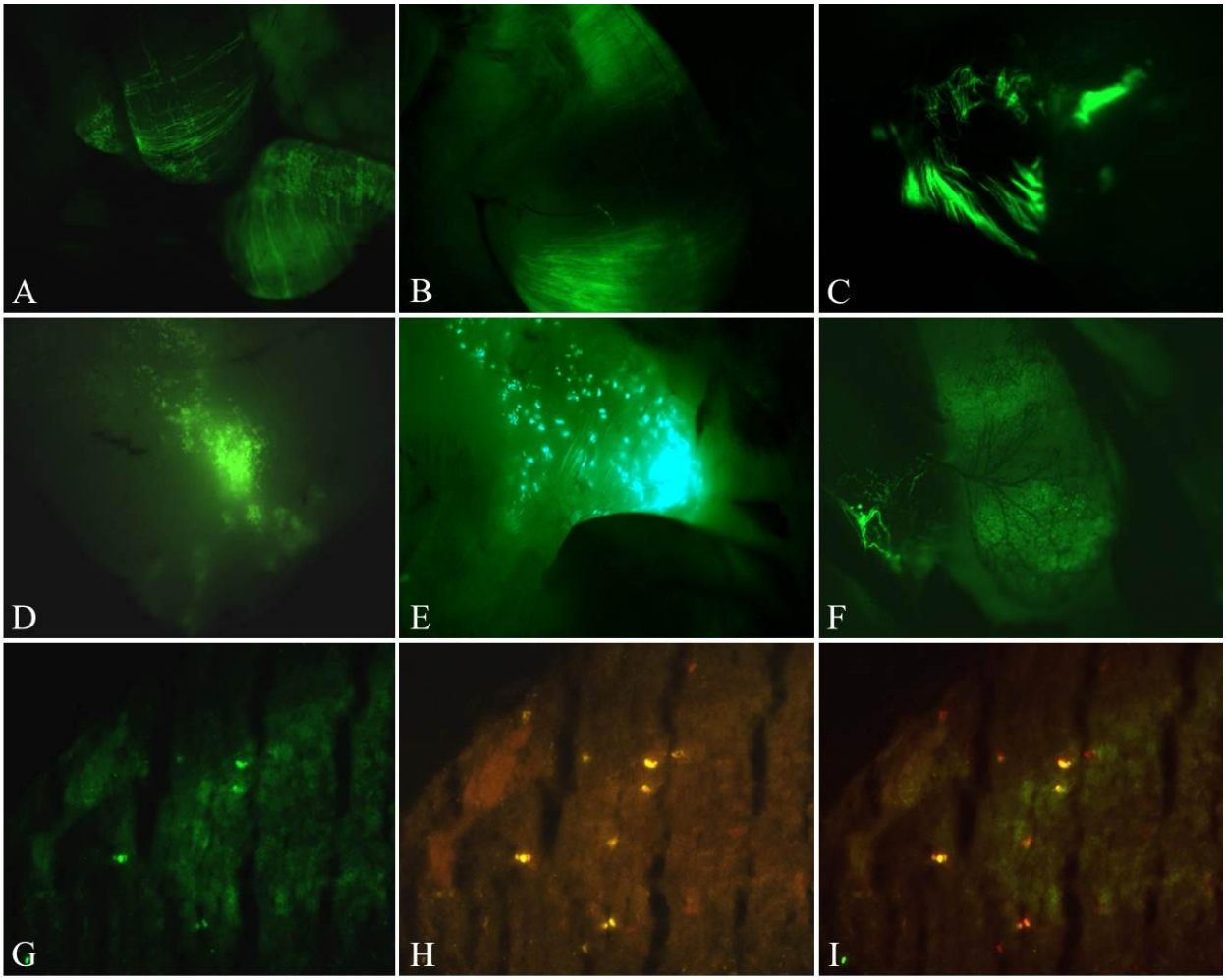

Figure 3.4. GFP expression in hatched chicks from Stage X injections. (A-I) Freshly laid Stage X embryos were injected with CAG-mPB/5'-CAG-GFP-3'/JetPEI and incubated until hatch. Chicks were euthanized and imaged to show robust and stable GFP expression in multiple tissues. (A) Intestine, (B) Gizzard, (C) Heart, (D) Brain, (E) Pelvic Bone, (F) Testes. (G-I) Representative sections of testes from (F) show GFP expression (G) and positive labeling with germ cell specific SSEA-1/EMA-1 antibodies (H). Overlaying these images confirms stable GFP expression in hatched chick testes germ cells.

CHAPTER 4

ANALYSIS OF *PIGGYBAC* INTEGRATION

INTRODUCTION

Throughout the development of the *piggyBac* transposon as a transgenic tool in the chicken, validation experiments were carried out to confirm integration of *piggyBac* into the genome of chick cells both *in-vitro* and *in-vivo*. These validations are necessary to claim the effectiveness of *piggyBac* as a transgenic tool in the chick, as only recently has one report been published to support this idea (Lu, et al., 2009). Initial analysis of integration was carried out *in-vitro* using a *piggyBac* transposon cassette, 5'-dsRED-3', which is differentially expressed only when integrated into an active gene. This method of integration screening has been previously used to analyze transposon insertion events and effectively shows transposition (Cadinanos and Bradley, 2007). Stable expression of the constitutive green fluorescent protein (GFP) transgene used to create chimeric chicks was also demonstrated *in-vitro* through transfection of chick fibroblast cell lines maintained for at least 10 days. Together, these experiments provide evidence of efficient integration and stable expression in chick cells.

Upon production of chimeric chicks expressing green fluorescent protein in multiple tissue and cell types, Southern blot analysis was performed using genomic DNA isolated from tissues from a subset of euthanized chicks to confirm the presence of the GFP gene. Since the development of the Southern blot technique in 1975 (Southern, 1975), it has become universally accepted as the standard protocol for detection of specific DNA sequences (Mellars and Gomez, 2011). Inverse PCR was also performed on these tissues for further integration analysis

(Ochman, et al., 1988). Three males chicks were grown to sexual maturity and polymerase chain reaction (PCR) was performed from genomic DNA isolated from mature, ejaculated spermatozoa to assess the presence of the GFP gene. Positive reactions were subsequently subjected to nucleotide sequence analysis to confirm the presence of the GFP gene. Confirmation by PCR and nucleotide sequencing of the GFP gene in sperm indicates the ability to transmit the gene to progeny. One positive rooster was cross-bred to wild-type hens in an attempt to produce transgenic offspring and thereby quantify transmission rate. Breeding was suspended before any positive chicks could be obtained due to rooster injury and seemingly inefficient transmission. Mature chimeric roosters were euthanized and their testes were sectioned and stained with germ cell anti-body to analyze co-expression of germ cell specific antigen and GFP. Results of Southern blot, PCR and sequencing analysis and testes sectioning and immunohistochemistry lead to the conclusion that *piggyBac* did integrate into somatic tissues and germ cells but at levels that varied in all chimeric chicks.

MATERIALS AND METHODS

Plasmid Construction

Plasmids CAG-mPB (transposase) and 5'-CAG-GFP-3'have been previously described in Chapter 3 (Figure 3.1). Integration plasmid 5'-dsRED-3' was previously described by Cadinanos and Bradley (2007) as 5'-PTK-3' with modifications. The puro-delta-TK gene sequence was removed by NcoI and SpeI restriction digest. The dsRED fluorescent protein gene was amplified from plasmid pRetroX-IRES-dsREDExpress (Clontech) with primers dsREDexprF: 5'-AACAACCCATGGATGGCCTCCTCCGAGGACGT-3' and dsREDexprR: 5'-ACACACACTAGTACAGGTGGGGTCTTTCATTCC-3' that contain NcoI and SpeI restriction enzyme sites added to the 5' ends of each primer respectively. PCR fragment was ligated into the

open 5'-PTK-3' vector to give plasmid 5'-dsRED-3' (Figure 4.1c). Cloning was confirmed by restriction digest and nucleotide sequencing.

Cell Culture

Chick embryonic fibroblast cell line (DF-1) was obtained from ATCC (Manassas, VA) and cultured as per manufacturer's recommendations. Briefly, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS), with the addition of chick serum added to a final concentration of 2%, and PEN/STREP antibiotics to minimize the risk of bacterial contamination. Cultures were grown in 6-well culture plates to 80% confluency prior to transfection.

In-Vitro Transfection

Chick embryonic fibroblast cells (DF-1) were transfected using the Lipofectamine 2000 transfection system (Invitrogen) per manufacturer's protocol with slight modifications. Amounts detailed are on a per well basis. 4µg total plasmid DNA was added to 250µl Opti-MEM I Reduced Serum Medium (Invitrogen) and mixed gently. Lipofectamine 2000 Transfection Reagent (Invitrogen) was mixed and 10µl were diluted into 250µl of Opti-MEM I. This solution was incubated for 5 minutes at room temperature. Diluted DNA and diluted Lipofectamine were combined, gently mixed and incubated at room temperature for 20 minutes. DNA/Lipofectamine solutions were then added to each well of the six-well plate and cells were incubated at 37°C in a CO_2 incubator for at least 24 hours before visualization.

