GGTA-1 TARGETING EFFICIENCY
WITH A XENOGRRAFT TRANSGENE

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Master of Science

by
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GGTA-1 TARGETING EFFICIENCY WITH A XENOGRFT TRANSGENE

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A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

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Dr. Eric Walters

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Dr. Monique Lorson
DEDICATION

This is dedicated to my wife, Whitney Beaton, whose love, companionship, support and patience have made the rough times bearable, and the good times memorable. I could not be where I am today without you and appreciate your continued support of my future endeavors.
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<table>
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<tr>
<td>attB</td>
<td>attachment Bacteria</td>
</tr>
<tr>
<td>attL</td>
<td>attachment Left</td>
</tr>
<tr>
<td>attP</td>
<td>attachment Phage</td>
</tr>
<tr>
<td>attR</td>
<td>attachment Right</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CB</td>
<td>Cytochalasin B</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxycytidine triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds-DNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>DSB</td>
<td>Double Strand Break</td>
</tr>
<tr>
<td>DT-α</td>
<td>Diphtheria toxin-α</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>E-Promoter</td>
<td>Endogenous promoter</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cells</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G418R</td>
<td>G418 resistance</td>
</tr>
<tr>
<td>GE</td>
<td>Genetically Engineered</td>
</tr>
<tr>
<td>GGTA-1</td>
<td>α1,3 Galactosyltransferase gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>HA</td>
<td>Homologous arm</td>
</tr>
<tr>
<td>hCD55</td>
<td>Human Decay Accelerating Factor for Complement</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous Recombination</td>
</tr>
<tr>
<td>ICSI</td>
<td>Intra Cytoplasmic Sperm Injection</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal Ribosome Entry Site</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>mg</td>
<td>Microgram</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mNeo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Mammalianized neomycin resistance</td>
</tr>
<tr>
<td>Neo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Neomycin resistance</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end-joining</td>
</tr>
<tr>
<td>NS</td>
<td>Negative selection</td>
</tr>
<tr>
<td>NSRRC</td>
<td>National Swine Resource and Research Center</td>
</tr>
<tr>
<td>p</td>
<td>Plasmid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCR&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Polymerase chain reaction positive</td>
</tr>
<tr>
<td>PNS</td>
<td>Positive-negative selection</td>
</tr>
<tr>
<td>PS</td>
<td>Positive selection</td>
</tr>
<tr>
<td>rAAV</td>
<td>Recombinant Adeno-Associated Virus</td>
</tr>
<tr>
<td>rDNA</td>
<td>Recombinant DNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>RADIL</td>
<td>Research Animal Diagnostic Laboratory</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SCNT</td>
<td>Somatic Cell Nuclear Transfer</td>
</tr>
<tr>
<td>ss-DNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>tDT</td>
<td>Truncated diphtheria toxin</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
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CHAPTER I

INTRODUCTION

Technology and animal modeling in regard to genetic manipulations has grown exponentially over the past 30 years. In genetically engineered (GE) animals, exogenous DNA has been added (transgenic) or modifications have been performed on the endogenous genome. With technologies continuing to grow, the use of GE animals could become a powerful tool with applications in animal agriculture, medicine, and industry. Use of GE animals has allowed for the ability to explore both cellular and physiological processes. Genetic engineering has undergone growth and evolution, with expansive growth in the early 1980’s.

The first transgenic animals were reported in the early 1980’s, and then the 1990’s brought about somatic cell nuclear transfer (SCNT) technologies with the utilization of genomic and bioinformatics technology. For an animal to be considered transgenic or genetically modified the genome of such animal must be modified to either contain exogenous DNA or a modification to the endogenous genome is applied. The term transgenic was first described by Gordon and Ruddle (1981) in their experiments to generate transgenic mice; since that time the term “transgenic” has become synonymous with “knockin” and “knockout” animals. Since the early 1980’s a number of methods have been developed to perform gene transfer in several mammalian species (Lipid-
Mediated Transfer; Calcium-Phosphate Mediated; Retroviral and Viral Infection; Electroporation; and Microinjection).

