INSERTION OF A FUNCTIONAL COPY OF SIX2 TO GENERATE A TRANSGENIC
MOUSE VIA PIGGYBAC TRANSPOSASE

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Cara A. Chang

Thesis Committee:

Scott Lozanoff, Chairperson
Stefan Moisyadi, Chairperson
Ben Fofelgren

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ABSTRACT

Identification of gene function based on known mutations remains an integral objective in the field of basic science. The adult Br heterozygous mouse displays a reduced number of nephrons in association with chronic renal failure, hypertension and reduced Six2 expression during embryonic renal morphogenesis. The purpose of this study was to determine whether a functional copy of Six2 could be inserted into the Br mouse genome in an effort to overexpress Six2. After processing and isolation by restriction enzyme digestion and pulse field gel electrophoresis, 26kb Six2 BAC DNA fragment was then cloned into the mGENIE-3-BAC transposon vector via the in vitro Gibson Assembly method. The mGENIE-3-BAC-Six2 construct was subsequently confirmed by colony PCR, restriction enzyme digestion; the construct was also tested for functionality and expression in human cell lines and transgenic mice. Our data indicates that our single plasmid transposase-mediated approach to transgenesis requires fewer embryos, while capable of incorporating 42kb of exogenous DNA into the mouse genome.
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<tr>
<td>Amp</td>
<td>Ampicillin</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Br</td>
<td>Brachyrrhine</td>
</tr>
<tr>
<td>ccdB</td>
<td>Control of Cell Death B</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>Cited</td>
<td>CREB-binding protein/p300-interacting transactivator with Asp/Glu-rich C-terminal domain</td>
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<tr>
<td>Cm</td>
<td>Chloramphenicol</td>
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<tr>
<td>Dach</td>
<td>Dachshund homolog</td>
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<tr>
<td>E</td>
<td>Embryonic Day</td>
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<tr>
<td>Eya</td>
<td>Eyes Absent</td>
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<td>HA</td>
<td>Homology Arms</td>
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<td>HD</td>
<td>Homeodomain</td>
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<td>Hox</td>
<td>homeobox</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
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<tr>
<td>Kan</td>
<td>Kanamycin</td>
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<tr>
<td>kb</td>
<td>Kilobases</td>
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<tr>
<td>Lef</td>
<td>Lymphoid enhancer-binding factor</td>
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<td>Pax</td>
<td>Paired Box</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcription Polymerase Chain Reaction</td>
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<td>RH</td>
<td>Renal hypoplasia</td>
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<td>RT</td>
<td>Room temperature</td>
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<td>Six</td>
<td>Sine oculis</td>
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<td>Wnt</td>
<td>Wingless-type MMTV integration site family</td>
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<td>WT</td>
<td>Wild-type</td>
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CHAPTER 1:

INTRODUCTION

When the human genome was made public at the beginning of the millenium, it provided scientists with the first comprehensive data to ultimately elucidate the complexities of DNA. Now, twelve years later with DNA and protein sequences in hand, researchers are still attempting to identify gene expression and mechanisms that allow a set of nucleotides to create a fully intact and functional organism. Bridging the gap between sequence and function is no easy undertaking, most scientists employ either loss of function, gain of function, or a combination of both to determine the role of genes in an organism (Alberts et al., 2008).

Although seemingly counterintuitive, one of the clearest ways to ascertain gene function is by modifying gene production in either a gain or loss of function experiment. The alteration of the genetic makeup of an organism through mutagenesis, thereby reducing the actions of a particular gene by either inactivating it or rendering it ineffective, is characterized as a loss of function mutation (Alberts et al., 2008). The creation of loss of function mutations can be done through viral introduction, electroporation, homologous recombination, and RNA interference. The most common application of these techniques in practice today is the generation of a knockout mouse. By using gene targeting events, scientists can completely delete or deactivate both alleles of the same gene (Alberts et al., 2008). This disruption in the normal operations of the organism will reveal the true value of this specific gene. Based on the same principles, increasing the production of a gene, adding novel features to that gene, or misexpressing
the gene in the organism will result in an altered phenotype for scientists to evaluate. These gain of function mutations are created using similar techniques but additional DNA sequences are added to either enhance the promoter sequence of a particular gene or incorporate multiple copies of the same gene into the organism’s genome (Alberts et al., 2002). Alone, both techniques provide important inferences about the role of a particular gene in the framework of an organism.

However, when both techniques are combined, the resultant genetic rescue experiment provides far more substantial evidence for clarifying gene function. In a genetic rescue experiment, the endogenous gene of interest is underexpressed in a loss of function experiment and the phenotype is characterized and analyzed. Through the incorporation of an exogenous protein or DNA fragment by the techniques described above into the knockdown organism, the underexpression of the protein product is compensated for artificially, in the hopes of rescuing the wild type phenotype. By rescuing the original phenotype, it confirms that the effects of the knockdown are the result of the target gene itself, rather than the interaction of the knockdown agent with another gene. While it is not always possible to rescue a gene of interest in a developing organism due to the necessity for proper temporal and distributional expression, when accurately executed, a genetic rescue experiment can provide important insights about previously unknown gene function and mechanisms.

The gene of interest that is profiled in this thesis is that of Six2. Six2 is a transcription factor that plays an important role in the embryonic development of the growing organism. Having been first identified in the Drosophila genome as sine oculis, these genes have been conserved and duplicated to form the Six gene family that is
present in various metazoans, including mice, humans, and chickens (Kawakami et al., 2000). In general, the Six gene family functions in vertebrate embryogenesis and organogenesis, especially in the head and renal systems, by promoting cell proliferation and survival at key time points in the developing organism (He et al., 2010, Christensen et al., 2008, and Kawakami et al., 2000). When their normal patterns of expression are disrupted, it will contribute to afflictions involving both the head and kidney, such as branchio-oto-renal disease (Fogelgren et al., 2008). Six2, in particular, is expressed in the head mesoderm, branchial arches, fore- and midgut, nephrotomes, genital eminences, and limb mesenchyme but the majority of its characterization is based upon its functions in craniofacial and renal development (Kawakami et al., 2000).

1.1. Six2 Craniofacial Expression and Function

The expression pattern of Six2 by in situ hybridization and section and whole mount immunohistochemistry in the craniofacial development of the mouse has been defined by multiple groups (Oliver et al., 1995, Ohto et al., 1998, and Fogelgren et al., 2008), all resulting in similar conclusions. The presence of Six2 arises early in the development of the mouse and develops in an anterior and posterior manner. By E8.5, Oliver et al. (1995), later confirmed by Ohto et al. (1998), and Fogelgren et al. (2008), described the initiation of Six2 expression in a restricted class of cells in the head mesoderm, hindbrain and the first branchial arch. Expression of Six2 continues to spread to other areas of the head and by E9.5, Six2 is expressed in the mesenchymal cells extending from the hindbrain to the spinal cord, first and second branchial arches, ectoderm of the mandible and maxilla, and non-neuronal derivatives of the neural crest origin that later contributes to the skeletal and connective tissues in the head (Oliver et
al., 1995). At E10.5, expression of Six2 continues in the head and reaches the neuroectoderm and a small cell population in the anterior hindbrain (Oliver et al., 1995). The presence of Six2 was still detected by E11.5, where Fogelgren et al. (2008) showed expression in the forebrain and facial prominences. By E12.5, Six2 expression remains in the facial prominences and the midface of the developing embryo (Fogelgren et al., 2008). However, by E14.5-E16.0, much of the staining for Six2 had receded except for some weak expression in the head bones and muscles, derived from neural crest connective tissue (Ohto et al., 1998 and Oliver et al., 1995).

While the expression pattern of Six2 in the developing head is clearly defined, its complete physiological relevance is still ambiguous. However, it is certain that without the presence of Six2, the mouse embryo would not survive very long past birth. Recent experiments using Six2 knockout mice have shown that while Six2 plays no role in the initial development of the cranial base, including the migration of the cranial neural crest and condensation and differentiation of mesenchymal progenitors, it plays an important role in the correct elongation of the cranial base and bone fusion (He et al., 2010). Further experiments involving the Brachyrrhine (Br) mouse mutant, whose mutation was mapped to the distal region of chromosome 17, where only Six2 was located, indicated that misexpression of Six2 was coupled with defects in the chondrocranial morphology, signified by the truncated snout, an underlying wide nasal capsule with a midline cleft, and an incomplete anterior cranial base (Fogelgren et al., 2008). Thus, because Six2 is prominently expressed in proliferating neural crest cells, tissue that are neural crest derived, such as the anterior cranial base, would have a more severe phenotype and would be more affected than other tissues.
In the developing head system, successful head formation is dependent upon the interactions between Six2 in Six-Eya-Dach-Pax network and Six2 and the Hox proteins. Six2 participates in the development of the neurogenic placodes in participation with the Six-Eya-Dach-Pax regulatory network, conserved from *Drosophila* compound eye development (Schlosser, 2006). Eya, which lacks a DNA binding site, acts as a transcription coactivator of Six; when bound, the Six-Eya complex translocates to the nucleus, where its function is dependent on other cofactors present. Dach and Pax work as transcription factors of Six and are coexpressed in various tissues. However, the primary interaction is between the Six and Eya families that contribute to differentiate the cranial placodes from the adjacent epidermis by the anti-apoptotic effects of Eya to regulate placode size, the delamination of sensory ganglion cells from neurogenic placodes and morphogenetic movements, and the promotion of certain cytodifferentiation pathways to promote a proliferative neurogenic precursor state (Schlosser, 2006). Conversely, in the neural crest-derived mesenchyme, Hoxa2 of the Hox family of proteins functions in the repression of Six2 during the branchial arch development, specifically in the second arch (Kutejova et al., 2005 and Yallowitz et al., 2009). Hoxa2 recognizes two GAATAAT motifs near the transcription start site of the Six2 promoter and physically interacts to promote repression (Kutejova et al., 2005).

1.2. *Six2 Renal Expression and Function*

As with the head, the expression pattern of Six2 in the kidney has been extensively characterized by multiple authors. Expression of Six2 actually precedes the development of the metanephric kidney and is observed, through in situ hybridization experiments, in the metanephric blastema before uteric bud ingrowth (Self et al., 2006).
The expression of Six2 in the developing kidney appears at E10.5 in the developing cervical nephrotomes, the precursor to the kidney (Oliver et al., 1995). As the kidney continues to grow, the mesonephros lengthen to form tubules, which are surrounded by mesenchymal cells expressing Six2. Pronounced staining continues for another two days until E12.5, when it is localized to the mesenchyme surrounding the urethric buds of the metanephric kidney (Oliver et al., 1995). Although the exact time point at which Six2 expression had diminishes is in contention, it is at a time point between E14.5 (Ohto et al., 1998) and E16.0 (Oliver et al., 1995).