Cell Culture Imaging

Post-transfection, cells were imaged using an Olympus IX70 inverted microscope with an attached MagnaFire SP digital camera in order to detect fluorescent protein expression. EGFP and dsRED fluorescent proteins exhibit excitation peaks of 489 and 558nm respectively and

emission peaks at 508 and 583nm. The microscope was equipped with both EGFP and dsRED filters for effective visualization of fluorescence.

Southern Blotting

Southern blotting was carried out according to published protocols with minor changes. In short, 10 µg of genomic DNA from eight different chimeric chick tissues was restricted with BamHI and EcoRV (Fermentas) restriction endonucleases for two hours. Plasmid transposon DNA and DNA isolated from non-transgenic embryos were used as a positive and negative control. Restricted DNA was electrophoresed on a 1% (w/v) agarose gel at 60V for 270 minutes. The gel was denatured, dupurinated and neutralized through a series of buffer washes and the transfer of DNA onto a (+) charged nylon membrane was performed overnight with 20X SSC buffer. The membrane was subsequently UV irradiated to crosslink the DNA fragments to the membrane. A DNA probe was made using the PCR DIG Probe Synthesis Kit (Roche) with Digitonin (DIG) labeled UTP nucleotides. PCR conditions were recommended by the manufacturer with annealing temperature being 58°C. Primers used to create the probe were EGFP Test F: 5'-CGCCACCATGGTGAGCAAG-3' and EGFP Test R: 5'-GCTTTACTTGTACAGCTCGTCC-3', which yielded a 729 base pair fragment corresponding to the full length EGFP gene. The probe was hybridized in Easy Hyb buffer (Roche) to the membrane overnight and subsequently washed free of any unbound probe. The membrane was blocked with Blocking buffer for 2 hours and incubated with anti-DIG antibodies conjugated with alkaline phosphatase (AP) diluted in Blocking buffer for 30 minutes. The membrane was then repeatedly washed with 1X PBST (PBS with Tween 20) to remove excess and unbound antibody and developed with BM Purple (Roche) substrate for 20 minutes. Wash and block buffers were part of the Wash and Blocking Buffer Kit (Roche).

PCR, iPCR and Sequencing

PCR on sperm genomic DNA was performed as follows. DNA was extracted from 30µl of washed sperm cells using the cultured cells protocol of the DNeasy Blood and Tissue Kit (Qiagen) in quadruplicate. Extracted DNA was ethanol precipitated to concentrate and further purify genomic samples. Samples were pooled to achieve larger volumes of concentrated DNA for PCR analysis. 1µg of genomic DNA was used in the PCR reaction with a 1X final concentration of DreamTaq (Fermentas) polymerase master mix. Primers used for detection were EGFP Test F: 5'-CGCCACCATGGTGAGCAAG-3' and EGFP Test R: 5'-CTTTACTTGTACAGCTCGTCC-3', amplifying the 729 base pair full length GFP gene. Reaction conditions were 1 cycle at 95°C for 2 minutes, 35 cycles of 95°C for 30s, 56°C for 15s and 72°C for 90s. Reactions were verified on a 1% agarose gel, gel purified using a Gel Purification Kit (Fermentas) and then ligated into the pJet1.2 cloning vector (Fermentas) following manufacturer's "sticky end cloning" protocol. In short, PCR fragments were blunted using "Blunting enzyme" included in the cloning kit before ligation into pJet1.2. Positive clones were sequenced using pJet1.2 Forward and Reverse sequencing primers by the Georgia Genomics Facility with a 3730xl 96-capillary DNA Analyzer for Sanger sequencing and fragment analysis (Applied Biosystems). pJet1.2 F: 5'-CGACTCACTATAGGGAGAGCGGC-3', pJet1.2 R: 5'-AAGAACATCGATTTTCCATGGCAG-3'. Sequences were compared to original EGFP sequence to confirm maximum identity to the original gene.

Inverse PCR (iPCR) was performed on genomic DNA isolated from EGFP positive tissues from hatched chicks. Genomic DNA was first isolated and purified as described before. 2µg of genomic DNA were restricted with AluI FastDigest Restriction endonuclease (Fermentas) in a 20µl volume for 1 hour. Endonuclease was deactivated by heating at 65°C for 20 minutes.

The volume of the reaction was increased to 200µl and 10 units of T4 DNA Fast Ligase (Fermentas) was added to anneal restricted ends together and form circular DNA fragments. PCR was performed under the same condition previously described with primers PB-Sequence-5' R: 5’-GAGAGAGCAATATTTCAAGAATGCATGCGT-3’ and PB-Sequence-5' (2) F: 5’-GTCGCTGTGCATTTAGGACATCTCAG-3’ and analyzed on a 1% agarose gel. Fragments were ligated into the pJet1.2 cloning vector (Fermentas) as described above and positive clones were sequenced by the Georgia Genomics Facility in the same manner as before. Sequences were compared to original *piggyBac* transposon plasmid sequence for identity comparison.

Sectioning

Mature rooster testes were removed by dissection and further sectioned into 3-5mm thick horizontal sections. Sections were placed in 4% formaldehyde and stored at 4°C overnight to fix the tissue. Sections were then washed with 1X PBS and then incubated in 5% sucrose for 30 min (room temperature). Sections were then transferred to 15% sucrose for two hours (RT). Sections were warmed to 37°C as was the gelatin (300 bloom, Type-A, Sigma) to be used for embedding. Sections were placed into the gelatin at 37°C for at least 30 min. Tissue sections were then placed into a specimen block and allowed to set. Tissue blocks were frozen with liquid nitrogen and mounted onto a sectioning chuck. Blocks were allowed to freeze again in the cryostat at -32°C for at least 30 minutes prior to further sectioning. Sectioning chuck containing the frozen tissue blocks was placed into the cryosectioning mount and tissues were sectioned to ~20µm thickness. Sections were mounted on Superfrost Plus glass slides (Menzel-Glazer). Slides of sections were stored at 4°C for processing.

Immunohistochemistry

Slides of testes sections were removed from 4°C and placed into a container of 1X PBS. The container was placed in a 37°C water bath to warm the slides and melt the gelatin. Slides were washed with pre-warmed 1X PBS and then again with room temperature 1X PBS. Slides were removed to a sealable slide box and mouse anti-MC-480 (SSEA 1)/EMA-1 primary antibodies were added. These antibodies were purchased as supernatant from the Developmental Studies Hybridoma Bank at the University of Iowa and diluted 1:300 in PBS with 0.1% BSA and 0.1% Triton-X100. Goat anti-mouse Alexafluor 532 secondary antibody (Life Technologies) was used for fluorescent labeling. Slides were viewed using an Olympus BX61 microscope with filters at 488 and 532 nm for emission wavelength of EGFP and YFP respectively.

RESULTS

piggyBac Will Integrate into Chick Fibroblast Cell Genome

The initial experiment in this study was designed to assess the efficacy of transposition of the *piggyBac* transposon system into the genome of chick cells *in-vitro.* Transposon plasmids used for this initial experiment were designed to contain splice acceptor (SA), internal ribosomal entry sequence (IRES) and red fluorescent protein gene (dsRED) sequences flanked by the inverted repeats of *piggyBac* (Figure 4.1). Using this plasmid ensures that expression of dsRED can only occur from an integration event into an actively expressed gene. Upon transposition into an active gene, transposon mRNA will be spliced into mature mRNA at the splice acceptor site. Native ribosomes will bind at the IRES site and begin translating functional dsRED protein. Without integration into an active gene, transposon mRNA would not be transcribed and, therefore, no dsRED protein would be translated. Test transposon and transposase plasmids were transfected using Lipofectamine 2000 (Invitrogen) into chick embryonic fibroblast DF-1 cell lines along with control EGFP plasmids to compare transposon integration to total cell uptake of

DNA. Figure 4.2A shows cells expressing the EGFP control plasmid. 4.2B shows those same cells also expressing the dsRED transposon plasmid, verifying integration into an active gene in the chick cell line. 4.2C is an overlay of the two images.

While effective at verifying transposition, this *piggyBac* plasmid construct only yields positive dsRED expression when integrated in the forward orientation into an active gene. This scenario would greatly decrease the efficiency of visually analyzing integrations. Since the goal of this project was to validate the use of *piggyBac* as a transgenic tool in the chick, we modified the transposon plasmid to constitutively express GFP for easier identification of insertion events (for plasmid map and description of 5'-CAG-GFP-3' see Ch. 3). Transposon plasmid 5'-CAG-GFP-3' was transfected into chick DF-1 fibroblast cells with transposase plasmid using the Lipofectamine 2000 transfection system (Invitrogen) as previously described. Analysis of GFP positive cells as a percentage of total cell populations at 3, 6 and 10 days post-transfection shows an approximately 2-fold decrease in GFP expression (Figure 4.3). Control cells transfected with plasmid pCIG (Addgene), which only yields a transient transfection, show an approximately 10 fold decrease in GFP expression after only 7 days. Maintenance of GFP expression in transposon transfected cells along with dsRED expression from insertion into active genes in prior experiments confirms that *piggyBac* will insert into the chick genome.

piggyBac Transposon Sequence can be Identified by PCR but not Southern Blot in the Genome of Hatched Chicks

While cell culture data confirms *piggyBac* integration *in-vitro* and long-term expression of GFP as seen in tissues of hatched chicks (Chapter 3) infers integration *in-vivo*, analysis must be performed to positively identify transposon sequence in the chick genome. Initial experiments used the inverse PCR (iPCR) method to amplify transposon inverted repeat sequence from DNA

isolated from visually GFP positive tissues. iPCR was successful in all tissue samples tested from multiple rounds of injections, yielding the expected multiple-size band pattern indicative of different insertion sites (Figure 4.4, A-C). Fragments from three different tissues (Figure 4.4, D) were ligated into the pJet1.2 cloning vector (Fermentas) and nucleotide sequenced for analysis. Analysis identified *piggyBac* 5'-inverted repeat sequence (Figure 4.5), indicating that green fluorescence seen in the tissues tested was from expression of an integrated *piggyBac* transposon sequence. No insertion site analysis was performed due to lack of genomic sequence data.