Transgenic technology was originally derived out of the desire to understand gene function in mice and has evolved into a tool to investigate phenotypic impact of the addition of transgenes. Recombinant DNA has allowed for the introduction of new (exogenous) genes into organisms. Transgenesis, the process of introducing an exogenous gene, allows for the construction and subsequent study of a functional transgene. However, the complications related to proper transgene construction and integration of transgenes has limited the application of this promising technology. Transgene construction requires understanding the components of a gene that make it functional in its natural state. This understanding must then be used to incorporate similar principles during the engineering of transgenes. Once engineered, the transgene integration site becomes an important factor. There are two integration methods: 1) random integration whereby the transgene integrates randomly into the genome, and 2) site-specific integration where the transgene is inserted into a specific location either as a knock-in or by the facilitation of a recombinase. The ability to choose a genomic location for transgene insertion that provides for predictable transgene expression as well as minimal effects on the expression of neighboring genes will also aid in utility of transgenesis.

These studies are aimed at examining several key aspects of transgenesis. All objectives are in relation to pigs, and are as follows: 1) examine the functionality and predictability of transgene expression, 2) study gene targeting via homologous recombination (HR) and its ability to efficiently integrate transgenes in a site-specific
manner, and 3) to examine the principle of stacking/linking multiple modifications.

Herein, I have outlined several issues related to transgene expression, gene targeting, and the ability to stack or link multiple modifications.
CHAPTER II

LITERATURE REVIEW

*Transgenic Animal Production*

The ability to produce an animal that has been GE, whether it is with the addition of exogenous DNA or a genetic manipulation of endogenous DNA, enables several scientific endeavors. GE organisms can be used to study cellular processes, produce “pharm” animals for the production of biomedical proteins or drugs, produce organ donors for xenotransplantation, improve livestock production traits, examine human disease, and evaluate therapeutic efficacy. To produce GE animals capable of aiding with any of the aforementioned scientific advances, manipulations of genes and their expression is required. A GE animal can be an animal whose genome contains DNA of exogenous origin that is introduced through experimental manipulations or can be an animal whose endogenous genome is manipulated in some fashion to create a desired effect. Those animals that harbor exogenous DNA are also denoted as “transgenic.”

The initial production of transgenic animals in the early 1980’s stemmed primarily from the work of five laboratories that employed the technique of gene transfer (Gordon et al., 1980; Wagner et al., 1981; Harbers et al., 1981; Brinster et al., 1981; Costantini and Lacy, 1981; Gordon and Ruddle, 1981). The animals were produced
using a microinjection method that had been generated 15 years prior (Lin, 1966). DNA microinjection is a gene transfer method in which DNA is directly microinjected into the pronucleus of a fertilized zygote. Though microinjection was the first technique used to generate transgenic animals, over time additional techniques were developed to generate transgenic animals. In viral gene transfer, foreign DNA is transferred into the cell with the use of viral particles (Chan et al., 1998). In embryonic stem cell transfer, genetically altered embryonic stem cells (ESC) are used to produce germline chimeras (Capecchi, 1989), which then transmit the genetic manipulation to their progeny. In somatic cell nuclear transfer (SCNT) (Wilmut et al., 1997), genetically altered nuclear donor cells are used to reconstruct embryos that are subsequently transferred to recipient females (Schneike et al., 1997). Somatic cell nuclear transfer can be used as an alternative to ESC. At the present, ESC have not been produced from livestock species therefore preventing their use for transgenic animal production in such species (Miyoshi and Sato, 2000). Thus, all transgenic mammalian studies performed with ESC have been limited to the mouse and rat.

Transgene Expression

Just as the development of a method to inject exogenous DNA was a requirement for the production of the first transgenic animals, recombinant DNA (rDNA) technologies were necessary to further facilitate the production of transgenic animals to allow a broader range for the study of various manipulations. rDNA technologies allow for one or more fragments of DNA to be recombined with other fragments of DNA. With the ability to combine two or more different fragments through various molecular biology
techniques, scientists have been able to engineer completely new fragments of DNA that contain various sequences from different origins (transgene expression cassette). In general, a transgene expression cassette is designed to contain all of the necessary components of a functional transgene. The minimum requirements to construct a recombinant gene include three main components, the first being the promoter with the purpose to drive transcription of the transgene. The second component is a region of DNA that encodes the desired gene product. And, the third component is a termination signal, which terminates transcription of the transgene. These three components are the minimal requirements for a transgene. Additional components (i.e., regulatory elements) and the site of integration can have a profound effect on transgene expression. In reality, regulatory sequences can be found outside of characterized promoter regions and at distant locations. In many transgene designs, the expression cassette does not include all of the surrounding components that may be important for proper regulation of the transgene. Often the more distant regulatory elements provide functions that prevent “cross-talk” with surrounding genes (Wells and Wall, 1999).