Because of Six2’s integral role as a progenitor for embryonic renal development, its function in the kidney is better understood. As in the head, normal mammalian kidney growth, even embryo survival, is dependent upon appropriate Six2 activity. At a very basic level, the role of Six2 is to maintain the renal epithelial stem cells in order to prevent the differentiation of blastemal cells (Self et al., 2006). However, when Six2 fails to act in this manner, it can have very deleterious effects on the developing mammalian kidneys in terms of osmoregulation, nephron number, and tubulogenesis, ultimately leading to renal failure (Somponpun et al., 2011, Fogelgren et al., 2009, Self et al., 2006). In experiments using either a Six2 knockout mouse or defective Six2 Brmouse, when the lack of Six2 reduces the stem cell progenitor population, accelerated nephrogenesis occurs. This results in mispatterning and aberrant glomeruli formation and duct branching and early termination of nephrogenesis (Fogelgren et al., 2009 and Self et al., 2006). The lack of adequate nephrons inherently affects normal kidney function, reducing its reabsorption capabilities and resulting in hypertension and chronic renal failure (Somponpun et al., 2011 and Fogelgren et al., 2009).
While the regulation of the physiological actions of Six2 is still undefined, its involvement in other embryonic gene networks to contribute to initial kidney formation and maintain the undifferentiated metanephric mesenchyme has been documented. Similar to the development of the head, maturation of the embryonic kidney is in part determined by Six2 interaction with Pax, Eya, and Hox gene families. Specifically, Hox11 paralogs, with cofactors Pax2 and Eya1, bind to an enhancer element on Six2, thereby activating it in the metanephric mesenchyme in order to stimulate the ureteric bud ingrowth (Yallowitz et al., 2009 and Hendry et al., 2011). When the kidney is further along in its development, sections emerge that are partitioned based on the expression of either Six2 or Cited1. To promote epithelial differentiation in the pretubular aggregate, Wnt9b/β-catenin act on Six2-only compartments to trigger the expression of Lef1 and Wnt4. As a result, Lef1 in the pretubular aggregate undergoes epithelialization through noncanonical Wnt signaling (Brown et al., 2013 and Hendry et al., 2011). Because the Cited1-expressing cell populations are not affected by the Lef1 and Wnt signaling, this compartment preserves its ability for additional nephrogenesis (Brown et al., 2013).

1.3. Six2 as a Transcription Factor

Six2, as with all members of the Six family, has two conserved domains that mediate its interactions with the above cofactors. The Six-type homeodomain is comprised of approximately 60 amino acid residues that enables Six2 to bind to specific DNA sequences. This particular homeodomain is conserved throughout evolution, being classified in the K50 class, but is distinctive in the loss of arginine at position 5 and glutamate at position 12 in the first helix and the replacement of asparagine with a lysine
at position 50 (Kawakami et al., 2000 and Hu et al., 2008). Both the asparagine and the arginine residues are typically involved in DNA binding to a specific TAAT sequence. However, because the Six-type homeodomain lacks these amino acids, Six proteins do not bind to DNA containing the TAAT core sequence (Kawakami et al., 2000 and Hu et al., 2008). 110-123 amino acid sequence comprising the Six domain, located N-terminal to the homeodomain (Hu et al., 2008) plays a more active role in executing Six2’s abilities as a transcription factor. The Six domain allows Six2 to bind to specific DNA sequences, in conjunction with the homeodomain, and then directing the translocation of Six2 back to the nucleus with Eya family cofactor (Kawakami et al., 1996, Ohto et al., 1999, and Brodbeck et al., 2004). Few regulatory targets of Six2 are known but through the interaction of the homeodomain and the Six domain, Six2 binds to the ARE regulatory element of Na,K-ATPase α1 subunit gene and MEF3 site in the proximal promoter of myogenin (Kawakami et al., 2000 and Hu et al., 2008).

1.4. Other Models of Six2 Transgenesis

Successful transgenesis of Six2 alleles into a mouse was achieved by Kawakami et al. in 2008 specifically for the purpose of mapping the cell fates of Six2+ cells, not for genetic rescue experiment. They created four Six2-Cre alleles (approximately 14.5kb): 1) Tet-off-eGFPCre (Six2-TGC^{tg/+}) and knock in alleles 2) with TGC (Six2^{TGC/+}), 3) with CreER^{T2} (Six2^{CF/+}), and with eGFPCreER^{T2} (Six2^{GCE/+}) that were inserted into at the Six2 start site (Kawakami et al., 2008). These vectors were introduced by gene targeting into embryonic stem cells, which were screened by PCR and subsequently injected into blastocysts (Kawakami et al., 2008). These mice strains were maintained and fully functional in their examination of nephron progenitor cell populations and nephron tubule
formation. This model specifically looked at Six2 gene interaction with other previously described gene networks, rather than the regulation of Six2 at the DNA sequence level.

Because of the necessity of the interaction between different domains within the Six2 protein, it underscores the importance of including all of the domains and regulatory elements when creating a rescue transgenic mouse. In independent experiments, Brodbeck et al. (2004) and Kutejova et al. (2005) cloned an approximately 900bp sequence, located upstream of the Six2 transcription start site, ahead of a lacZ reporter gene to identify if it contains the regulatory elements necessary to drive transcription. The resultant β-galactosidase staining in the transgenic embryos was consistent with the pattern of tissue-specific Six2 expression in the kidney and branchial arches (Brodbeck et al., 2004 and Kutejova et al., 2005). While this does imply that this promoter region is necessary to drive a basal level of Six2 expression, it lacks any other significant Six2 regulatory elements that may make the gene functional (Fogelgren et al., 2008).

When the Br mouse was first characterized by Ma and Lozanoff in 1993, a semidominant mutation was discovered that was eventually linked to Six2 (Fogelgren et al., 2008). The Br mutation was linked to the distal region of chromosome 17 (McBratney et al., 2003), where the only candidate gene possible in the 171kb critical region was Six2 (Fogelgren et al., 2008). As mentioned earlier, the Br mutation exhibits a comparable phenotype to that of a Six2 knockout, resulting in frontonasal dysplasia and renal hypoplasia (Fogelgren et al., 2008). After the completion of thorough sequence analysis by Fogelgren et al. (2008), no mutation was discovered in the entire Six2 coding region, intron region, 5’ and 3’ untranslated region, and 1.8kb upstream of the start codon, which includes the predicted transcription start sites and both of the demonstrated
promoter binding sites, implying that the mutation must lie in a cis-acting regulatory region. Consequently, this justified a need for the creation of a transgenic rescue mouse that reexpressed Six2 in the Br colony that was able to express not only Six2 gene itself but also its corresponding regulatory regions.

1.5. The Use of Viral and Non-viral Vectors for Transgenesis

In the postgenomic era, research efforts have largely been focused on the manipulation of the genome in order to increase efficiency, overcome barriers to cellular entry and enhance cargo capacity through the use of viral and non-viral vectors. According to Khan et al. (2012), The optimal vector must have the following characteristics, including the abilities to 1) evade degradation by the immune and lysosomal systems, 2) bypass the cell and nuclear membrane, and 3) enhance the ability of its cargo to be transcribed and translated. Because of the inherent role of the cell membrane as the gatekeeper for entry, it acts as a considerable obstruction to the insertion of foreign genes (Atkinson and Chalmers, 2010). The delicate balance between temporarily interrupting the membrane and keeping the cell intact, while still maintaining the integrity of the DNA has necessitated multiple methods of gene delivery. Furthermore, intracellular barriers for successful gene delivery also exist ranging from destructive nucleases and lysosomes to the larger problem of chromosomal integration in the nucleus (Al-Dosari and Gao, 2009). These challenges pose great threats to the success of large-scale gene manipulation to create stable cell lines and colonies. Neither viral nor non-viral vectors have been identified as the absolute solution, though both do have their own unique set of advantages and disadvantages.
Taking advantage of their natural abilities to highjack and control cellular processes through DNA manipulation, the use of viral vectors emerged as an obvious choice for genomic alteration. There are numerous viruses, including the retrovirus, adenovirus, adeno-associated virus (AAV), pox virus, herpes simplex virus, vaccine virus, and the lentivirus (Atkinson and Chalmers, 2010 and Witlox et al., 2007) that have been modified for this purpose. The primary advantage for the use of the viral vector is its ability to safely and efficiently move genetic cargo across the plasma membrane. Natural mechanisms, such as the retroviral-specific enzyme integrase or AAV-specific endonuclease and helicase, integrate the foreign DNA into the genome to ensure replication in multiple cell cycles for long term expression (Atkinson and Chalmers, 2010 and Witlox et al., 2007). Despite the initial promise of viral vectors, especially that of the retrovirus, adenovirus, and AAV, their pathogenicity prohibited their exclusive use for gene manipulation. Using viral vectors elicited a major inflammatory response from adenoviruses and increased the potential of oncogenes due to preferential retroviral insertion sites upstream of transcription start sites, research efforts were directed away from viral vectors and towards non-viral methods of gene delivery (Dobbelstein, 2003 and Atkinson and Chalmers, 2010).

While synthetically designed non-viral vectors have eliminated some of the health risks associated with viral vectors, future improvements in technique and technology will hopefully yield success in this field. Because of the lack of intrinsic ability to cross the plasma membrane and integrate into the chromosome safely, the use of non-viral vectors involves multiple barriers to entry that involves modification of the gene delivery method, DNA packaging, and the gene of interest itself. The process of gene delivery
using a non-viral vector occurs one of two ways: physically, which involves transient puncture of the cellular membrane, or chemically, which involves packaging the DNA into a cationic lipid or polysaccharide based polymer (Al-Dosari and Gao, 2009). As a result, these vectors have low immunogenicity and can carry vast genetic information but have low efficiency and the potential to be genotoxic due to their methods of integration into the genome (Atkinson and Chalmers, 2010 and Khan et al., 2012). Although methods exist for successful conditional gene manipulation through the Cre-loxP recombinase system and bacteriophage C31 integrase, the lack of cellular control of genomic insertion sites associated with these enzymes can result in unexpected excision, epigenetic effects and chromosomal aberrations (Atkinson and Chalmers, 2010). Given these disadvantages, according to Atkinson and Chalmers (2010), the future of long term gene integration seems to lie with a transposon based system.