Southern blot analysis was also performed on genomic DNA from the same tissues tested by iPCR to provide evidence of *piggyBac* transposon sequence in the genome. Analysis was done using a PCR amplified DIG labeled DNA probe corresponding to the 729 base pair full length EGFP gene. Figure 4.6 shows the blot with the positive control lane being the only positive reaction for GFP. No positive bands in the negative control lane confirm the specificity of the probe. No positive bands in the sample lanes indicate that the level of GFP gene sequence is below the detectable level by Southern blot, when taken in conjunction with the previous data.

EGFP Gene Sequence can be Positively Identified in the Sperm of Adult Roosters, but No Chimeric Chicks Were Produced

Three male chicks hatched after Stage X injection of the *piggyBac* transposon system were grown to sexual maturity and sperm was collected for PCR analysis. Genomic DNA was extracted from washed sperm cells and PCR was done to detect the presence of the GFP gene, which would indicate the ability to transmit the gene to progeny. Sperm genomic DNA from Rooster 1 consistently tested positive by PCR for the GFP gene (Figure 4.7). Nucleotide sequencing data from this PCR fragment confirmed the presence of the GFP gene when compared to original sequence (Figure 4.8). Genomic DNA extracted from roosters 2 and 3 only

yielded sporadic positive results (data not shown), indicating a very low level of GFP positive germ cells. All three roosters were bred to wild-type hens to produce GFP positive transgenic offspring and provide more data on transmission rate. Unfortunately, rooster 1 had to be euthanized before any positive chicks could be produced and analyzed. Roosters 2 and 3 sired over 300 negative chicks each before breeding analysis was stopped due to apparently low transmission rate.

Testes Sections Reveal Low Percentage of Germ Cell Integration in Adult Roosters

After the breeding analysis program was halted, it was decided that direct testes analysis of Roosters 2 and 3 would provide the best data to quantitatively assess germ cell integration and possible transmission rate since positive PCR data was not repeatedly attainable. Roosters 2 and 3 were euthanized and the testes were extracted, sectioned and stained with germ-cell specific antibodies to examine germ cells within seminiferous tubules. We were unable to section and stain Rooster 1 testes due to regression and degradation of testicular tissue. In all of the sections viewed, Figure 4.9 shows the only instance of identifiable GFP expression that coincides with positive staining by germ cell specific antibodies. While these images do not encompass all germ cells present in the seminiferous tubules, they do give a sample population large enough to conclude an integration rate of much less than 1% in the two roosters tested.

DISCUSSION

Analyzing integration of *piggyBac* into the genome of chick cells is necessary to evaluate the efficacy of using this system for producing transgenic chickens. Initial cell culture experiments confirm that *piggyBac* will integrate in the chick genome *in-vitro* and maintain expression in these cells. PCR analysis of genomic DNA from both tissue and sperm samples confirm the presence of transposon sequence and GFP sequence respectively, again confirming

integration of the *piggyBac* transposon into these cells. The lack of surrounding genomic data from sequencing iPCR reactions was most likely due to the fact that endonuclease AluI restricts the genome very frequently, giving small restriction fragments to sequence from. Negative Southern blot data, however, provides an interesting clue to the integration profile of *piggyBac* in these cells. Using Southern blots to confirm DNA sequence identity among genomic DNA is a classical analysis technique but does have limits. Even under completely optimized conditions and maximum labeling of the probe being used, Southern blots lack the sensitivity to identify single copy genes in a genome (Brown, 2001). Tissue samples analyzed by Southern blot in this experiment were visually verified to express GFP and were dissected out carefully, but many cells were excised that did not contain GFP due to the mosaic nature of GFP distribution in these tissues. This is a current drawback to all transgenic strategies in the chick, not just transposons. When this is taken into consideration, it is no surprise that Southern blot analysis was unable to detect the GFP gene as it could be present at less than one copy per genome. It is also no surprise then, that PCR was able to detect transposon sequence, since it has a much higher sensitivity level.

Sectioning and immunohistochemistry of adult Rooster testes completes the profile analysis of *piggyBac* in the chick. As stated earlier (Ch. 3), 6/18 male chick testes viewed were expressing GFP. When sectioned, it was shown that populations of germ cells within these testes were also expressing GFP (Figure 3.4). This implies that 33% of hatched male chicks have the ability to transmit the GFP transgene to progeny, with varying efficiency. PCR analysis of genomic DNA isolated from sperm from three adult roosters gave a consistent positive result from 1 out of 3, following the same pattern. Although rooster 1 could not be effectively analyzed for transmission, the ability to consistently PCR amplify the GFP gene from his sperm indicates

a higher level of germ cells with transposon integration. Analysis of roosters 2 and 3 did show GFP germ cells (Figure 4.9), but the presence was so low that it was below a quantifiable level.

Interestingly, it seems that somatic tissue expression and germ cell expression are correlated and both directly influenced by injection efficiency. Chicks that expressed GFP in testes and germ cells also expressed GFP in other somatic tissues. Chicks that were not positive in testes did also express in somatic tissues but at a much lower level. This pattern held true in adult roosters as well. Rooster 1 had more somatic GFP expression than roosters 2 and 3. These observations lead to the theory that the limiting factor in *piggyBac* transposon transgenics is the initial injection. Indeed, targeting of the Stage X blastoderm, depth of the needle into the blastodisc during injection and volume of injected reagent all directly affect the efficiency and consistency of injections. With the application of more consistent injection protocols the transposition ability of *piggyBac* can be utilized to its full potential.

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FIGURES AND LEGENDS

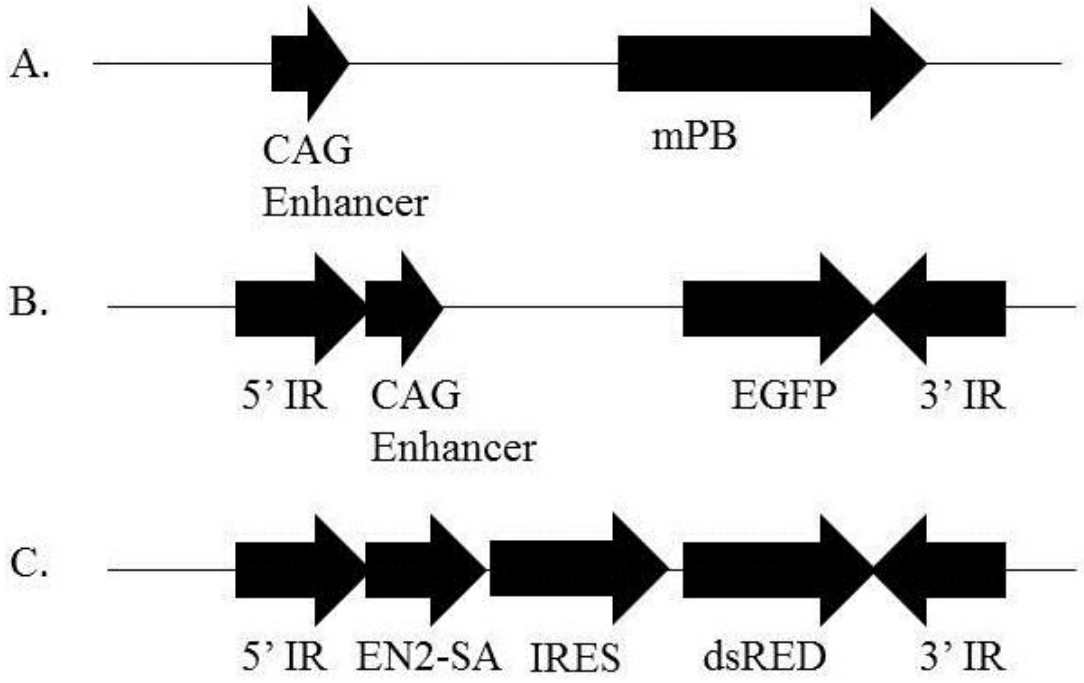


Figure 4.1. *PiggyBac* plasmids. (A) *piggyBac* helper plasmid encoding the *piggyBac* transposase (mPB) gene driven by a constitutive chick β-actin/CMV fusion promoter (CAG). (B) *piggyBac* transposon modified to contain the enhanced green fluorescent protein gene (EGFP) driven by the CAG promoter between the *piggyBac* inverted repeats (IR). (C) *piggyBac* transposon containing the EN2-splice acceptor (SA), internal ribosomal entry sequence (IRES) and dsRED fluorescent protein gene flanked by *piggyBac* inverted repeats (IR).