DNA constructs integrate randomly and the site of integration can have an effect on the level, location, or timing of expression. Some integration sites may partially or completely suppress transgene expression, whereas other sites may actually facilitate expression (Clark et al., 1994; Bonifer et al., 1996). In addition to misregulation of the transgene expression, in some cases the loss in function of an endogenous gene occurs. In this instance, the site of integration could knock-down or knock-out an important regulatory gene that is required for development, for an example, mice resulting in male sterility (Magram and Bishop, 1991; Walters et al., 2009).
Gene Targeting

Gene targeting involves the replacement of an endogenous gene by the way of HR with an alternative copy of that gene which has been constructed in vitro. Evans and Kaufman (1981) and Martin (1981) showed that murine ESC are pluripotent, thus providing the potential to introduce precise modifications to the mouse genome by gene targeting (Smithies et al., 1985; Capecchi, 1989). Smithies et al. (1985) was the first group to report gene targeting of an endogenous gene in mammalian cultured cells, thus proving that gene targeting is possible. The gene targeting method begins with a modification to a cloned copy of a gene using rDNA technology. Once the modification has been produced in vitro, HR then transfers the new version of the region into the genome between the exogenous DNA and the endogenous DNA. The efficiency of gene targeting in the mouse using ESC is approximately 1X10⁻⁶ (Reid et al 1991, Templeton et al 1997). Gene targeting is an extremely rare event because a double strand break (DSB) must occur in order for a double crossover event to occur where the exogenous DNA is inserted. When a DSB occurs, two competing pathways are activated in the cell, the HR pathway (site-specific integration) and the non-homologous end-joining (NHEJ) pathway (random integration). The NHEJ pathway appears to be the favored pathway for integration of exogenous DNA in mammalian cells.

Targeting vectors are often designed to include a long and short arm. The primary driving force behind this design is to help facilitate analysis of the targeting event. In general, a targeting vector is composed of two targeting arms that range in length of 1 to 6 kb, both of which flank a selection cassette and/or a transgene cassette.
For the most part, previous experiments have used targeting arms in the range of length of 1 kb to 8 kb (Hasty and Bradley, 1993a). However, successful HR has been reported using less than 0.5 kb of homology on one arm (Hasty et al., 1991a). With overall sequence homology of the arms being a factor in HR efficiency, the length of contiguous sequence identity is the major determinant of HR efficiency (Waldman and Liskay, 1988). As additional support, Hasty et al. (1991a) has shown that the success or frequency of HR could be increased when the length of homology arms was increased. Along with targeting arm design length, Deng and Capecci (1992) have shown that when comparing non-isogenic DNA and isogenic DNA (same genetic composition), the frequency of HR increased when using isogenic DNA.

Gene targeting is a relatively rare event in mammalian cells. It is the less favored reaction when compared to random insertion or integration. Random integration occurs approximately 30,000 to 40,000 times more frequently than HR in mouse ESC (Hasty et al., 1991b). Considering the inefficiency of HR, several enrichment strategies have been developed to improve the likelihood of finding such a rare event including positive selection, negative selection, and trapping endogenous elements. Positive selection utilizes a marker such as a drug resistant gene to facilitate the isolation of targeting events. The addition of a positive selection marker enriches for cells that have received the targeting construct allowing only cells that express the marker to grow under selection. However, a standard positive selection cassette does not differentiate a targeting event from a random integrant. Another enrichment strategy uses the addition of a negative selectable marker, such as Thymidine Kinase or Diphtheria Toxin. The negative marker flanks one of the homology arms and provides selection against random
integrants by inducing death of such cells. Since the negative marker does not integrate during HR, cells that have undergone a targeting event remain alive. The simultaneous use of both positive and negative selection strategies is referred to as positive-negative selection (PNS, Mansour et al., 1988). In addition, some other strategies attempt to “trap” functions that are provided by the endogenous gene but are omitted from the targeting vector (promoter trap, poly (A) trap, etc). Figure 1 shows a graphical representation of a few basic designs of gene targeting vectors.