The transposon based system utilizes the natural ability of the transposase to integrate large sets of genes into the chromosome and applies that toward the generation of transgenic animals. First, identified in maize in the 1950s, transposon-based mutagenesis has proven effective for genetic analysis in both prokaryotes and eukaryotes (Ding et al., 2005). There are two main types of transposases: DNA transposons that use DNA intermediates and retrotransposons that use RNA intermediates (Atkinson and Chalmers, 2010). Both employ a simple cut and paste mechanism that can move multiple genes across the genome while neither triggering a host immune response nor resulting in genotoxic effects (Atkinson and Chalmers, 2010). However, further utilization of the transposase is limited by previously lagging technology in gene delivery methods, which can result in low efficiency and potential toxicity from the chemical-based vectors (Li et
Furthermore, due to the integration sites chosen by the selected transposase, genes of interest may be inadvertently silenced through retrotransposon integration in heterochromatin and integration as concatemers, which are suppressed due to repeat-induced gene silencing (Li et al., 2013 and Atkinson and Chalmers, 2010). Nevertheless, further research into this field has identified a few classes of transposases, including the Sleeping Beauty and piggyBac families, which eliminate the majority of concerns associated with transposon based genetic manipulation.

1.6. piggyBac Family Transposons

Despite the challenges associated with non-viral vectors, the piggyBac family of transposons has found initial success in the generation of stable transgenic animals. Isolated from the cabbage looper moth, *Trichoplusia ni*, piggyBac is a DNA transposon that has been effectively used for genetic analysis to transform the germline of more than a dozen species and is known to be particularly efficient in mammalian cells, (Burnight et al., 2012 and Ding et al., 2005). Consisting of a 2472bp transposon with 313bp 5’ terminal repeat and 235bp 3’ terminal repeat to create 594 amino acid transposase, it is capable of incorporating 100kb with varying levels of efficiency (Ding et al., 2005, Kim and Pyykko, 2011, and Li et al., 2011). Its integration mechanism is unique amongst transposases as it inserts cargo DNA, flanked by terminal repeat sequences, most commonly at tetranucleotide TTAA sequences in mammalian chromosomal DNA (Li et al., 2013 and Burnight et al., 2012). piggyBac transposases seem to have a preference for transcriptional units and multiple sites of integration, which ensures increased transcription of the gene of interest without the ill effects of heterochromatin silencing.
and position effects (Ding et al., 2005 and Li et al., 2013). Attempts as recent as March 2013 by Li et al. required the co-transfection of three plasmids in order to generate stable cell lines with consistently increased expression levels without the cell cloning steps, normally needed with traditional gene delivery. Based on its ability to carry multiple genes and insert them in ideal locations within the chromosome with no genotoxic effects, piggyBac family of transposons has established itself as an effective tool for insertional mutagenesis and phenotypic characterization (Ding et al., 2005).

1.7. Objectives

Based upon the discovery by Fogelgren et al. (2008) that the Br mutation results in an aberration in the cis-regulatory elements of Six2, the primary objective of this research is to create a transgenic rescue mouse that includes the integration of the Six2 gene as well as all of its regulatory elements. The resultant transgenic mouse will then be analyzed for the reexpression of Six2 to rescue the renal and craniofacial phenotype. In the process, we will attempt to optimize mGENIE-3-BAC non-viral vectors using piggyBac transposase to increase the efficiency and stability of the transgenic animal colonies. In order to accomplish these goals, we will generate a single vector that will have selectivity in both bacterial and mammalian cells, include Six2 and its regulatory elements, and will be self-inactivating. Verification of these vectors will require the analysis of restriction enzyme fragments, pulse field gel electrophoresis, and qPCR. Ultimately, this thesis will test the hypothesis that piggyBac transposase can integrate large genomic fragments, using a single vector and self-inactivating transposase configuration for functional Six2 expression. Additionally, this thesis will provide
further insight into the factors and mechanisms regulating the insertion of large genomic fragments via piggyBac transposase, including insertion sites and copy number.
CHAPTER 2:
MATERIALS AND METHODS

1 Materials

1.1 Bacterial/Mammalian cells used in this study:
- *E. coli* DB 3.1 (Invitrogen; Cat#: 11782-018) for propagation of the pENTR1A plasmid;
- *E. coli* DH10B (Invitrogen; Cat#: 18290-015) for propagation of pBacGENIE-3 plasmid;
- *E. coli* MDS42 (Scarab Genomics; Cat#: E-0742-10) for propagation of Six-2 BAC clone and pBac GENIE-3_26kb Six-2 plasmid
- HEK293 T-REx - Human Embryonic Kidney cell line (Invitrogen; Cat#: R710-07) for the validation of the engineered construct for transgenesis.

1.2 Cultivation Media for *E. coli* cells:

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luria-Bertani broth (LB)</td>
<td>10g tryptone; 5g yeast extract; 10g NaCl; ddH2O to 1L; pH 7.5.</td>
</tr>
<tr>
<td>Luria-Bertani agar</td>
<td>LB broth supplemented with 20 g / liter Bacto agar.</td>
</tr>
<tr>
<td>Sucrose media</td>
<td>10g tryptone; 5g yeast extract; ddH2O to 880ml; sterile 50% sucrose up to 1L</td>
</tr>
<tr>
<td>Sucrose agar</td>
<td>440ml of sucrose media; 7g of agar; 60ml of sterile 50% sucrose</td>
</tr>
<tr>
<td>Terrific broth (TB)</td>
<td>12g bacto-tryptone; 24g bacto-yeast extract; 4 ml glycerol; 2.3g KH2PO4; 12.5g K2HPO4; ddH2O to 1L; pH 7.5.</td>
</tr>
<tr>
<td>SOC media</td>
<td>0.5% Yeast Extract; 2% Tryptone; 10 mM NaCl; 2.5 mM KCl; 10 mM MgCl2; 10 mM MgSO4; 20 mM Glucose</td>
</tr>
</tbody>
</table>

1.3 Antibiotics for selection were used in the following working concentrations:
- Ampicillin: 100 μg/ml
- Chloramphenicol: 12.5 μg/ml
Kanamycin: 30 μg/ml
Hygromycin: 100 μg/ml

1.4 Plasmids used in this study are listed in Table 2 below:

<table>
<thead>
<tr>
<th>Plasmid/BAC clone</th>
<th>Reference</th>
<th>Selection marker</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pENTR1A</td>
<td>Invitrogen, CA</td>
<td>Kan</td>
<td>Donor vector</td>
</tr>
<tr>
<td>RPC 23-397-J6</td>
<td>Bac Pac repository clone</td>
<td>Cm</td>
<td>Six-2 gene source</td>
</tr>
<tr>
<td>pENTR1A-6-2 HA</td>
<td>Designed/constructed for this study</td>
<td>Kan</td>
<td>Intermediate donor vector</td>
</tr>
<tr>
<td>pBac GENIE-3</td>
<td>Designed/constructed for this study</td>
<td>Amp/SacB</td>
<td>Destination vector</td>
</tr>
<tr>
<td>pBac GENIE-3_Six-2 HA</td>
<td>Designed/constructed for this study</td>
<td>Amp/hygro/dsRed</td>
<td>Intermediate destination vector</td>
</tr>
<tr>
<td>pBac GENIE-3_26kb Six-2 gene or GA#15</td>
<td>Designed/constructed for this study</td>
<td>Amp/hygro/dsRed</td>
<td>Final destination vector used for transgenesis</td>
</tr>
</tbody>
</table>

1.5 Restriction Endonucleases and DNA Modifying Enzymes:

EcoRV, Sall, PmeI, BamHI, HindIII, ClaI, BsrGI, PvuI, SnaBI restriction enzymes were purchased from New England Biolabs (NEB). Desphosphorylation of DNA was performed using Shrimp Alkaline Phosphatase, purchased from USB (Cat# 70092Y). RNA was removed from the cDNA samples using RNaseH from New England Biolabs. Ligation reactions were made with Fast Link DNA Ligation kit from Epicentre Biotechnologies (Cat #LK6201H). Gateway recombination reactions were completed using Gateway LR Clonase II enzyme mix (Invitrogen Cat#: 11791-100). Final assembly of the construct was completed using the Gibson Assembly Master Mix (Cat#: E2611S) from New England Biolabs.
### 1.6 Primers used in the study were ordered from Integrated DNA Technologies and listed in Table 3.