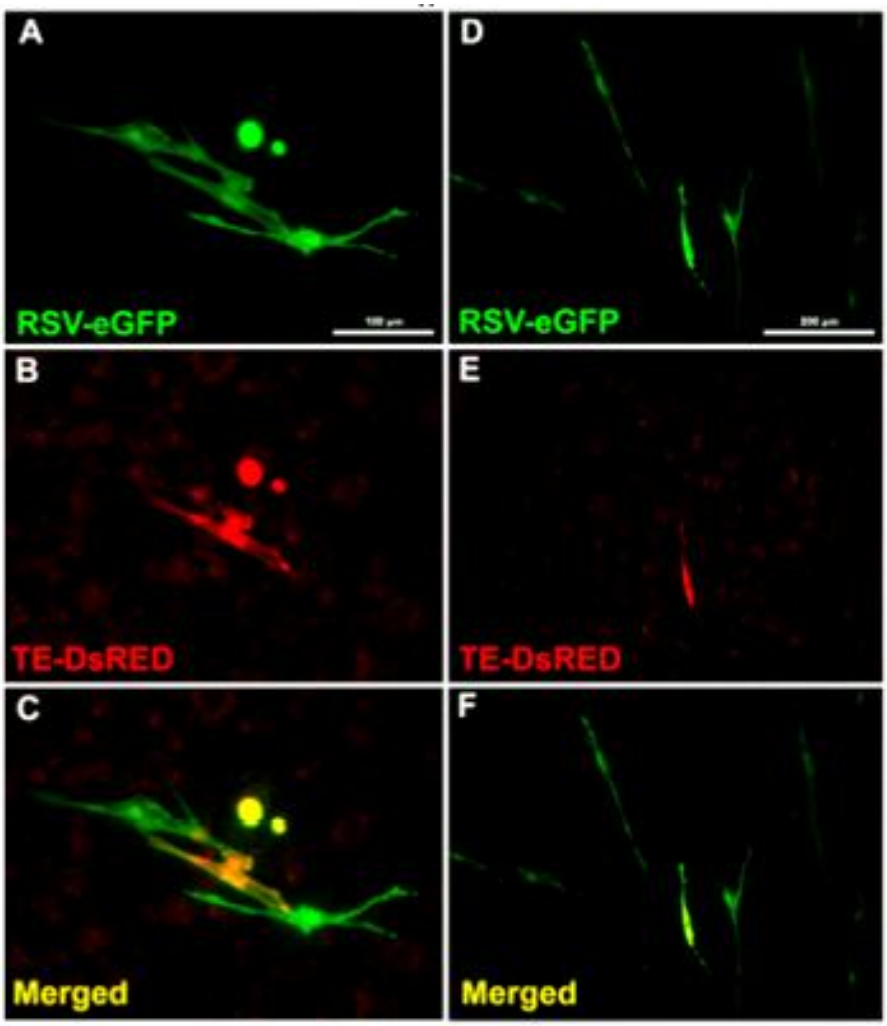
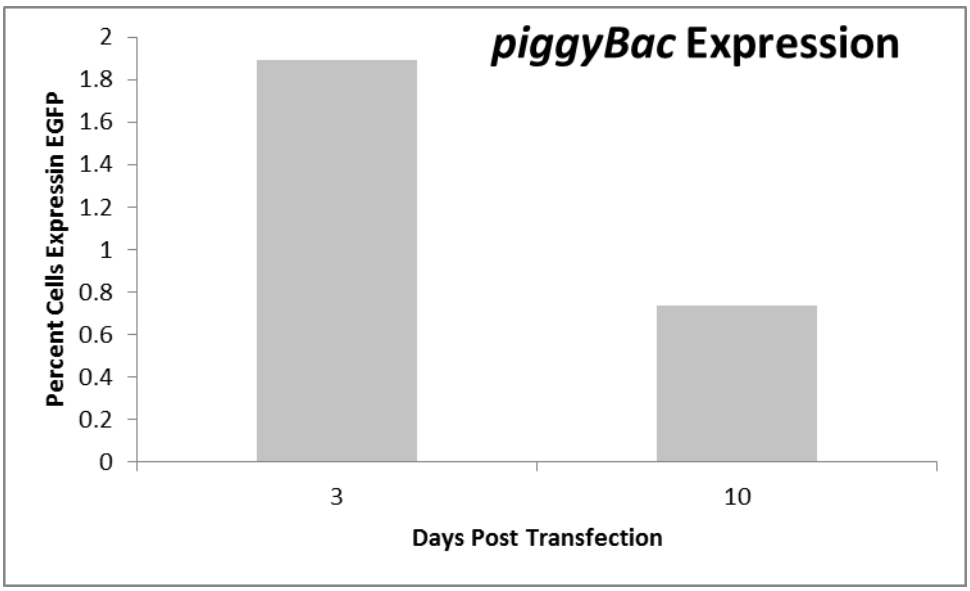


Figure 4.2. Integration of the *piggyBac* transposon is confirmed by dsRED expression. Panels (A) and (D) show transiently expressed GFP expression to indicate cells that were transfected with DNA. Panels (B) and (E) show those same cells expressing dsRED, verifying that *piggyBac* integrated into actively expressed genes and was spliced into mature RNA in those cells. Panels (C) and (F) show merges of these images. Scale bar = 100µm left column, 200µm right column

A



B

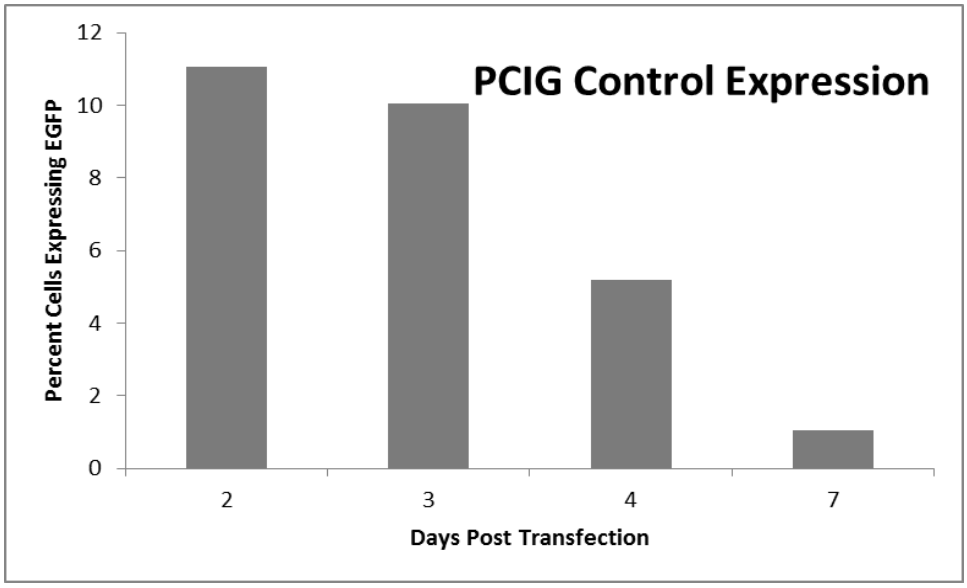


Figure 4.3. Cells transfected with *piggyBac* maintain long-term expression. Comparison of the number of cells expressing GFP between transposon transfected cultures (A) and transient pCIG transfected cultures (B) shows cultures transfected with the *piggyBac* system maintain expression levels over longer periods, indicating expression from an integrated transposon.