Gene targeting by HR is typically used to disrupt or knockout a particular gene either by removing coding exons or by an interruption in the transcription of the RNA. After recombination, the RNA transcript cannot be translated to generate the proper protein for the gene of interest. Both methods normally result in either a knocked down or knocked out version of the targeted gene. In addition, HR can also be used to introduce subtle mutations into a target gene or replace the endogenous gene with a new gene. This process produces what is known as a gene knock-in.

A prerequisite for gene targeting is that an efficient method to introduce sufficient amounts of the targeting vector DNA into the cells, which would allow for translocation to the nucleus where it can interact with the host genome, recombine, and stably integrate (Laible and Alonso-Gonzalez, 2009). In early studies, nuclear microinjection of the DNA construct was performed and was shown to be a useful method for gene targeting in mammalian cells (Thomas et al, 1986). However today, the two main methods of introducing the DNA construct are by lipofection, a chemical method based upon liposomes, and electroporation. These two methods are more commonly used because of the ability to transfect a much larger number of mammalian cells. Both methods have
shown to be effective in delivering DNA vectors. However, gene targeting efficiency is primarily determined by the gene being targeted and the recipient cell line.

Predictability of phenotype and transgene expression could be much greater with the use of gene targeting via HR instead of random integration. The predictability is increased because transgenes are placed into a known, targeted location and thus the endogenous gene or genes it affects are predetermined. After a predictable location in the endogenous genome is recognized, all future transgenes could potentially be located in the same location with the same method of HR.

One of the most important factors to consider when evaluating transgene expression is where the integration of a transgene occurs. The term, position effect, is used to describe the impact of the site of integration on transgene expression. There can also be an unintended impact of the transgene on genes at or near the site of integration. The effect of the neighboring genes at the site of integration includes, variegated expression, ectopic expression, and impact on transgene expression level. As mentioned by Wells and Wall (1999), another factor involved with expression is that the transgene may lack certain control elements necessary for transgene expression. Since this time, it has been shown that some sites of integration are dependable permissive for transgene expression. In particular, the ROSA26 locus in the mouse has been widely used for achieving transgene expression. Soriano (1999) was one of the first to demonstrate the ability of this locus (ROSA26) to support expression of transgenes. Subsequently the ROSA26 locus has proven to dependably support transgene expression after transgene placement by HR. Over the past decade HR has proven to be an efficient strategy to knock-in several transgenes into this locus and have resulted in a generalized expression
level. One could infer that the ROSA26 locus is a safe-harbor in the mouse genome for transgene expression. Further, no phenotypic impact has been observed for insertions at this locus.

It is clear that targeted genetic manipulations have played and continue to play a major role in understanding the role of endogenous genes as well as the function of transgenes. Gene targeting may have a major impact on biotechnology and understanding human disease and development.
Figure 1. Graphical representation of basic designs for gene targeting vectors. E-Promoter, Endogenous Promoter; HA, Homologous Arm; IRES, Internal Ribosome Entry Site, (which functions as a translation initial site for the PS cassette); PS, Positive Selection; PNS, Positive-Negative Selection; NS, Negative Selection.
Gene-Linking/Stacking

In traditional transgenesis only one modification is attempted at a time, whether it is the knockout of an endogenous gene or the addition of a single transgene. However, with some applications several transgenes may be required. The classical approach to produce an animal with multiple manipulations is to achieve the end result through separate additions, at different times, and potentially through different techniques. This process is labor and time intensive, and does not always provide a reliable vehicle for producing a sustainable line of animals for several reasons; 1) each integration will segregate independently if propagated through breeding, 2) the predictability of transgene expression is unknown based upon the same premises, and 3) gene knockout would not be linked to additional modifications if not on the same allele. The most obvious solution would be to stack transgenes in a single location and/or to add multiple transgenes simultaneously that are linked together. Based on observations in other systems (Keravala and Calos, 2008), it seemed reasonable that we could adapt a site-specific recombinase system to facilitate gene stacking.