**Table 3**

<table>
<thead>
<tr>
<th>Sequence Name</th>
<th>Use</th>
<th>Sequence</th>
<th>Tₘ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA pENTR1A_F</td>
<td>Introduction of Six2 Homology Arms into pENTR1A</td>
<td>TTG CCC CAG GAT GAT ATC TAG ACC CAG CTT TCT TGT AC</td>
<td>68.2</td>
</tr>
<tr>
<td>GA pENTR1A_R</td>
<td></td>
<td>ATT TGG GAA GAA GCC AGA GT CGA TGG TAT TGG AGA TAA GCA GAC AGT TTT ATT GTC C</td>
<td>67.8</td>
</tr>
<tr>
<td>dsRed detect_F</td>
<td>Colony PCR</td>
<td>CGA GGA CGT CAT CAA GGA GT</td>
<td>56.4</td>
</tr>
<tr>
<td>dsRed detect_R</td>
<td></td>
<td>GCT TCT TGT AGT CGG GGA TG</td>
<td>55.3</td>
</tr>
<tr>
<td>attL1_ZS</td>
<td>Colony PCR and sequencing for pENTR1A</td>
<td>CAG GAC GCC CGC CAT AAA C</td>
<td>59.1</td>
</tr>
<tr>
<td>attL2_ZS</td>
<td></td>
<td>GTA TTA CTG TTT ATG TAA GCA GAC AGT TTT ATT GTC C</td>
<td>56.7</td>
</tr>
<tr>
<td>detect_Hygro_F579</td>
<td>Detection of Hygromycin Resistance Gene</td>
<td>GAT GTA GGA GGG CGT GGA TA</td>
<td>55.9</td>
</tr>
<tr>
<td>detect_Hygro_F104</td>
<td>qPCR</td>
<td>ATT TCG GCT CCA ACA ATG TC</td>
<td>53.5</td>
</tr>
<tr>
<td>detect_Hygro_R</td>
<td></td>
<td>GAT GTC GGC GAC CTC GTA TT</td>
<td>55.0</td>
</tr>
<tr>
<td>detect 3’TRE 177_F</td>
<td>Detection of 3’TRE Gene</td>
<td>CGC ATG TGT TTT ATC GGT CT</td>
<td>53.2</td>
</tr>
<tr>
<td>detect 3’TRE 177_R</td>
<td></td>
<td>TGT CTG GGA GTC CCT CTC AC</td>
<td>58.1</td>
</tr>
<tr>
<td>detect 5’TRE_F103</td>
<td>Detection of 5’TRE Gene</td>
<td>CAC GGC GTC TTT AAA GTC CA</td>
<td>54.8</td>
</tr>
<tr>
<td>detect 5’TRE_R103</td>
<td></td>
<td>GTC GCT GTC CAT TTA GGA CA</td>
<td>55.4</td>
</tr>
<tr>
<td>6-2 E1 colF</td>
<td>Detection of Exon1 of Six2 for Colony PCR</td>
<td>AAA GGA TAC CGA GCA GAC CA</td>
<td>55.8</td>
</tr>
<tr>
<td>6-2 E1 colR</td>
<td></td>
<td>GCA GTT CCG AGG ATG AGA AG</td>
<td>55.3</td>
</tr>
<tr>
<td>6-2 E2 colF</td>
<td>Detection of Exon2 of Six2 for Colony PCR</td>
<td>TGA ACC AGT TGC TGA CTT GC</td>
<td>55.9</td>
</tr>
<tr>
<td>6-2 E2 colR</td>
<td></td>
<td>AGG ACG GAT CTG TGT GAC TC</td>
<td>56.6</td>
</tr>
<tr>
<td>6-2 QPCR – F</td>
<td>qPCR Detection of Six2</td>
<td>CTC ACC ACC ACG CAA GTC AGC AAC</td>
<td>62.5</td>
</tr>
<tr>
<td>6-2 QPCR – R</td>
<td></td>
<td>CAC CGA CTT GCC ACT GCC ATT GAG</td>
<td>62.5</td>
</tr>
<tr>
<td>HPRT_hF</td>
<td>qPCR Detection of HPRT</td>
<td>TGA CAC TGG CAA AAC AAT GCA</td>
<td>56.0</td>
</tr>
<tr>
<td>HPRT_hR</td>
<td></td>
<td>GGT GGT TTT CAC CAG CAA GCT</td>
<td>58.0</td>
</tr>
<tr>
<td>GAPDH_hF</td>
<td>qPCR Detection of GAPDH</td>
<td>TGA CAA CTT TGG GTA TCG TGG AAG G</td>
<td>57.1</td>
</tr>
<tr>
<td>GAPDH_hR</td>
<td></td>
<td>AGG GAT GAT GGT CTG GAG AGC C</td>
<td>58.6</td>
</tr>
<tr>
<td>18S_hF</td>
<td>qPCR Detection of 18S</td>
<td>CAT TCG TAT TGC GCC GCT A</td>
<td>55.8</td>
</tr>
<tr>
<td>18S_hR</td>
<td></td>
<td>TGC TTT CGC TCT GGT TCG</td>
<td>55.6</td>
</tr>
<tr>
<td>CYPH_hF1</td>
<td>qPCR Detection of CYPH1</td>
<td>GCT GTG GCC GTC TTA GCC</td>
<td>57.1</td>
</tr>
<tr>
<td>CYPH_hR1</td>
<td></td>
<td>GCA AAC AGC TCA AAG GAG AGCA</td>
<td>56.6</td>
</tr>
<tr>
<td>CYPH_hF2</td>
<td>qPCR Detection of CYPH2</td>
<td>CCA AGA CTT AGT GCC TGG ATG</td>
<td>57.8</td>
</tr>
<tr>
<td>CYPH_hR2</td>
<td></td>
<td>TCC ACA ATG CTC ATG CCT TC</td>
<td>55.6</td>
</tr>
</tbody>
</table>
2 Methods:

2.1 Bacteria culturing conditions:

*E. coli* strains were cultivated in LB/TB/sucrose or SOC media at 37°C on an orbital incubator shaker at 240 rpm with appropriate antibiotic for selection. Alternatively, agar plates with antibiotics incubated in a 37°C incubator overnight were used for screening.

2.2 Mammalian cell culture and transient transfections

HEK293 T-REx cells were maintained by standard tissue culture techniques in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (Life Technologies). Cells were incubated in 5% CO₂ at 37°C. In accordance with Roche transfection protocols, cells were maintained at 50-70% confluence in a 24-well plate and subsequently transfected using the X-tremeGENE-9 reagent (Roche Cat#06365787001), with 2000ng of the expression construct plasmid DNA per well.

2.3 Electro-competent *E. coli* cells were prepared using the NEB protocol and 50µl aliquots were stored at -80°C:

2.4 Electroporation

An aliquot of the frozen electrocompetent cells was thawed on ice and 1µl of plasmid DNA or ligation mix was added to the cell, mixed gently, and transferred into electroporation cuvette (all procedures carried in ice). The BTX ECM630 Electro Cell Manipulator was adjusted to the following settings:

- Voltage: 1750V
- Resistance: 200Ω
- Capacitance: 25µF

After electroporation, 2ml of room temperature SOC media was added, and cells were transferred to a 15ml culture tube. After 1h of constant shaking (240rpm) and incubation at 37°C cells were plated on LB agar plates with the appropriate antibiotic for selection.

2.5 DNA/RNA Isolation and Purification

2.5.1. Miniprep Isolation

In small scale testing for positive clones, both the Zyppy Plasmid Miniprep Kit (Cat#: D4019) and ZR BAC DNA Miniprep Kit (Cat#: D4049) were used according to procedures outlined by the manufacturer unless stated differently.

2.5.2. Maxiprep Isolation

In large scale production of the plasmids for transfection, the Macherey Nagel NucleoBond Xtra Maxi Kit (Cat# 740414.10) was used to isolate DNA in accordance with manufacturer protocols with slight modifications where required (described in text).

2.5.3. RNA Isolation

RNA isolation from HEK293T cells transfected with the pBac GENIE-3_26kb Six-2 construct as well as from non-transfected cells (control sample) was performed using the Qiagen RNeasy Mini Kit (Cat#: 74104) and Qiagen QIashredder (Cat#: 79656). Treatment with DNase I (Qiagen) was performed during the RNA isolation, and the RNA was eluted using nuclease free water.

2.6 PCR Amplification

For PCR amplification we used a high fidelity polymerase, PrimeSTAR GXL Cat# R050A or GoTaq green master mix from Promega (Cat#: M7123). PCR runs were completed in the Bio-Rad C1000 Touch Thermal Cycler, duration and program were
dependent upon associated primers (See Table 3).

2.7 **cDNA synthesis**

We used High Capcity cDNA Reverse Transcription Kit from Applied Biosystems (Cat#: 4368814) for cDNA synthesis according to manufacturer’s instructions, followed by an RNaseH treatment and further incubated at 37°C for 30 min.

2.8 **Reverse Transcription PCR**

cDNA was diluted to a final concentration of 50ng/ul with nuclease free water, and 100ng were used as a template for qPCR per 20ul reaction mix. The BioRad 2x SIBR Green master mix and the primer sets listed in Table 3 were used to examine the mSix-2 gene expression. The following housekeeping genes were tested: mHPRT, mCPH1; mCPH2, and GAPDH. The expression of the mSix2 gene was normalized to HPRT and calculated using the $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen, 2001).

2.9 **DNA Manipulations**

2.9.1. **Digestion of DNA by Restriction Endonucleases**

Although individual digestion reactions were dependent upon the clone and its application, per the standard provided by New England Biolabs, approximately 1µg of DNA was added to a reaction mixture containing nuclease free water, the restriction enzyme, and its associated buffer. Unless otherwise specified by the manufacturer, incubations proceeded for one hour at 37°C. Success of the digestion was verified through analytical gel electrophoresis (continuous field or pulse field).

2.9.2 **LR II clonase recombination reaction**
Gateway recombineering reaction was carried out using Gateway LR Clonase II enzyme mix, according to Gateway system protocols. Sample volume was calculated to ensure desired manufacturer ratios of entry clone and destination vector. Reaction was incubated at room temperature for one hour or overnight. The addition of Proteinase K to each reaction mixture ensured the termination of the reaction. One µl of the LR reaction mix was electroporated into an E. coli cells. Success of the recombineering experiment was verified through colony PCR, restriction enzyme digest and sequencing.

2.9.3 Separation of DNA Digested Fragments

The chosen method of electrophoresis was dependent upon the size of the fragment. Fragments larger than 30kb required the use of pulse field gel electrophoresis that varied the direction of the electric current to obtain a higher resolution of large DNA bands (Schwartz and Cantor, 1984). Smaller fragments required the use of the continuous field electrophoresis.

2.9.3.1 Separation of DNA Digested Fragments in Continuous Field Electrophoresis

Agarose gel electrophoresis was used for analytical and preparative separation as well as purification of DNA. The size of the DNA fragments to be separated determined the concentration of agarose in the TAE gel. For fragments with sizes of 1-10kb, 1.0% agarose gels were used and 0.8-0.9 % agarose gels for fragments of 10-20kb. The agarose gels, were run at a voltage of 1-5V/cm for a duration dependent upon the size of the fragment being separated. The following DNA molecular weight markers were used: 100bp (Cat# N3231S), 1kb (Cat# N3232L), 2log (Cat# N3200L) and λDNA digested by HindIII (Cat#: N3012S) from New England Biolabs.

2.9.3.2 Separation of DNA Digested Fragments in Pulse Field Gel Electrophoresis
PFGE was similarly used for analytical and preparative separation as well as for purification of DNA fragments larger than 15kb. PFGE machinery included Bio-Rad Chef Mapper, Cooling Module, Variable Speed Pump, and Electrophoresis Cell chamber. We used 1% agarose gels in 0.5X TBE, under the Auto-Algorithm Protocol and the following PFGE parameters: molecular weight (low: 1kb; high 300 kb); calibration factor 1.00; 14°C; gradient: 6.0V/cm; run time: x hours; included angle: 120°; Int. Sw. Tm: 0.06s; Fin. Sw. Tm: 26.29s; Ramping Factor: Linear. After the completion of the PFGE run, gel was run in 0.5X TBE buffer in continuous field electrophoresis for approximately 15 minutes in both forward and reverse directions. After the final electrophoresis, the agarose gel was stained with ethidium bromide solution (0.5µg/ml in TBE buffer) for 15-20 minutes. Mid-PFG Marker (Cat#: N3551S), Low-PFG Marker (Cat#: N0350S), 2log (Cat# N3200L) and λDNA digested by HindIII (Cat#: N3012S) from New England Biolabs were used as DNA molecular weight markers.