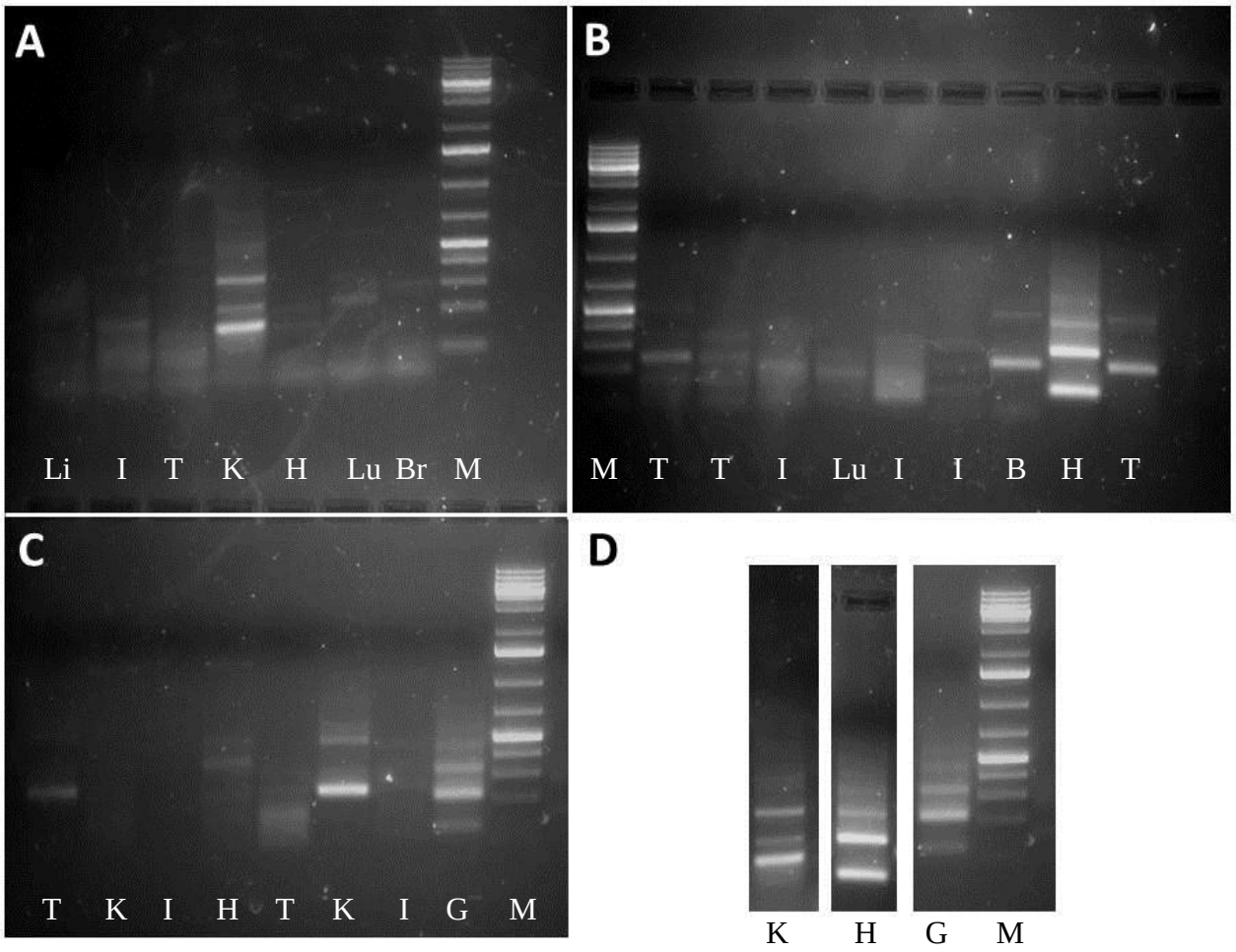


Figure 4.4. Inverse PCR amplification of *piggyBac* inverted repeat sequence. Panels (A), (B), and (C) show positive amplification of *piggyBac* inverted repeat sequence in nearly every tissue tested. Multiple bands indicate multiple insertion events. Panel (D) shows the three reactions that were subsequently nucleotide sequenced to identify inverted repeat. (A) L-R: Liver, Intestine, Testes, Kidney, Heart, Lung, Breast, Marker (B) L-R: Marker, Testes, Testes, Intestine, Lung, Intestine, Intestine, Brain, Heart, Testes (C) L-R: Testes, Kidney, Intestine, Heart, Testes, Kidney, Intestine, Gizzard, Marker (D) L-R: Kidney from (A), Heart from (B), Gizzard and marker from (C).

Figure 4.5. Nucleotide sequence analysis of iPCR product. Yellow highlighted area corresponds to *piggyBac* 5'-inverted repeat sequence. Red highlighted sequences correspond to pJet1.2 Forward and Reverse sequencing primers.

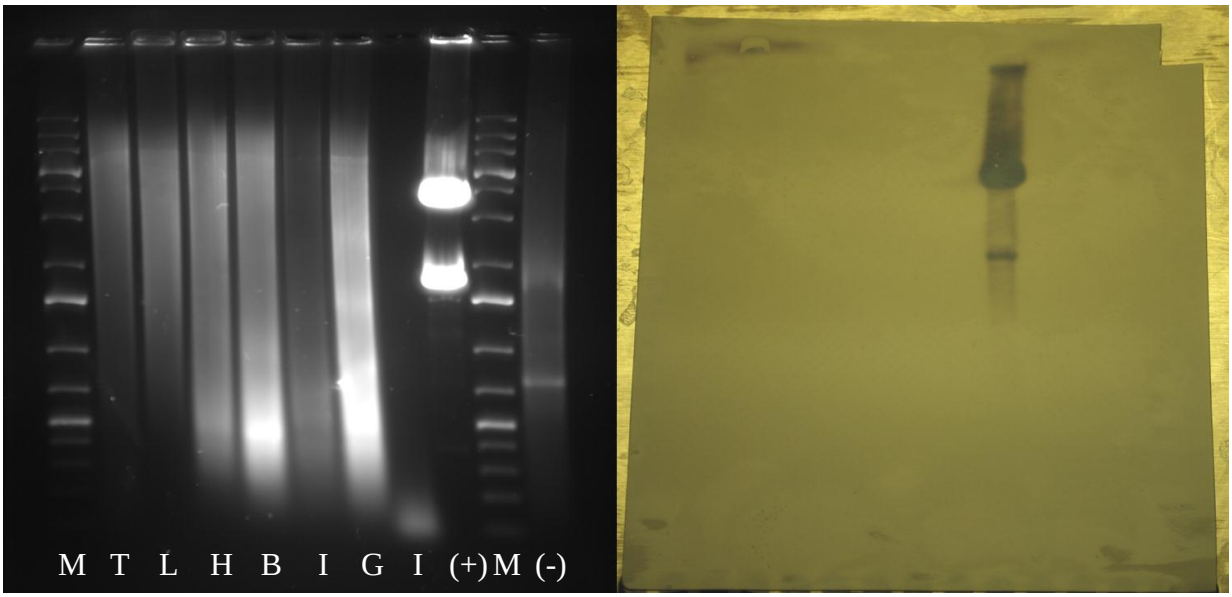


Figure 4.6. *PiggyBac* transposon sequence is not confirmed by Southern blot analysis. Southern blot was performed on selected tissues but no positive bands were seen when hybridized to a full length EGFP DNA probe. Positive reaction is EGFP positive plasmid control. Lanes L-R: Marker, Testes, Lung, Heart, Brain, Intestine, Gizzard, Intestine, (+) C, Marker, (-) C Genomic DNA

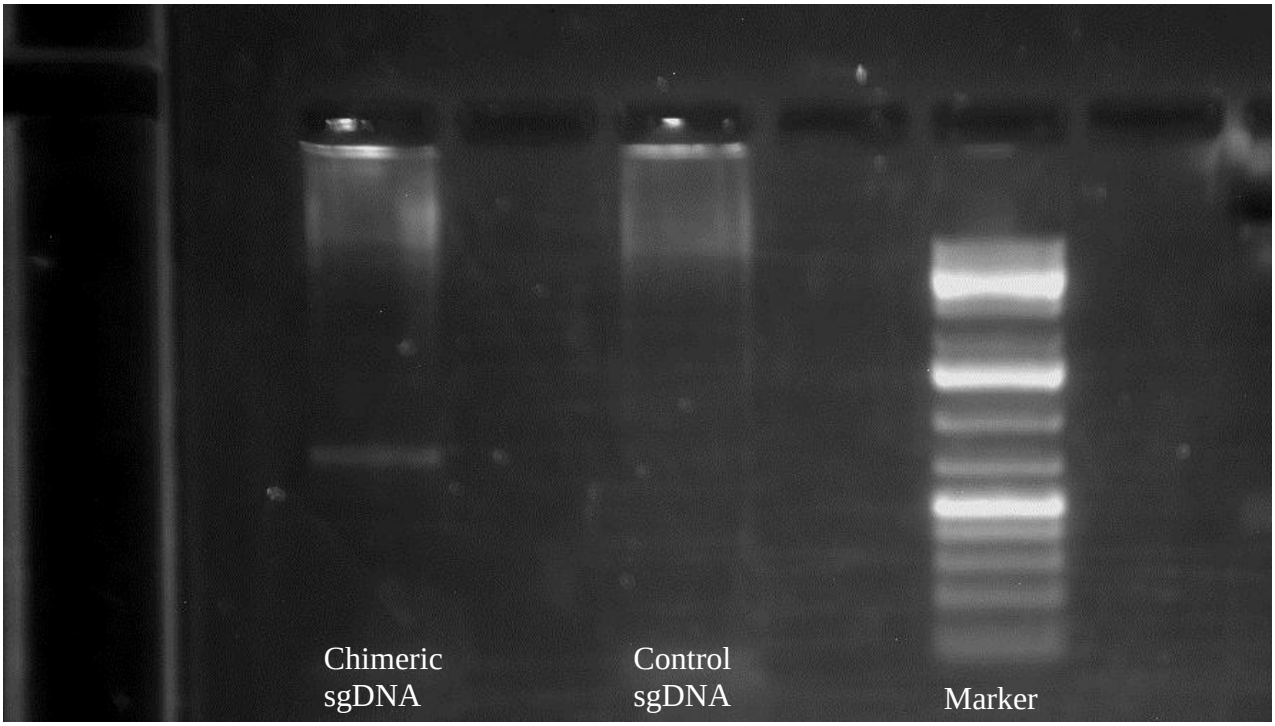


Figure 4.7. EGFP can be PCR amplified from sperm genomic DNA of chimeric Rooster 1.

Lane 1 shows positive amplification of the 729 base pair EGFP gene from sperm genomic DNA.

Lane 3, negative control genomic DNA. Lane 5, Marker.