Site-specific recombination systems mediate control of a variety of biological functions by catalyzing precise excision, inversion, or integration of defined DNA fragments. Due to the accuracy of recombination and relative simplicity of use, site-specific recombination systems have been the focus of studies in plants, flies, and mammals. These systems are generally denoted by the name of recombinase protein, followed by the name of the DNA recognition sequence. A few of the most commonly used site-specific recombination systems with proven utility include Cre-\textit{loxP}, FLP-FRT, and \textit{φC31-att} systems. These systems are characterized by their ability to function
independently of DNA topology, through the interaction of a single recombinase with a pair of specific recognition sites. In the case of \textit{loxP} and FRT, the recombination site required on both DNA substrates is a 34 bp palindromic sequence comprised of 13 bp inverted repeats surrounded by an 8 bp asymmetric spacer region that confers directionality to the site and thus to the recombination reaction (Figure 2). In contrast to identical recombination target substrates of the Cre-\textit{loxP} and FLP-FRT systems, the \textit{φC31-att} system recognizes non-identical \textit{attB} (attachment \textit{Bacteria}) and \textit{attP} (attachment \textit{Phage}) sequences and upon recombination generates two new sites denoted \textit{attL} (\textit{att Left}) and \textit{attR} (\textit{att Right}). The newly generated sites are chimeric compositions of \textit{attP} and \textit{attB} sites (Figure 3). Thus, \textit{attP} and \textit{attB} are destroyed upon recombination and cannot further serve as substrates for the \textit{φC31} integrase. This type of specificity and unidirectionality allow for \textit{φC31-att} system to be used as a tool for site-specific genome manipulations.

Utilization of a recombinase to stack genes as part of mammalian transgenesis could improve the ability to predict transgene expression as well as provide a means to link multiple transgenes. This strategy requires that a chromosomal location be known to facilitate appropriate transgene expression levels for the transgene promoter of interest and that the target locus is “tagged” with an appropriate recombination sequence. The \textit{ΦC31} integrase for example is a site-specific recombinase that normally performs precise, unidirectional recombination between two attachment or \textit{att} sites called \textit{attB} and \textit{attP}. Keravala and Calos (2008) demonstrate that when an \textit{attP} site is pre-integrated into a mammalian chromosome it can serve as a target for integration of an introduced plasmid carrying an \textit{attB} site. Once recombination occurs, it leads to the precise
integration of the $attB$ plasmid into the chromosome at the $attP$ site and will provide a high efficiency of net integration. Previous work by the Calos lab showed that the $\Phi C31$ integrase works in human, murine, and drosophila (Groth et al., 2000; Thyagarajan et al., 2001; Olivares et al., 2002; Groth et al., 2004; Bertoni et al., 2006; Chalberg et al., 2006; Fish et al., 2007). In principle, site-specific integration can be used to deliver a second transgene next to a previously inserted transgene, therefore genetically linking the transgenes. To enable this process the first transgene would have to include an appropriate recombination site. To retain the ability to add additional transgenes, each new transgene must contain an appropriate recombination site. Figure 3 depicts a generalized strategy for adding the first plasmid using attachment sites and the $\Phi C31$ integrase.

In traditional gene targeting, a selectable marker remains in the targeted genome. Constructs can be designed to contain internal homology that allows a second HR event to remove the selectable marker while retaining precise genome modifications (Abuin and Bradley, 1996). However, this method is cumbersome, requires plasmids that are unstable during propagation and is very inefficient. Site-specific recombinases offer an alternative to this strategy. Site-specific recombination systems have been used to remove the selectable marker after an insertion event (Meyers et al., 1998; Nagy et al., 1998). The removal of a selectable marker is advantageous for two main reasons: 1) to circumvent any concerns that the presence of a selectable marker cassette may affect the expression of neighboring genes (Fiering et al., 1995), and 2) to “recycle” the original targeting vector (or the marker gene contained within it) to create a homozygous knock-out cell line. In the case of Cre-$loxP$ mediated deletion, the vector must be designed to
include loxP sites that flank the selectable marker. The use of this strategy to produce a homozygous knockout cell line requires three separate rounds of selection and transfections (Figure 4). Round 1: Target the gene of interest and identify the heterozygous-targeted cell line by PCR analysis and Southern blot analysis. Perform SCNT to restore a fresh somatic cell line due to the finite lifespan of somatic cells. Round 2: Remove the selectable marker by transient expression of the Cre recombinase. Perform SCNT to generate a fresh somatic cell line that has the selectable marker Cre-deleted. Round 3: Re-target the new fetal cell line with the same targeting vector to knockout the remaining wild-type allele and render the cell line homozygously targeted. To achieve a live animal that has a gene homozygously knockout, SCNT must be performed and allow the pregnancy go to term and produce live offspring.
Figure 2. The Cre-loxP and FLP-FRT system. A. Sequence of a loxP and an FRT site. Both sites consist of a 34 bp double strand in which each terminal base may vary. Both recognition sites contain an 8 bp core sequence, which is flanked on each side by a 13 bp palindromic sequence. B. Graphical representation of a strategy to introduce a single, markerless loxP site. B1) A selectable marker that is flanked by loxP sites is introduced into the genome by HR. B2) The marker can be deleted by Cre recombinase to leave a single loxP site at a predetermined location in the genome. The event is being represented by a Cre-driven reaction but would be the same for FLP.
A)  \(attB\): 5’ - GGTGCCAGGGCGTGCCC TT GGGCTCCGGGGCGCG - 3’