2.9.1 Extraction of DNA Fragments from TAE/TBE Gel
DNA fragments were excised from the gel in accordance with the manufacturer protocols associated with the Zymoclean Large Fragment DNA Recovery Kit (Cat# D4046) and the Zymoclean Gel DNA Recovery Kit (Cat# D4008). The appropriate kit was chosen dependent upon the size of the fragments being isolated. Minor modifications were made to the standard protocols to ensure complete elution of DNA.

2.9.5 Dephosphorylation of DNA
The dephosphorylation of the 5’ ends of the DNA fragments by shrimp alkaline phosphatase (SAP) was necessary to prevent self-ligation of the linearized vector. 1µl of
SAP was added to the restriction enzyme mixture and reaction was further incubated at 37°C for 30 min. The DNA fragments were purified using Invitrogen micro kit or separation on agarose gels and gel extraction.

2.9.6 **Microkit Purification of DNA**

In order to purify the DNA from any environmental contaminants or reaction byproducts, the Invitrogen PCR Purification Kit (Cat# K3100-02) was used in accordance with manufacturing protocol.

2.9.7 **Ligation of DNA Molecules**

The DNA molecules of pENTR1A-6-2 HA amplified via PCR with GA_pENTR1A forward and reverse primers, were subject to self-ligation in order to generate the final entry clone. About 50-100ng of the 5’ phosphorylated and micro kit purified DNA fragments were mixed with ligation buffer, ATPs, T4 DNA Ligase, and nuclease free water. After incubation for 30 minutes at room temperature, the 1µl of the ligation mixture was electroporated. 0.5µl of T4 DNA Ligase was added to the remaining mixture and further incubated at 16°C overnight.

2.9.8 **Gibson Assembly Reaction**

To assemble the PmeI linearized pBac GENIE-3_Six-2 HA vector and the 26kb Six-2 DNA fragment we used Gibson Assembly Cloning Kit (Cat# E5510) from New England Biolabs. An equimolar ratio of both vector and fragment were mixed with the same volume of the Gibson Assembly master mix and incubated in a thermocycler at 50°C for 1 hour. The 1µl of the 1:5 diluted Gibson Assembly reaction was electroporated into MDS42 cells according to the previously described procedure. Success of the assembly was verified according to the screening procedures below.
2.10 Screening of the recombinant pBacGENIE-3_26kb_Six2 gene bacterial clones

2.10.1 Screening by colony PCR

This protocol is designed to quickly screen for plasmid inserts or testing the orientation of the inserted fragment directly from *E. coli* colonies. A single colony was resuspended in 10µl of nuclease free water and 1ul of this suspension used as a template per 15 ul reaction.

For efficiency, a PCR master mix was prepared in the following ratio: 25µl of Go Taq Green master mix (2x) from Promega; 1µl of Forward primer (10 µM); 1µl of Reverse primer (10 µM); 23µl of nuclease free water. The PCR master mix was further scaled to include all potential clones. Positive controls are specific to the construct being tested and specified below. Negative controls contained no DNA, only nuclease-free water.

PCR was conducted according to the following thermal profile:

**Table 4**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hold</td>
<td>95</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Cycle (35 cycles)</td>
<td>95</td>
<td>15 seconds</td>
</tr>
<tr>
<td></td>
<td>Primer T\text{m} - 2°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>Varies based on the amplicon’s size (1 minute per kilobase)</td>
</tr>
</tbody>
</table>

Completed PCR reactions were loaded on 1.0% agarose gel, and results analyzed against the positive and negative controls, in addition to the 100bp and 1kb DNA molecular markers.

2.10.2 Screening via Restriction Enzyme Digests

Once the positive colonies were identified, they were subsequently cultivated to extract the DNA for further testing. The positive recombinant clones were digested by
restriction endonucleases and the pattern of the obtained bands was analyzed in comparison with the predicted band (calculated using Vector NTI software). The appropriate conditions for digestion were described by the manufacturer data sheets and completed as such. The digested DNA was separated via agarose gel electrophoresis as per instructions above.

2.10.3 Verification of Positive Clones via Sequencing

The sequences of the positive clones were obtained through the submission of 400-700ng of DNA and 3.2pmol of associated primer in a final volume of 5µl to the Biotechnology Sequencing Facility at the University of Hawaii. Sequences were analyzed using Chromas software, and BLAST at the National Center for Biotechnology Information Website.

2.10.4 Screening for Expression of dsRed and Hygromycin in HEK cells

HEK cells, transfected with pBac GENIE-3_26kb Six-2 plasmid, were examined under UV light through Olympus 1X70 microscope to visualize expression of the dsRed gene. After 48 hours the culture media was exchanged to DMEM plus 100µg/ml hygromycin and cells were further cultured to test for expression of hygromycin resistance gene. Ability of the cells to survive the hygromycin selection was considered as expression of hygromycin resistance gene.

2.10.5 Screening for Expression of Six2 in HEK Cells by RT-PCR

2.10.5.1 Harvesting of Cells for Isolation of RNA

HEK cells were harvested 48 hours post-transfection. After aspiration of the medium from the well, cells were washed with 1ml of 1X PBS. 1X PBS was then aspirated and then another 1ml of 1X PBS was added to resuspend the cells. After HEK
cells were transferred to 1.8ml tube, cells were centrifuged at 2000rpm for 7 minutes at 4°C. 1X PBS was aspirated from the tube and cells were stored at -80°C.

2.10.5.2 Screening for Six2 expression by RT-PCR
Once the transfected and non-transfected (negative control) cells were harvested, total RNA was isolated and the associated cDNA was synthesized as described above. Six2 expression was then evaluated in the Applied Biosystems StepOne Plus Real Time PCR System. See above for additional details.

2.11 Generation of Transgenic Mice Overexpressing Mouse Six2 gene

2.11.1 Pronuclear microinjection for transgenesis
Integration of the 26kb DNA fragment encoding the Six2 gene into the mouse genome was executed by pronuclear microinjection at Transgenic Core Facility, at the University of Hawaii at Manoa, as described by Marh, et al. (2012). Plasmid concentrations of 15ng/µl, 20ng/µl, and 25ng/µl, were injected at the 2 pronuclei stage embryos in order to determine the optimum concentration for the highest efficiency and lowest mortality. Embryos are derived from B6D2F2 mice and CD1 female mice were used as surrogate mothers. All transgenic mice were screened at birth for expression of the dsRed reporter gene using the FluorVivo Imaging System, INDEC Biosystems.

2.11.2 Isolation of chromosomal DNA from mouse tails
At day 14, the tip (0.6 – 1.0cm) of the mouse tail was removed and frozen at -80°C for the isolation of chromosomal DNA. Two different kits, Qiagen DNeasy Blood and Tissue Kit (Cat#: 69506) and Roche High Pure PCR Template Preparation Kit (Cat#: 11796828001) were tested to evaluate quality and efficiency of chromosomal DNA
isolation. Treatment with RNase I (Qiagen) was performed during the chromosomal DNA isolation. All manufacturer protocols and procedures were followed. Chromosomal DNA was stored in +4°C until needed for PCR and copy number assay.

2.11.3 Validation of the Transposon Insertion by PCR

Genomic DNA isolated from the transgenic mice was tested by PCR for the presence of genes associated with pBac GENIE-3_26kb Six-2, including dsRed, Hygromycin Resistance, 5’TRE, 3’TRE, and m-piggyBac. Negative control samples contained no DNA, while positive control samples used the pBelloGENIE-3 template. PCR data was compiled as described above.

2.12 Software used

Specialized software: Vector NTI, Chromas; Applied Biosystems SDS software for the RT-PCR data analysis

Web-based software programs for DNA and RNA sequence analysis: NCBI
The following results involve the cloning and insertion of multiple fragments into their appropriate destination vectors. Figure 1 aims to condense each cloning reaction in a single figure to provide the reader with a greater understanding of each reaction and its purpose. The latter part of the figure summarizes how the functionality of the final construct was tested in both human cells and in the generation of a transgenic mouse.
Figure 1: Strategy for the Generation of Six2 Transgenic Mice. (A) The pBG3_26kb Six2 plasmid is generated through multiple stages of cloning that results in a final construct that contains the Six2, self-inactivating piggyBac transposase, and multiple selection factors. attL1 and attL2 represent sites of plasmid recombination. (b) One mode of verification of the functional construct was transfection into HEK293T-Rex cells. (c) Once validated, the final construct was used to integrate the plasmid into the mouse genome by pronuclear injection.
1. Construction of the pENTR1A with Six2 homology arms vector for transgenesis.

1.1. Generation of the pENTR1A vector with homology arms for the Six-2 gene.

The pENTR1A was used as an intermediate vector to clone mouse Six-2 gene homology arms. They were contained within the primers used to amplify the pENTR1A vector backbone: the forward primer included the 52bp 3’ homology arm (HA) and the reverse primer – contained the 45bp 5’HA (Fig. 2). Each homology arm also included a partial sequence for the PmeI restriction endonuclease at their 3’end. (Fig 2). Annotated sequence of the primers and plasmid/insert regions are shown in Fig. 2.

Figure 2: Primer design and genomic sequence of the 26 kb EcoRV fragment encoding the Six-2 gene. The 31bp sequence preceding the EcoRV and including the SalI/BamHI sites overlaps with the pENTR1A vector; the 29bp sequence following the EcoRV (2nd row) towards the attL2 also overlaps with the pENTR1A, while the sequences in between the yellow highlighted EcoRV site belongs to the genomic insert encoding the Six-2 transcription factor. The 45bp sequence (in blue) represents the 5’ homology arm (HA) for cloning; the 52bp sequence (in red) represents the 3’HA; the gray highlighted region is the sequence of the Pmel restriction endonuclease site constructed after the self-ligation of the PCR amplified pENTR1A vector.
A high fidelity GXL Polymerase was used to amplify the pENTR1A plasmid with above described primers. The PCR product was purified by continuous field electrophoresis and subsequently gel extracted to isolate the 2367bp fragment (Fig.3). The fragment was phosphorylated using the blunting kit (NEB) and subjected to self-ligation which gave rise to the pENTR1A-Six2 HA vector (Fig.4).