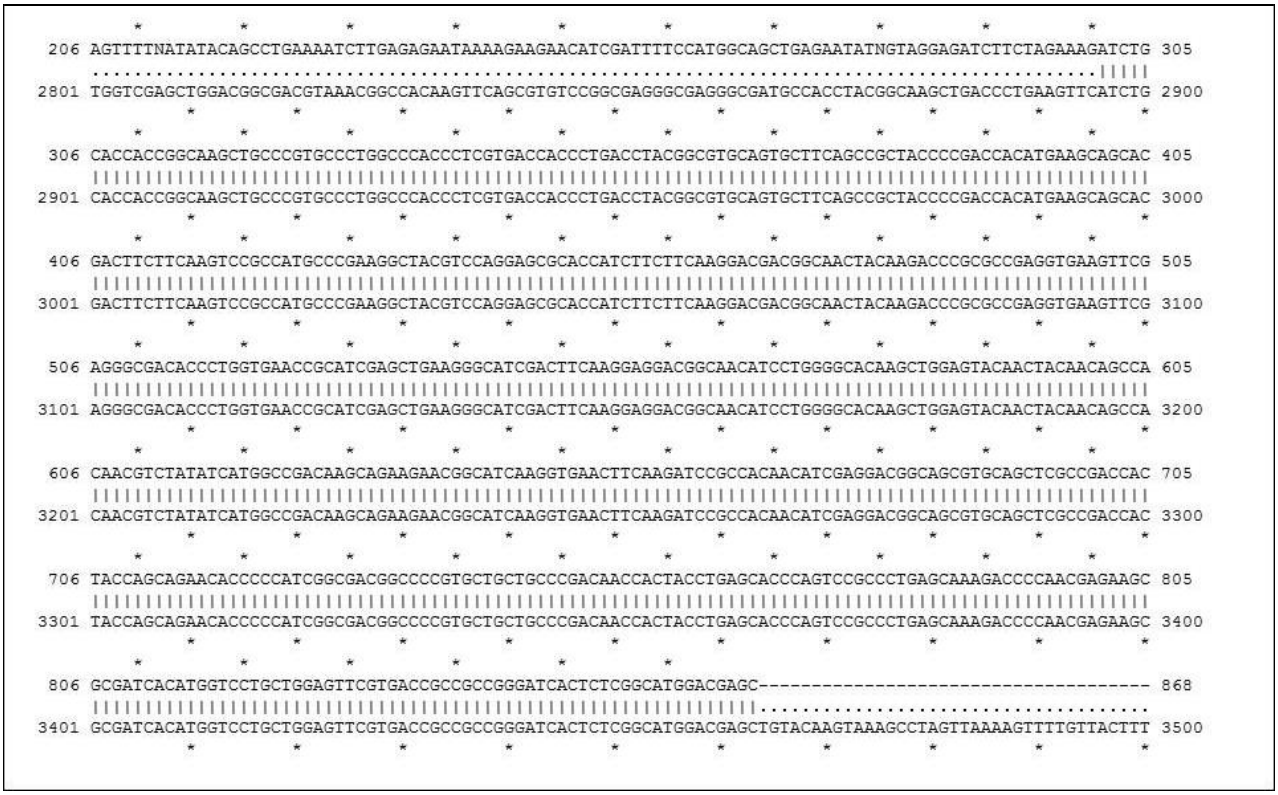


Figure 4.8. Nucleotide sequence analysis and comparison of PCR fragment to EGFP plasmid sequence. PCR fragment from 4.7 was nucleotide sequenced and that sequence was compared to EGFP plasmid sequence using A Plasmid Editor (APE) to confirm sequence identity. Comparison shows no mismatches, confirming the presence of the GFP gene in sperm.

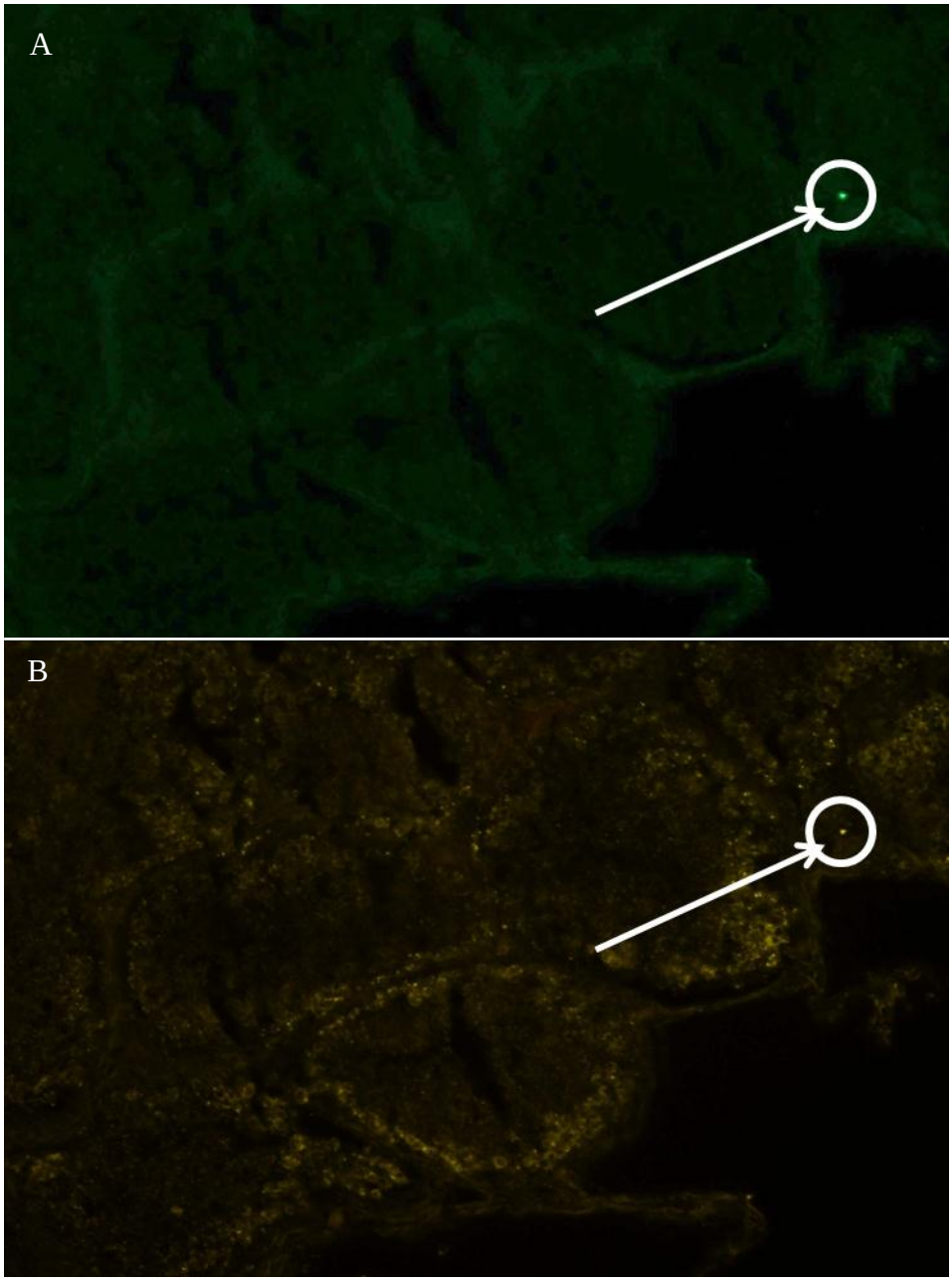


Figure 4.9. Sectioning reveals low number of GFP expressing germ cells in mature Rooster testes. (A) shows a single cell expressing GFP in the outer rim of a seminiferous tubule. (B) shows the same field stained with anti-SSEA-1 antibodies to label germ cells. We can see from comparing these images that the GFP positive cell is a germ cell.

CHAPTER 5

SPERM MEDIATED GENE TRANSFER IN CHICKEN USING A DEHYDRATION/REHYDRATION PROTOCOL[1]

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[1]Brian J. Jordan and Robert B. Beckstead. To be submitted to *Poultry Science*. 5/30/2012

ABSTRACT

Recent evidence has shown that sperm can take up exogenous DNA and RNA and transmit a cDNA copy to the developing embryo at fertilization (Giordano, et al., 2000). While this technique has met with some success in other systems, it has not been widely used in the chick due to instability of chicken sperm outside of the bird. A sperm mediated gene transfer technique has recently been reported with high success in fish (Collares, et al., 2010), whose eggs are similar to chickens with very high yolk content. A preliminary project was designed to recapitulate this technique using chicken sperm to test the effectiveness of sperm to deliver exogenous DNA to embryos.

Keywords: Sperm mediated gene transfer, chicken, sperm, transgene, embryo

INTRODUCTION

Transgenic animal research has become the gold standard for both basic and applied studies over the past twenty years. Despite the advantages of easy embryo access, short development time and ability to produce large numbers of progeny (Stern, 2005), the chick model has lagged behind other systems in transgenic analysis. The predominant method of chick transgenesis uses viral particles carrying the transgene of interest to indiscriminately infect cells of the early chick embryo. This technology has been adversely affected by low infection and integration rates (Rapp, et al., 2003), mosaic distribution of transgene expression upon successful integration and nucleotide sequence size restraints that make it unsuitable for many applications (Scott, et al., 2010). Other chick transgenic methods that are not as prevalent, such as transposons and germ cells, are met with the same mosaic expression making it necessary to cross-breed chimeric animals to obtain progeny that are uniformly transgenic. The ability to either target specific cells of the early chick embryo or target embryos at an earlier stage of development would be a great advantage for the chick system.