\(attP\): 5’ - ccccaactgggaacct TT gagttctctagttgggg - 3’

\(attL\): 5’ - GGTGCCAGGGCGTGCCC TT gagttctctagttgggg - 3’

\(attR\): 5’ - ccccaactgggaacct TT GGGCTCCGGGGCGCG - 3’

B) Targeted Allele containing \(attB\) site

Integrase \(\Phi\)C31-Mediated Allele

**Figure 3.** Sequence and schematic representation of \(\Phi\)C31 integrase-mediated recombination between \(attB\) and \(attP\) attachment sites. A. Sequence of the nonidentical \(attB\) (35 bp; upper case) and \(attP\) (39 bp; lower case) sites. The attachment sites are imperfect inverted repeat sequences comprising the binding sites for the integrase, which flank the dinucleotide core sequence TT where recombination occurs (Groth et al, 2000). The recombination products are an \(attL\) and an \(attR\) site. B. Graphical representation of a \(\Phi\)C31 integrase-mediated recombination between a plasmid-borne \(attP\) and a chromosomal \(attB\).
Figure 4. Genetic engineering strategy to target genes using a single targeting vector and Cre recombinase. To generate a homozygous knock-out porcine primary cells, a 3 round strategy could be applied. Round 1 would include isolating porcine fibroblast cells, followed by introducing the exogenous targeting vector (being represented by an electrical pulse). Upon isolation of a heterozygous targeted cells, somatic cell nuclear transfer (SCNT) would be performed by transferring a targeted cell and injecting into a porcine enucleated oocyte followed by transferring reconstructed embryos into a surrogate gilt. To retrieve replenished fetal fibroblasts, a day 28-35 fetus is collected and the fibroblast cells are propagated. The propagation of heterozygous targeted cells begins round 2. The same steps ensue in round two as in round one except for a mammalianized Cre-expression vector is introduced and through transient expression of the Cre recombinase, DNA within the loxP sites are removed from the targeted sequence. SCNT is performed on isolated Cre-deleted cells. Once again to retrieve fresh fetal fibroblasts, a day 28-35 fetal collection is performed. Round 3 begins by propagating the Cre-deleted fetal fibroblast cells. In round 3, round 1 steps are duplicated. The heterozygous targeted cells that have the selectable marker Cre-deleted have the exogenous targeting vector DNA introduced. SCNT is performed on isolated homozygous targeted cells and the embryos are allowed to go to term and live homozygous gene-targeted animals are born.
**Project Objectives**

The site of integration can have a profound effect on transgene expression affecting expression level, ectopic expression and variegation (Wells and Wall, 1999). Therefore, we hypothesized that the genome contained “safe harbor” sites for predictable transgene expression and that such sites could be used for transgene stacking. For these studies, the context was within the porcine α1,3 Galactosyltransferase (GGTA-1) gene and its relevance to pig-to-human xenotransplantation. It is known that in order for such xenotransplantation to become plausible, the porcine GGTA-1 locus must be knocked out and that integration of multiple human transgenes will be required. Due to the interest of our lab in potential of stacking multiple transgenes in a safe location in the porcine genome, we chose to experiment with the porcine GGTA-1 locus as that potential location. Within these studies we also evaluated several methods to increase gene targeting efficiency and determine the functionality of a human transgene placed within the GGTA-1 gene.
CHAPTER III