1. Excised PCR product
2. PCR product (shown for size)
3. Log2 ladder (NEB) - in kb
4. 100bp ladder (NEB) – in kb

**Figure 3: Amplification of the pENTR1A backbone to introduce the HA’s and the PmeI site.** The fragment of interest was excised from the gel (red dotted box); the subsequent row was untouched to demonstrate exact size. Picture was taken after extraction of the fragment to reduce the exposure to UV light.
1.2. Validation of Positive pENTR1A Clones

The clone was verified via restriction enzyme digest with PmeI and BamHI. Because both enzymes are single cutters, the resultant digest pattern will linearize the plasmid. During gel electrophoresis, an undigested plasmid will migrate in different positions dependent upon its structure. In relation to one another, the supercoiled plasmid will migrate further, followed by the linear plasmid, then the nicked plasmid. As seen in Figure 4, the undigested plasmid is observed below both of the linearized plasmids, consistent with previous research. Digestion with BamHI serves as a linear control to validate that the PmeI restriction site is functional and linearizes the plasmid. For further validation, the clones were submitted for sequencing.

Unlike the pENTR1A plasmid, the modified entry clone lacks ccdB expression. The ccdB gene product blocks gyrase, an enzyme involved in DNA replication. DH10B electrocompetent cells do not support the growth of bacteria containing the ccdB gene, which ensures that bacteria containing the pENTR1A clone will not survive while those containing the entry clone with homology arms will.
Figure 4: Construction of the pENTR1A vector with the Six-2 homology arms.

(A) The pENTR1A plasmid encodes for the kanamycin resistance (KanR) and the ccdB survival genes (orange arrows), which are used for selection. It utilizes the pUC origin of replication and contains the attL1 and the attL2 recombination sites. The latest were used to transfer the “3HA_PmeI_5’HA cassette” from the intermediate vector to the destination vector pBGENIE-3 via LRII clonase reaction. (B) shows virtual PCR amplification; primer annealing sites and homology arms: 5’HA (in blue) and 3’ HA (in red). The PmeI recognition site is split between the two homology arms (gray boxes). (C) Self-ligation of the pENTR1A amplified with above primers resulted in generation of the unique PmeI site and the Six2 homology arms replaced the ccdB gene.
The destination vector includes the sequences for a self-inactivating piggyBac transposase to ensure single copy integration into the mouse genome. The sequences between the attL sites in entry clone, generated from the previous reaction, we integrated into the destination pBeloBac11Genie3dsRed vector using LR clonase II reaction. After overnight incubation at room temperature, recombination via LR clonase results in the generation of two different molecules, a DNA byproduct and the vector of interest. Thus, the reaction mixture must be screened to eliminate the DNA byproduct and isolate the vector of interest.

### 2 Construction of pBAC GENIE3 Six2 HA

#### 2.13 Generation of the pBacGENIE3 Six2 HA vector

The destination vector includes the sequences for a self-inactivating piggyBac transposase to ensure single copy integration into the mouse genome. The sequences between the attL sites in entry clone, generated from the previous reaction, we integrated into the destination pBeloBac11Genie3dsRed vector using LR clonase II reaction. After overnight incubation at room temperature, recombination via LR clonase results in the generation of two different molecules, a DNA byproduct and the vector of interest. Thus, the reaction mixture must be screened to eliminate the DNA byproduct and isolate the vector of interest.
Figure 6: Map of pBac GENIE-3 plasmid. This plasmid encodes the genes for the piggyBac transposase (mPBase) as well as all of the genes necessary for successful integration into the mammalian genome. There are multiple selection markers in this plasmid including, ampicillin resistance (AmpR), chloramphenicol resistance (CmR), hygromycin resistance (HygroR), and dsRed expression. The region between attR1 and attR2 sites serve as the point of recombination for Gateway System Recombineering.

Figure 7: pBAC GENIE-3 with Six2 Homology Arms Map. The vector of interest includes selection markers for both bacteria and mammalian cells, including Ampicillin resistance, dsRed fluorescence, and hygromycin resistance. This plasmid is the result of the LR Clonase II reaction that incorporates the 3’ and 5’ Six2 Homology Arms that will be used for the insertion of the Six2 gene fragment in the final recombination. The homology arms are separated by a PmeI restriction site that will linearize the plasmid, allowing the homology arms to be accessible to the Six2 gene.
2.14 Validation of Correct pBAC GENIE3 Six2-HA Clone

The final vector of interest was verified through antibiotic selection, restriction enzyme digest and sequencing. The destination vector (pBacGENIE-3) contains the gene for Cm resistance between the attR1 and attR2 sites. Because recombination replaces the region between the attR1 and attR2 sites with the Six2 homology arms, the final vector will no longer survive the LB+Cm selection. Thus, when the colonies were screened by antibiotic selection, the colonies that were able to survive in Amp but not in Cm were expected to be positive and were tested further. After isolation of the miniprep DNA, it was digested with restriction endonucleases, PmeI, HindIII, and BsrGI, for restriction pattern analysis. Based on the plasmid map (Figure 5), the total size of the correct vector is 16489bp. As before, digestion with HindIII served as a positive linear control to validate that the PmeI restriction site was functional. When comparing the restriction digestion patterns of both pBacGENIE-3 and vector of interest, pBacGENIE-3_Six2HA, PmeI will linearize only pBacGENIE-3_Six2HA and not pBacGENIE-3. By identifying the clones that have been linearized, it was possible to select for the vector of interest. Clones that were positive by restriction enzyme digestion test were submitted for sequencing.
3 Gibson Assembly of the final construct for transgenesis

3.1 Linearization and purification of the pBacGENIE-3 with Six-2 homology arms vector in preparation for GA

Modifications to the vector, as described below, were necessary to ensure proper alignment and complete assembly of the final construct. PmeI, restriction endonuclease, cuts at a single site in the circular vector, resulting in its linearization. Approximately 1μg of DNA was digested with 20U of PmeI restriction endonuclease in a mixture containing NEBuffer 4, BSA, and nuclease free water. The restriction enzyme digest was incubated at 37°C overnight. Following the completion of the digest, the mixture was
purified using PFGE in order to separate the digested linear from circular vector that may have not been digested. The linear vector would run at 16.8kb. This fragment is excised from the gel and the DNA is purified using the large fragment DNA recovery kit. In order to prevent recircularization of the vector, 1µg of linear vector DNA was further treated with shrimp alkaline phosphatase to dephosphorylate the 5’ termini. Because inactivation of the Shrimp Alkaline Phosphatase requires heating at 65°C, which would possibly affected the quality of the DNA, the SAP- treated fragment was purified using the microkit to remove the SAP from the DNA. The quality of the DNA was verified using the NanoDrop to monitor the concentration, 260/230 ratio, and 260/280 ratio.

3.2 Preparation of the 26kb genomic DNA fragment encoding the mouse Six-2 gene (the insert)

The BAC clone, RP23-397J6, was used as the source of the Six2 gene (Figure 9). After electroporation into DH10B or S42 cells, this clone was propagated and maxi prep DNA was isolated using the Machery-Nagel kit. The maxi prep underwent multiple round of digestion with EcoRV to ensure sufficient amounts of fragment were obtained (Figure 10). The Six2 gene is encoded by the 26kb fragment which will be used to complete the assembly of the final construct.
Figure 9: Map of the RPC23-3976-J6 Bac clone. The pictured BAC clone contains the sequences for the genomic DNA of Six2 gene (192kb) along with selection markers for expression of Cm resistance. The Six2 gene is isolated by EcoRV restriction enzyme digest, resulting in a 26kb gene fragment; there are a total of 15 restriction sites by EcoRV digestion (not shown). This 26kb DNA fragment includes the sequences for exons 1 and 2 of the Six-2 gene flanked at its 3’ and 5’ ends by homology arms. The plasmid uses F1 origin of which would contribute to expression of the BAC as a single copy per cell and chloramphenicol resistance for selection are also shown. The genomic DNA separates the SacB promoter from the SacB gene, which allows the clone of interest to survive on sucrose media, adding an additional layer for selection.
PFGE was required in order to separate the DNA fragments digested by EcoRV to get the better resolution. After analysis of restriction map revealed that the 26kb fragment would contain Six2, this fragment is excised from the gel and the DNA is isolated. Due to the size of the Six2 26kb fragment, efficiencies for DNA gel extraction were generally below 20ng/μl, insufficient for Gibson Assembly protocols. As a result,

**Figure 10: Digestion of Six2 BAC by EcoRV.** This figure shows the restriction pattern, consistent with the expected fragments from restriction map. The red dotted box indicates the gel fragment that was excised and used for Gibson Assembly. *indicates fragments (8.3 and 8.2 kb) that have migrated together, note the intensity of the band compared to others. **indicates a fragment that was cut by EcoRV as a result of star activity; it is also likely that there is an additional restriction site not indicated in the genomic sequence of the BAC DNA. Additional band does not affect region of Six2 gene.
restriction digestion and gel extraction steps were completed in multiple rounds to ensure that enough insert was prepared to recombine with the pBacGENIE3 Six2 HA. As a result, the gel extracted DNA from multiple digestions were combined and then concentrated using speed vacuum centrifugation to reach a DNA concentration similar (~100ng/µl) to that of the isolated vector.

3.3  

**Gibson Assembly of pBacGENIE3 Six2-HA and 26kb Six2 gene**

The Six2 BAC DNA digested by EcoRV was combined with the vector digested by PmeI and calculated to result in an equimolar ratio. The reaction (Table 9) was incubated at 50°C for one hour, diluted in a 1:5 ratio and used for electroporation. The final construct is shown below (Figure 11).