A definite attribute of the chick model system for developmental study is easy access to embryos inside laid eggs. However, the egg is also a detriment to chick transgenesis as the chick embryos are already 24 hours developed and 60,000 cells (Spratt, 1963) at oviposition. Targeting embryos at this stage inevitably leads to mosaic distribution of transgene, even when using systems with very high infection or integration efficiencies. Researchers are not able to target chick embryos at earlier developmental time points because there is currently no system to culture eggs taken from the oviduct to viable chicks outside of a donor shell (Sang, 2004). The only alternative, then, would be to target the ovum at fertilization, when it is a single cell. This process requires that sperm used for fertilization would carry the transgene of interest and deliver

it to embryos at fusion of sperm and egg. This process is called sperm mediated gene transfer (SMGT) (Lavitrano, et al., 1989) and has been successfully used to create transgenic animals of multiple species (Smith and Spadafora, 2005).

It is now accepted that sperm take up exogenous DNA and can carry that DNA to an egg at fertilization (Spadafora, 2008). Reports of SMGT in other systems show low efficiency, primarily due to poor DNA uptake by sperm. Sperm possess defense mechanism against foreign DNA uptake including nucleases in seminal fluid (Zani, et al., 1995) and activation of apoptosis-like pathways when large quantities of exogenous DNA are detected in the sperm nucleus (Maione, et al., 1997). Early reports of SMGT in chick shared the same low efficiency of transmission as other systems. Recently, combining DNA with transfection reagents such as Lipofectamine (Invitrogen) (Harel-Markowitz, et al., 2009) (Churchil, et al., 2011) and cryoprotectants like DMSO (Collares, et al., 2011) have increased the transmission rate in the chick. Also, a report in catfish stated 40-60% transmission of transgene using only varying concentrations of sperm diluent to dehydrate and then rehydrate sperm cells, thereby increasing uptake of nutrients and DNA in solution (Collares, et al., 2010).

The aim of the present study was to re-create the experiment previously performed in catfish in the chick using SMGT. Using differing concentrations of diluent in solution with DNA is an easy and repeatable protocol that requires no special reagents or equipment. Early findings indicate that chicken sperm will take up exogenous DNA and transmit the transgene to progeny with high efficiency, although using this protocol negatively impacts fertilization rate.

MATERIALS AND METHODS

Plasmids

Plasmid used in this protocol was pEGFP-N3 (Clontech) and was unmodified from manufacturer.

Sperm Diluent Preperation

Sem-AID turkey sperm diluent (Poultry Health Laboratories) was used to dilute sperm samples to $1X10^9$ sperm per tube. For SMGT protocol, Sem-AID diluent was concentrated to a dry pellet using a SpeedVac Concentrator (Savant) and then re-suspended in half of the original volume to achieve a final concentration of 2X. Sem-AID diluent was also diluted to a final concentration of .5X by performing a 1:1 dilution of diluent in dH2O.

Incubation of Chicken Sperm and Plasmid DNA

The dehydration/rehydration protocol from Collares, et al., (2010) was followed as previously described with minor changes. Clean sperm samples were collected from mature, stock Athens-Canada Random Bred (ACRB) roosters and pooled to achieve a homogenous solution. Four hundred microliters were aliquoted into two tubes and concentrations were determined to be ~$1X10^9$ sperm. Samples were centrifuged at 600Xg for 10 minutes to pellet sperm cells. Seminal fluid supernatant was removed and 1ml of 2X diluent was added to dehydrate sperm cells. Sperm were sufficiently suspended into solution and then incubated at 5°C for 1 hr. After 1 hr., solution was centrifuged with same parameters and supernatant was again removed. Sperm cells were suspended with 1ml of .5X diluent suspension with 50µg of plasmid DNA to rehydrate the sperm cells and assist DNA uptake. Solution was incubated at 5°C for 1 hr. The solution was then quickly warmed and hens were inseminated with one solution by artificial insemination. The second solution was used for PCR analysis.

DNA Extraction

The solution prepared for PCR analysis was washed with 1X diluent as described above 4 times to remove any residual plasmid that may be bound to the outside of the sperm. DNA was extracted from washed sperm using the cultured cells protocol from the DNeasy Blood and Tissue DNA Extraction Kit (Qiagen). In short, sperm cells were ruptured in buffer with Proteinase K, protein contaminants including cell membranes were removed by centrifugation and DNA was isolated using a charged spin-column format. DNA was concentrated from individual washes by ethanol precipitation. DNA was extracted from dissected 3-day embryos using the tissue protocol of the DNeasy Blood and Tissue DNA Extraction Kit (Qiagen).

PCR and Sequencing Analysis

PCR was performed on sperm genomic DNA along with concentrated washes using primers EGFP Test F: 5'- CGCCACCATGGTGAGCAAG-3' and EGFP Test R: 5'- GCTTTACTTGTACAGCTCGTCC-3' under cycling conditions of 1 cycle at 95°C for 2 minutes, 35 cycles of 95°C for 15s, 56°C for 15s and 72°C for 90s. Reactions were resolved on a 1% agarose gel. PCR performed on embryo genomic DNA was done under the same conditions with primers RT EGFP F: 5'-GACACCCTGGTGAACCGCAT-3' and Primer Express R3: 5'-TCCAGCAGGACCATGTGATC-3'. Reactions were verified on a 1% agarose gel, gel purified using a Gel Purification Kit (Fermentas) and then ligated into the pJet1.2 cloning vector (Fermentas) following manufacturer's "sticky end cloning" protocol. In short, PCR fragments were blunted using "Blunting enzyme" included in the cloning kit before ligation into pJet1.2. Positive clones were sequenced using pJet1.2 Forward and Reverse sequencing primers by the Georgia Genomics Facility with a 3730xl 96-capillary DNA Analyzer for Sanger sequencing and fragment analysis (Applied Biosystems). pJet1.2 F: 5'-CGACTCACTATAGGGAGAGCGGC-

3', pJet1.2 R: 5'-AAGAACATCGATTTTCCATGGCAG-3'. Sequences were compared to original EGFP sequence to confirm maximum identity to the original gene.

RESULTS

Chicken Sperm Will Take up EGFP Plasmid DNA through Dehydration and Rehydration

Clean, homogenous chicken sperm samples were collected and aliquoted so that each of two tubes contained $1X10^9$ sperm. Both samples were subjected to the dehydration/rehydration protocol using 2X and .5X concentrated sperm diluents with plasmid EGFP-N3 (Clontech) DNA. Plasmid DNA was included at a concentration of 50µg per 10^9 sperm cells. Previous reports have shown that the threshold of toxic DNA is ~50ng per 10^6 cells. One solution of sperm was used directly for insemination. The other solution was centrifuged and washed 4 times to remove any residual plasmid. DNA was either extracted from sperm cells or concentrated from individual washes and PCR was performed to determine DNA uptake. Figure 5.1 shows a positive PCR band corresponding to the 729 base pair GFP gene present in the sperm, indicating that chicken sperm will take up plasmid DNA using this protocol. The GFP band is present in washes 1 and 2 but not 3 or 4, verifying that all plasmid bound to the external surface of the sperm cells had been sufficiently washed away. This data shows that sperm will take up exogenous DNA after being dehydrated and rehydrated. This also indicates that if these sperm fertilize an embryo, the GFP transgenes should be introduced at that time.

The GFP Gene can be Detected by PCR in Embryos Fertilized with SMGT

The second SMGT solution described above was used to directly artificially inseminate hens for embryo production. Twenty SMGT hens and 10 control hens were inseminated to evaluate fertilization rate and assess the ability of sperm to transmit the GFP transgene to progeny. Table 5.1 shows the fertilization rate comparison of SMGT to control hens. It is easily

seen that this SMGT protocol had an overall negative impact on fertilization. When analyzed by days after fertilization, the SMGT protocol did not maintain fertile eggs as long as the control insemination (Table 5.2) which indicates that more frequent inseminations are needed to maintain a prolonged level of fertilization.

All 6 fertile embryos produced by the SMGT protocol were incubated for 3 days and visually analyzed under the fluorescent microscope for GFP expression. None of the embryos showed fluorescence. The embryos were dissected away from yolk and membranes and DNA was extracted as previously described. A negative control embryo was also extracted at the same time for PCR analysis. All six SMGT embryos were positive for a 317 base pair band corresponding to a portion of the GFP gene (Figure 5.2), while the control embryo gave no band. Two PCR products were purified and ligated into the pJet1.2 cloning vector for nucleotide sequencing. Analysis and comparison of the sequence showed perfect alignment to the original GFP plasmid sequence (Figure 5.3), verifying that the sperm did in fact transmit the EGFP transgene to embryos at fertilization and the embryos maintained the gene for at least three days of incubation.