TARGETING EFFICIENCY USING A GGTA-1 PROMOTE-TRAP IS NOT FURTHER ENHANCED BY SINGLE-STRANDED DNA OR NEGATIVE SELECTION

Abstract

Gene targeting by HR is a powerful tool to direct site-specific modifications to mammalian genomes. The technology to target specific genes in murine germ cells at high efficiencies has been available for several decades. However, the advancements of somatic cell gene targeting in livestock has lagged in comparison and could be attributed to the relatively short life span of somatic cells and the ratio of homologous integrants to random integrants. With the lack of embryonic stem cells (ESC) for livestock, we wanted to evaluate two independent enrichment strategies to determine whether we would obtain viable targeted porcine somatic cells at similar targeting efficiencies as targeted murine ESC. The first strategy compared introduction of exogenous plasmid DNA in either single-stranded or double-stranded conformations. The second strategy utilized a promoter-trap vector as a method of increasing targeting efficiency and evaluated if further enrichment could be achieved with the addition of a negative selectable marker, a truncated diphtheria toxin cassette (tDT). These studies demonstrated for the targeting vector used herein, single-stranded DNA and double-stranded DNA yielded similar targeting efficiencies. In addition, tDT inclusion within
the vector or as a co-transfectant did not enrich gene targeting provided by the promoter trap strategy alone. Targeted colonies were confirmed by PCR analysis. Gene targeting was confirmed by PCR and Southern blot analysis using fibroblast cells from the subsequent fetal collection and live piglets.

Introduction

Homologous recombination (HR), commonly referred to as gene targeting, provides the ability to modify any gene in a predetermined and precise manner. To date, the most widely used approach for gene targeting has been in embryonic stem cells (ESC) in mice. Due to the lack of established ESC in livestock species, HR in livestock requires techniques that utilize somatic cells instead of ESC for gene targeting experiments (Miyoshi and Sato, 2000). When it first became clear that somatic cell nuclear transfer (SCNT) may offer an opportunity for HR in livestock species, it appeared that somatic targeting efficiency may be prohibitive when compared to mouse ESC. The efficiency of targeting genes in mouse ESC averages approximately $1 \times 10^{-6}$ (Reid et al 1991, Templeton et al 1997) while initial gene targeting studies in somatic cells occurred at frequencies two to three orders of magnitude lower (Sedivy and Dutriaux 1999). Thus, suggesting that gene targeting in species other than the mouse was inefficient. In recent years, several groups have targeted genes in cultured porcine somatic cells at frequencies similar to those observed in mouse ESC, $9.3 \times 10^{-5}$ to $8.3 \times 10^{-7}$ targeting events per
transfected cell (Dai et al, 2002; Harrison et al, 2002; Lai et al., 2002; Ramsoondar et al, 2003; Rogers et al, 2008; Mendicino et al, 2010; Lorson et al., 2011; Ramsoondar et al. 2011). Recent studies in several other livestock species supports those using porcine somatic cells, suggesting that the overall rate of HR in somatic cells is comparable to gene targeting in ESC (Laible and Alonso-Gonzalez, 2009). Thus, the application of HR technology to produce animals with genome modifications clearly extends beyond the mouse.

Experimentally induced HR is a relatively rare event and is a less favored reaction in comparison to the random insertion of the targeting vector DNA, which occurs 30,000 to 40,000 times more frequently than HR (as measured in mouse ESC, Hasty and Bradley, 1993b). Since the random integration of a selectable marker can give rise to non-targeted colonies during selection, the number of non-targeted colonies generally far exceeds the number of targeted colonies making the recovery of a targeting event inefficient. The efficiency of HR in relation to random integration creates the need for a selection strategy that can increase the likelihood of recovering a targeting event by reducing the random integration events that survive selection. Several strategies for targeting vector delivery and design have emerged to provide different forms of gene targeting enrichment. The most common delivery methods include direct microinjection of the DNA construct, chemical methods such as liposomes, electroporation, and the use of rAAV vectors (Lou and Saltzman, 2000). To achieve enrichment based on a vector design, several strategies have been used: promoter-trap (Denning et al 2001), poly(A) trap (Mendicino et al 2010), and positive-negative selection (PNS) vectors (Mansour et al 1988).
This study compares the efficiency of targeted colony production and recovery using two distinct enrichment strategies: 1) transformation of single-stranded and double-stranded DNA conformations, and 2) negative selection in the context of a promoter trap. We chose to evaluate the targeting efficiency of the porcine alpha1,3-galactosyltransferase (GGTA-1) gene because several groups have successfully targeted this locus in porcine cells (Denning et al., 2001; Dai et al., 2002; Harrison et al., 2002; Lai et al., 2002; Jin et al., 2003; Takahagi et al., 2005).