**Table 5: Composition of the Gibson Assembly Reaction**

<table>
<thead>
<tr>
<th></th>
<th>Volume (µl)</th>
<th>(ng)</th>
<th>(pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insert xEcoRV</td>
<td>3.6</td>
<td>474.24</td>
<td>0.0456</td>
</tr>
<tr>
<td>Vector xPmeI</td>
<td>6.5</td>
<td>772.00</td>
<td>0.0457</td>
</tr>
<tr>
<td>Gibson Assembly Master Mix</td>
<td>10.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20.2</td>
<td>1246.24</td>
<td>0.0913</td>
</tr>
</tbody>
</table>
3.4 Validation of Correct pBac GENIE-3 Six2 gene clone

The pBacGENIE-3 Six2 gene construct was electroporated into electrocompetent DH10B cells and then streaked on Amp+LB plates for overnight incubation at 37°C. Each of the resultant colonies was tested for the presence of the pBacGENIE-3 Six2 gene. The 304 colonies were individually tested by colony PCR for the presence of the Six2 gene using the primers for E1 and E2. The forty four colonies were found to be PCR positive, indicating the presence of the Six2 gene within the construct. Because PCR analysis can only validate the presence of a particular DNA sequence in the clone,
another layer of selection, through restriction enzyme digestion, must be completed to ensure that the entire plasmid was inserted.

Miniprep DNA was isolated from the forty four PCR positive colonies to further test for the presence of the final construct by restriction enzyme digestion. Based on the analysis of the restriction map of the final construct, restriction endonuclease digestion by

Figure 12: Screening for potential positive clones of pBacGENIE-3 Six2 gene by colony PCR. The figure illustrates a representative gel containing the PCR amplification products, using the Six2 E1col forward and reverse primers. Samples labeled with an asterisk represent potential positive clones. Positive clones are selected based on the presence of the 400bp band that comigrates with the positive control DNA. Six2Bac clone was used as a template of a positive PCR product. The negative control contains no DNA.
HindIII would result in three different fragments of 26kb, 12.6kb, and 3.3kb that could be visualized using continuous field electrophoresis. Out of the 44 PCR positive colonies, restriction enzyme digestion yielded three colonies that gave the desired digestion pattern, indicating the presence of the final construct. Those three colonies were further tested by digestion with HindIII and SnaBI to validate the expected digestion pattern.

Table 6

<table>
<thead>
<tr>
<th>Recombinant Plasmid</th>
<th>Restriction Endonuclease</th>
<th>Restriction Fragment Lengths (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>Clal/SnaBI</td>
<td>8935; 5216; 4060; 2600</td>
</tr>
<tr>
<td>pBB11G3dsRed62gene</td>
<td></td>
<td>20424; 8935; 4378; 4060; 2600; 1489</td>
</tr>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>Nhel</td>
<td>17163; 3648</td>
</tr>
<tr>
<td>pBB11G3dsRed62gene</td>
<td></td>
<td>17163; 14044; 6223; 4456</td>
</tr>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>Hpal</td>
<td>17436; 1728; 1647</td>
</tr>
<tr>
<td>pBB11G3dsRed62gene</td>
<td></td>
<td>17436; 14040; 5312; 3370; 1728</td>
</tr>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>Pmel</td>
<td>1</td>
</tr>
<tr>
<td>pBB11G3dsRed62gene</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>HindIII</td>
<td>16489</td>
</tr>
<tr>
<td>pBB11G3dsRed62gene</td>
<td></td>
<td>25969; 12661; 3337</td>
</tr>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>PvuI</td>
<td>8566; 4348; 3575</td>
</tr>
<tr>
<td>pBB11G3dsRed62gene</td>
<td></td>
<td>34044; 4348; 3575</td>
</tr>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>EcoRV</td>
<td>8313; 7856; 229; 91</td>
</tr>
<tr>
<td>pBB11G3dsRed62gene</td>
<td></td>
<td>25569; 8313; 7856; 229</td>
</tr>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>ClaI</td>
<td>16489</td>
</tr>
<tr>
<td>pBB11G3dsRed62gene</td>
<td></td>
<td>32048; 8430; 1489</td>
</tr>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>SnaBI</td>
<td>8935; 6660; 894</td>
</tr>
<tr>
<td>pBB11G3dsRed62gene</td>
<td></td>
<td>26372; 8935; 6660</td>
</tr>
<tr>
<td>pBG3dsRed62HasPmel</td>
<td>SpeI</td>
<td>1377; 12132; 10367; 5891</td>
</tr>
</tbody>
</table>
Figure 13: Screening of potential positive clones by HindIII restriction enzyme digestion. This figure shows a gel that is representative of clones that were screened by restriction enzyme digestion. While all of these clones contained a positive PCR signal, only 3 clones, #113, #114, and #117, revealed the expected digestion pattern of HindIII with fragments of 26.0, 12.6, and 3.3kb, consistent with the restriction map. UD refers to the lane containing the undigested miniprep DNA isolated from the associated clone. Each clone was digested using both 100ng and 800ng of DNA to ensure that we achieved a high resolution of each DNA fragment.

1. pBac GENIE-3_26kb Six-2 gene digested with HindIII
2. pBac GENIE-3_26kb Six-2 gene digested with SnaBI
3. 1kb ladder – MW ladder (NEB) in kb
4. λHindIII – MW ladder (NEB) in kb

Figure 14: Validation of correct clone by restriction endonuclease digestion. The restriction pattern when digested with HindIII or SnaBI yields the correct fragments, based on the digestion map of pBac GENIE-3_26kb Six-2 gene.
Transfection into HEK Cells

3.5 Verification of pBacGENIE-3_26kbSix2_gene Functionality in HEK Cells

2000ng of the pBacGENIE3_26kb_Six2_gene BAC clone was used for transfection into HEK293 T-REx cells. To establish a baseline of dsRed expression, HEK293 T-REx cells were also transfected with the empty vector, pBacGENIE-3 Six2HA. 24 hours post-transfection, the cells were evaluated for expression of dsRed fluorescence. Transfection with the empty vector indicated high levels of dsRed expression, while HEK cell expression of dsRed post-transfection with the pBacGENIE3_26kb_Six2_gene BAC clone was significantly lower. As seen in Figure 15, transfection with a large transposon greatly reduces efficiencies, as expected, though the transposon is still functional. 48 hours post-transfection, the HEK cells were further evaluated for hygromycin selection. HEK cells that were transfected with the pBacGENIE-3_26kb_Six2_gene survived hygromycin selection.

Figure 15: HEK dsRed Expression of pBacGENIE3-Six2HA and pBacGENIE2_26kb_Six2gene. (a) HEK293 T-REx cells 24 hours post-transfection with pBacGENIE3_26kb_Six2gene and (b) the corresponding phase contrast image. (c) HEK293 T-REx cells 24 hours post-transfection with pBacGENIE3_Six2HA and (d) the corresponding phase contrast image.
4.2 Validation of Six2 gene presence in transfected HEK cells

Total RNA from the HEK cell was isolated from both transfected and non-transfected cells in order to give a base line comparison for indication of plasmid integration within the genome. Mouse Six-2 expression was compared between transfected and non-transfected HEK cells. HEK294T-Rex cells retain a level of endogenous human Six2 gene expression. Transfection with pBacGENIE3_26kb_Six2 gene results in the expression of the mouse Six2 gene. The primers used in this were designed to differentiate between the mouse and human copies of the Six2 gene. Analysis of mouse Six2 gene expression revealed that mRNA levels in transfected HEK cells are increased relative to the non-transfected HEK cells, where only endogenous hSix-2 is present. qPCR data is normalized to HPRT to represent the relative fold amount of mSix2 mRNA in qPCR amplicons. mSix2 mRNA levels are 35 fold higher than HPRT, confirming expression of mSix2 in transfected HEK cells.
5 Preparation of Six2 Transgenic Mice

5.1 Generation of Transgenic Mice

In order to determine the optimal concentration for pronuclear microinjection, different concentrations of 15, 20, and 25 ng/µl were tested. All three concentrations were found to be capable of producing offspring, with 15ng/µl being the most optimal for our purposes. All animals were germline transgenic.

Table 7: Efficiency of producing Six2 transgenic mice using different concentrations of pBacGENIE-3_26kb_Six2 gene

<table>
<thead>
<tr>
<th>DNA Concentration (ng/µl)</th>
<th>No. of 2P embryos injected (replicates)</th>
<th>No. of 2PN embryos survived (% injected)</th>
<th>No. of 2-cell embryos (% injected)</th>
<th>No. of embryos transferred (# recipients)</th>
<th>No. of offspring (% transferred)</th>
<th>No. of Tg offspring (% injected) [% offspring]</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>14 (1)</td>
<td>14 (100)</td>
<td>-</td>
<td>14 (1)</td>
<td>5 (36)</td>
<td>3 (21.0) [60.0]</td>
</tr>
<tr>
<td>20</td>
<td>29 (1)</td>
<td>25 (86)</td>
<td>10 (40)</td>
<td>10 (1)</td>
<td>4 (40)</td>
<td>1 (3.4) [25.0]</td>
</tr>
<tr>
<td>25</td>
<td>25 (1)</td>
<td>25 (100)</td>
<td>8 (32)</td>
<td>8 (1)</td>
<td>3 (38)</td>
<td>2 (8.0) [66.6]</td>
</tr>
</tbody>
</table>

Figure 16: Confirming mouse Six2 expression in HEK cells. qPCR data indicates that mouse Six2 is expressed in HEK cells that were transfected with the pBacGENIE-3-Six2gene, compared to untransfected HEK cells.
5.2 Verification of dsRed Functionality in Transgenic Mice

Transgenic mice were viewed under UV light and were shown to express the dsRed construct, as expected based on positive PCR result for the dsRed gene, indicating that the gene is functional.

Figure 17: Evaluation of dsRed expression of Six2 transgenic mouse. In the inset, two mice, WT mouse (left) and the Six2 transgenic mouse (right) are viewed under white light. When the same mice are viewed under UV light, it is possible to see the expression of the dsRed gene product of the Six2 transgenic mouse.