DISCUSSION

The ability of sperm to take up exogenous DNA is now accepted as a general rule, despite the counter-intuitiveness of this idea. It is no wonder that researchers would want to utilize the inherent ability of sperm to develop a more successful mode of gene transfer for all model systems. Indeed, over 70 instances of transgenic animal production have been reported using this method (Smith and Spadafora, 2005). An underlying problem with this technique that must be addressed is the mechanism of DNA uptake and maintenance inside the sperm and embryos. The predominant theory is that once sperm take in foreign DNA, it is transcribed into

RNA and then reverse-transcribed into a cDNA copy by native reverse transcriptase (RT) activity (Giordano, et al., 2000). Using constructs containing intron sequence interrupting the EGFP gene, researchers have shown that sperm will transcribe RNA from the plasmid and then splice out the intronic sequence, thereby producing a "mature" RNA molecule that is reverse transcribed into the cDNA copy (Pittoggi, et al., 2006). It has also been shown that sperm will take in RNA as efficiently as DNA, and RT activity will create a cDNA copy of the RNA molecule (Giordano, et al., 2000).

Through this sequence of events, an extrachromosomal cDNA copy of a transgene is kept within sperm cells, as there is little evidence that the sequence is integrated into the genome (Zoraqi and Spadafora, 1997). This "episomal" gene copy is transmitted to progeny at fertilization and autonomously replicates inside the developing embryo. But because there is little to no integration, the cDNA copy is lost over time which ultimately yields a mosaic distribution of the transgene among cells in the embryo (Pittoggi, et al., 2006). And while PCR can detect the presence of the transgene, it is below the detection level of Southern blot (Spadafora, 2008). The transgene is also most often not expressed, with PCR being the only method of detection. The future of the SMGT protocol would seem to lie in the ability to pair this procedure with a genomic integration protocol that would stably integrate and express transgenes from all cells of fertile embryos.

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FIGURES AND LEGENDS

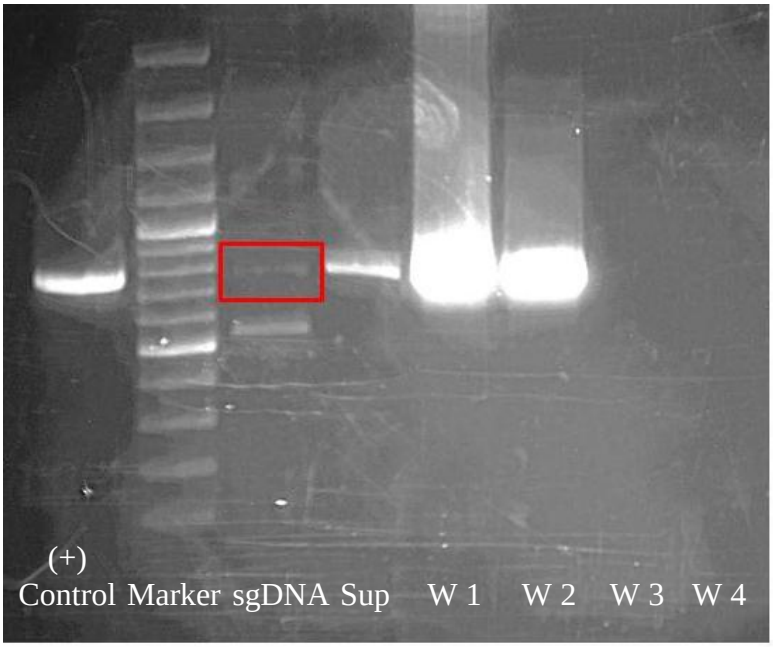

Figure 5.1. Chicken sperm will take up exogenous plasmid DNA through dehydration and rehydration. The gel above shows positive PCR amplification using DNA isolated from sperm after SMGT dehydration/rehydration protocol (Lane 3). From L-R: Positive plasmid control, Marker, Rehydration supernatant solution with plasmid DNA, Wash 1, Wash 2, Wash 3, Wash 4.

Table 5.1. Analysis of fertilization. Number of eggs and percent eggs laid by hens inseminated with SMGT sperm and control sperm over a six day period.

Trial	Total Eggs	Fertile	Percent Fertile
SMGT	94	6	6.40%
Control	57	50	87.70%

Table 5.2. Analysis of fertilization by day. Percent of fertile eggs laid by hens for six days following insemination with SMGT sperm or control sperm.

Trial	Day 2	Day 3	Day 4	Day 5	Day 6
SMGT Percent Fertile	9.50%	10%	9.09%	4.76%	0.00%
Control Pecent Fertile	100%	100%	75%	75%	84.60%

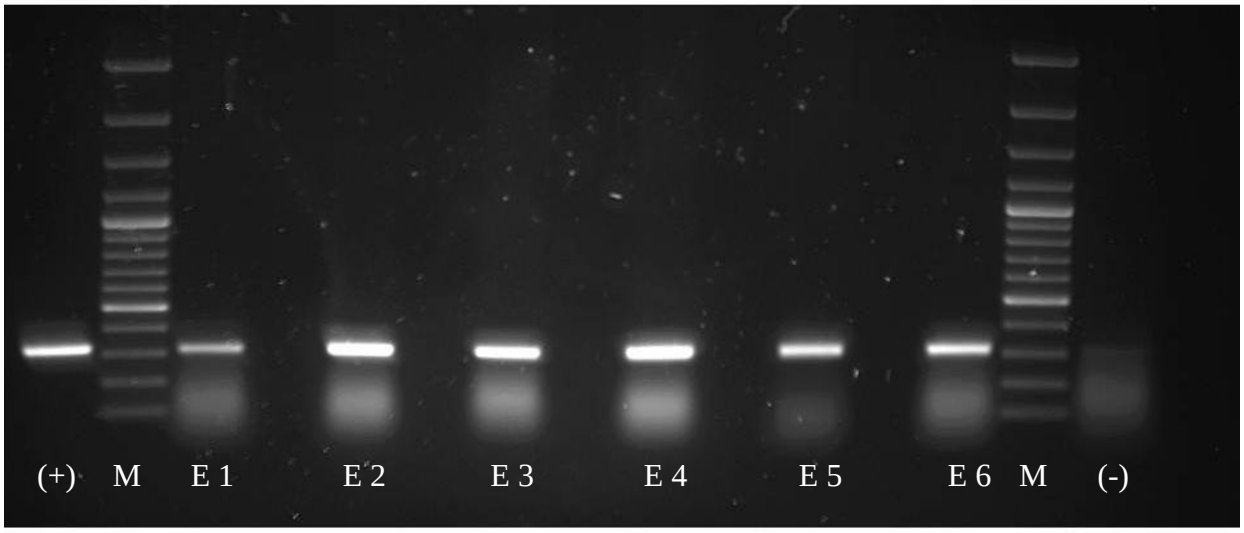

Figure 5.2. GFP gene can be detected by PCR in all fertile embryos. A 317 base pair fragment corresponding to a portion of the EGFP gene can be amplified by PCR from genomic DNA extracted from all fertile embryos. From L-R: Positive plasmid control, Marker, Embryos 1-6, Marker, Negative genomic DNA control.

Figure 5.3. Nucleotide sequence analysis of PCR product of SMGT genomic DNA from embryos. Sequence analysis and comparison verifies that PCR product obtained from embryo genomic DNA is a portion of the EGFP gene.

CHAPTER 6

CONCLUSIONS

The overall aims of this project were to assess the feasibility of producing transgenic chicks using the *piggyBac* transposon system paired with an *in-vivo* transfection reagent, JetPEI. Secondary to that was to explore an alternative protocol using sperm to carry transgenes for deposition into an embryo at fertilization, aptly named Sperm Mediated Gene Transfer. Our findings both *in-vitro* and *in-vivo* indicate that *piggyBac* is an effective molecular biology tool for integrating transgenes into chick cells. Cell culture data showed definite integration through expression of fluorescent protein in situations where only integration into active genes yielded fluorescence. Early embryo injections of *piggyBac*/JetPEI solutions yielded long-term expression of transgene, up to 6 months in adult rooster testes sections, as visualized by fluorescence. Also, PCR verified the presence of transposon sequence in genomic DNA isolated from positive tissues. Combined, this evidence shows the effectiveness of *piggyBac* in chick cells and suggests that its efficiency may only be limited by the mode of administration.

An introductory study into the effectiveness of SMGT found that chicken sperm will take up exogenous DNA and transmit that DNA to its offspring. While the protocol used here was detrimental to fertility, the groundwork has been laid to investigate other SMGT methods. Multiple methods of SMGT have been previously used in other systems and could be investigated in the chick. The simplicity of using a SMGT protocol combined with the possibility of transgene introduction at the single cell stage make this method an interesting alternative at the least, and a powerful transgenic tool at the most. The next step in SMGT evolution is

combining this protocol with a genomic integration system, whether active in the sperm itself or in the early embryo after fusion with the egg. Either way, transgene integration at this step would prove most beneficial to researchers in all areas.

In tandem with this idea, it seems very reasonable that the two aims of this project could be used in conjunction with each other in the future. Indeed combining these two protocols may overcome drawbacks associated with each method. *PiggyBac* has proven to be a very flexible and efficient integration system, and if paired with the proper SMGT protocol, could lead to very high rates of transgenesis. If proven, this combined method could be used to analyze the spectrum of developmental and applied research topics, bringing the chicken back to the forefront of model systems.