5.3 Verification of the Presence of pBacGENIE-3_26kb_Six2 Genes from Mouse Genomic DNA

After genomic DNA was isolated from the transgenic mice tails, genes that are solely related to the insertion of the transposon, mouse piggyBac, Hygromycin Resistance, 3’TRE, and 5’TRE, were tested by PCR. PCR results indicated that all samples were positive for their respective genes, migrating in parallel with the positive control, pBacGENIE-3_Six2HA. Presence of the genes surrounding the Six2 gene in the transposon (see Figure 11) serves as an additional indication that the Six2 transposon was integrated into the mouse genome.
**Figure 18: PCR amplification of plasmid genes from the transgenic mouse genomic DNA.** The following genes, mPiggyBac, Hygromycin Resistance, 3’TRE, and 5’TRE are found only associated with the insertion of the transposon and are not found endogenously in the mouse genome. When compared to the positive control, every sample from the mouse tail comigrates with the positive control, indicating confirmation by PCR that those genes are integrated into the genome.
CHAPTER 4:
DISCUSSION AND CONCLUSIONS

The advent of the first transgenic mouse, created by Dr. Jon Gordon and Dr. Frank Ruddle in 1980 (PNAS 1980, 1981) revolutionized the process of discovery of gene function through site-directed mutagenesis. Known as reverse genetics, breakthroughs in molecular biology technology have enabled scientists to alter the sequence of a known gene and characterize its corresponding phenotype, even in a tissue specific manner. As stated earlier, prior to the transgenic mouse, discovery of knowledge about gene function was accomplished through forward genetics, using random mutagens, such as radiation, to alter the phenotypes of a population and then conduct a genetic screen for a particular gene. Dr. Ruddle and Dr. Gordon’s scientific breakthrough has since set the standard for discovery in the field of functional genomics, as many scientists today continue to advance their original techniques in the generation of more efficient and complex transgenic mice. Mouse models have been established as a compelling means to identify gene function because many development pathways, such as those that involve the Six2 gene, have been retained throughout evolution for both species.

Previous studies employing transposon-mediated transgenesis require the coinjection of the circular helper plasmid containing the DNA of interest and the transposase donor plasmid or mRNA encoding the transposase (Katter et al., March 2013 and Kong et al., 2010). In this manner, the transposase mobilizes the transposon from the donor plasmid and integrates it into the genome. Thus, the only method of controlling the genomic integration involves adjusting the donor and helper plasmid ratios, which is
no easy undertaking considering controlling random chromosomal insertions is very complex (Kong et al., 2010). However, recent experiments in our laboratory have developed unique piggyBac plasmid vectors that encode both the transposon and the piggyBac transposase gene (Marh et al., 2012) in a single plasmid. To demonstrate proof of concept, we undertook the greater task of using a single plasmid that contained both the transposase and a large 26kb gene insert. Our PCR and dsRed fluorescence data suggests that not only is our transposon integrated into the mouse genome but it is also fully functional. As stated earlier, previous successful transposon mediated transgenesis using piggyBac could only functionally accommodate 100kb (Li et al., 2011). Although our transposon does not yet reach those standards, the insertion of a 42kb DNA is still rather significant. The technical aspects, including potential DNA shearing, involved with the microinjection of large transgene DNA have precluded others from successful transgenesis using larger plasmids (Urschitz and Moisyadi, 2013). Our success with a larger than normal transposon is most likely attributed to the use of the BAC system that is known to contain up to 300kb of genomic DNA (Giraldo and Montoliu, 2000). The Six2 gene was contained within the BAC vector, which is known for its stability with large constructs as well as its ability to encode for its own regulatory factors.

Creation of the mGENIE-3-BAC vector by the Moisyadi laboratory incorporated conclusions from a wide search of literature in order to obtain the highest efficiencies for transposon mediated transgenesis. Initially developed by our laboratory as a potential vector for gene therapy, the self inactivating piggyBac transposase is designed to reduce the genotoxic effects of multiple insertions into the animal genome (Urschitz et al., 2010). Seen in Figure 11, the 3’TRE is located between the CAG promoter and the
piggyBac transposase gene in the intron. When the transposase excises the transposon from the plasmid, it makes two incisions at the 3’TRE and the 5’TRE sites. Due to the placement of the 3’TRE site in the plasmid, transposition results in the separation of the CAG promoter and the piggyBac gene start from the piggyBac transposase, rendering it ineffective. piggyBac has been known to insert its associated transposon into TTAA transcriptionally active sites, with a preference for transcription start sites (Wilson et al., 2007). Thus, this knowledge necessitated the self-inactivation of the piggyBac to ensure that only a single copy of the transposon was incorporated into the mouse DNA.

Ensuring a single copy of Six2 gene expression is also significant, considering the involvement of Six2 in the maintenance of the Wilms Tumor. As explained earlier, Six2 plays an important role in the maintenance of the nephron progenitor population in the earliest stages of the developing kidney (Kobayashi et al., 2008). Six2 expression continues in the earliest stages of kidney development but is quickly inactivated in the later stages of kidney maturation. In the Wilms Tumor, a common childhood cancer localized to the kidney, the malignancy is generally related to the inhibited differentiation of the nephron population in the embryonic kidney (Murphy et al., 2013). Recently, Six2 and Cited1 have been identified as maintaining an active expression in the Wilms Tumor at all stages in the disease progression (Murphy et al., 2013). These authors also concluded that the mere detection of Six2 and Cited1 genes in differentiated mature nephron populations, suggested that the resurgence of embryonic expression pathways is fundamental to the development of the Wilms Tumor. In a similar way, one of the Six2 transgenic rescue mice that was generated in the Br mutated line, had an enlarged kidney likely due to the overexpression of Six2 from the transposon. It is unknown whether that
is related to the Wilms Tumor phenotype or simply excessive expression of Six2. At the time of this writing, copy number assays have not been optimized and therefore cannot identify whether additional copies of Six2 are present in this mouse’s genome. Thus, future studies should identify possible causes of the enlarged kidney and determine the genes involved in this misregulation of normal kidney development.

The ability to incorporate a large transposon into the mouse genome is especially critical for the generation of a transgenic rescue mouse that reexpresses Six2. Data presented by Fogelgren et al. in 2008 suggests the possibility that the Br mutation is the result of aberrant transcriptional regulation involving Six2, rather than an abnormal Six2 mRNA sequence. Their sequencing experiments found no mutations within the exons, introns, untranslated regions or 5’ promoter regions in the Br mouse but other experiments had causally linked the Br mutation to the Six2 gene. Their findings necessitated a transgenic rescue mouse that could not only incorporate the gene itself but also its regulatory elements that lie far beyond the Six2 gene. As it stands, the 26kb fragment does not yet accomplish these goals but it does provide a starting point for the generation of transposase mediated transgenic mouse using a large insert. Further experiments in current production have yielded single plasmids encoding the transposase and inserts of 106kb, 164kb, and 195kb, but further validation and testing is required to determine functionality and expression.

Modifications made to the intracytoplasmic sperm injection-based transgenesis (ICSI-Tr) procedure by the Moisyadi laboratory have further enhanced the efficiencies of generating transgenic animals. The standard procedure for the creation of transgenic animals is pronuclear microinjection (PNI), in which the male pronucleus of a zygote is
injected with linear transgene DNA (Marh et al., 2012). Identified as passive transgenesis method, DNA repair mechanisms of the zygote play an active role in incorporating the transgene DNA into the genome. Its efficacy is restricted not only through its dependence on the repair mechanisms but also through its random insertions, usually as concatamers (Marh et al., 2012). As a result, this method of transgenesis has very low efficiency rates of 3.2% of embryos injected, or 25% or animals born, with 70% of the founders being germline transgenic (Wall, 2001 and Nakanishi et al., 2002). Furthermore, initial studies using ICSI-Tr required the use of approximately 100 embryos in order to generate only two transgenic animals. However, improvements made by the Moisyadi laboratory to incorporate piggyBac transposase in a single vector with the transposon cargo have vastly improved efficiency rates while using fewer embryos (Marh et al., 2012). Results illustrating the efficiencies of transgenic animals employing the modifications to the ICSI-Tr procedure, as seen in Table 7, indicate that each concentration of the final construct was able to generate at least one transgenic animal. These experiments only required the use of less than 30 embryos for each trial and generated animals that were shown to be germline transgenic. Considering the large size of the transgene, these findings represent a significant improvement standard PNI methods.

Currently, the characterization of Six2 transgenic mice have produced an incomplete picture of Six2 gene function in relation to the Br mutation, comprised of loss of function or gain of function Six2 transgenic mice but no rescue transgenic mice. The first characterization of the loss of Six2 function in a transgenic mouse was completed by Self et al. in 2006. Through the selective replacement of the Six2 exon 1 gene, the
transcription start site, Six domain and homeodomain with pGK-Neo cassette, their transgene was electroporated into mouse embryonic stem cells. Based on linkage analysis and the similar phenotype of the Six2 transgenic knockout mice and the Br mouse, it can be inferred from the data that the Br mutation is likely due to misexpression of Six2 (Fogelgren et al., 2008). In contrast, the first characterization of the overexpression of Six2 in a transgenic mouse was generated by Kutejova et al. in 2005. Inserted into the fertilized mouse egg via pronuclear injection, the 900Six2-lacZ cDNA transgene was used to generate transgenic mouse lines. The conclusions drawn by Kutejova et al. (2005) determined that a 900bp promoter sequence upstream of the Six2 gene was capable of driving endogenous Six2 expression. However, in attempts by Fogelgren et al. (2008) to determine the cause of Six2 misexpression in Br mouse, no mutations were found in the 900bp promoter sequence. Thus, while these experiments have made great strides in the characterization of Six2 expression and function, neither Six2 transgenic mouse has provided adequate data to identify the location of the Br mutation in a currently unknown cis-regulatory region. As previously stated, ongoing attempts to generate a larger construct that will include said regulatory regions will further elucidate the relationship between Six2 misexpression and the Br mutation.

The generation of a Six2 transgenic rescue mouse has vast implications for further research. Future studies should include the following:

a. Further verification 26kb Six2 transgenic rescue mouse for transgene copy number and by Southern hybridization.

b. Determination of sites of genomic insertion of the Six2 gene in transgenic mice by nrLAM-PCR.
c. Further validation and expression studies of the Six2 and other related genes are necessary to characterize the phenotype of the transgenic rescue mouse.

d. Additional characterization of the Br mutation with respect to the Six2 transgenic rescue mouse.

e. Generation of the non-antibiotic backbone for mGENIE-3-BAC vector for gene therapy purposes. Although less practical in the generation of transgenic animals for research purposes, the creation of an antibiotic-free vector has profound ramifications for the use of the vectors for gene therapy in humans. Currently antibiotics have become the standard for screening multiple clones grown in bacterial hosts. Therefore, the use of antibiotic resistance genes in recombinant clones have the potential to not only contribute to the evolution of a multi-drug resistant bacteria but also integrate into the transgenic animal’s genome, possibly unintentionally inducing antibiotic resistance in the animal as well (Dong et al., 2010).
CHAPTER 5:

REFERENCES


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