The first enrichment strategy was based upon the observations of Lorson et al. (2011) regarding the efficiency of targeting the porcine SMN gene through the use of a single-stranded targeting vector. These experiments were designed to evaluate if the conformation of the targeting vector DNA affects the ability to recover targeted colonies [linearized single stranded DNA (ss-DNA) versus linearized double stranded DNA (ds-DNA)]. Previous studies demonstrated GGTA-1 gene targeting using ds-DNA (Denning et al., 2001; Dai et al., 2002; Harrison et al., 2002; Lai et al., 2002; Jin et al., 2003; Takahagi et al., 2005). The hypothesis was that when compared to ds-DNA, the ss-DNA conformation of the targeting vector was going to alter the ratio of HR and random integration by either increasing or decreasing the number of targeted colonies or antibiotic resistant colonies.

The second enrichment strategy was based on PNS in the context of a promoter-trap vector. Two questions were addressed in this experiment: first, does PNS offer additional enrichment beyond the promoter trap strategy; second, can a separate vector as opposed to a concatemerized construct provide the negative selectable marker. A
diphtheria toxin-α expression cassette (DT-α) was included in cis with the targeting vector or supplied in trans to provide the negative selection against random integration.

Herein, we report the production of GGTA-1 +/- porcine fibroblast cells through the use of two targeting enrichment strategies. Gene targeting was observed at an efficiency comparable to those observed for other genes in mouse ESC. Targeted cells supported development to term after SCNT and subsequent embryo transfer to surrogate gilts.

Materials and Methods

GGTA-1 Targeting Constructs

Four isogenic targeting constructs, pBB7, pBB8.1, pBB8.2, and pBB13 were assembled from DNA isolated from primary porcine fetal fibroblasts, male cells 104821 “Minnesota Miniature” breed (National Swine Resource and Research Center [NSRRC]). The targeting constructs are graphically represented in Figure 5. A 6,641 bp GGTA-1 genomic fragment which includes most of intron H and exon 9, was generated by long-range PCR using the TaKaRa LA system (Takara Bio Inc., Japan, Code No. RR02AG). The PCR product was cloned into pCR-XL-TOPO (Invitrogen) to produce the plasmid pBB4. The degree of sequence divergence, if any, generated by PCR between pBB4 and the target locus from 104821 primary cells was not determined. The pBB4 plasmid served as the template to isolate the GGTA-1 recombination arms. Plasmid pBB7 was generated to include a 5,740 bp loxP-IRES (internal ribosome entry site)-mNeoR-loxP-
CAG-hCD55-attB-SV40 poly(A) cassette, which was inserted into the unique EcoRV site at the 5’ end of exon 9.

Cassette components were sourced from commercial and lab-constructed plasmids. The loxP-IRES-mNeoR-loxP was isolated from an in-house plasmid (pKW2) that contained all four components. The neomycin resistance (NeoR) gene is based on mammalian codon usage. A 1,962 bp Pmel/BsrBI restriction fragment was isolated from pKW2 and cloned into pBB5 (a derivative of pBB4 that has an E. coli backbone modification) at the EcoRV site located in exon 9 of the GGTA-1 locus to build pBB6. The hCD55, lambda attB, and the SV40 poly (A) components were isolated from cDNA clone MGC:5192 IMAGE:3460621 (Open Biosystems #3460621). After lab modifications of cDNA clone 3460621 were performed to remove vector backbone restriction sites, the resulting plasmid was named pBB2. To isolate the CAG promoter, pCAG-Cre:GFP (Matsuda et al 2007; Addgene plasmid 13776) was cut with EcoRI and SalI, and the 3’ ends were extended with T4 DNA polymerase (New England Biolabs) to produce blunt ends. This fragment was subcloned into pBB2 between the Bsu36I and NotI restriction locations which had been treated with T4 DNA polymerase (New England Biolabs) to produce blunt ends. The resulting plasmid was named pBB3. A 3,804bp PvuI/SpeI restriction fragment containing the CAG-hCD55-attB-SV40 cassette was isolated from pBB3 and cloned into pBB6 at the compatible PacI/NheI sites, producing the plasmid pBB7.

Three additional plasmids were constructed. 1) Plasmid pBB7 was modified to replace the λ attB site with a φC31 attB site to produce pBB13. These two plasmids, pBB7 and pBB13 differ by a total of 70 bp. 2) A truncated diphtheria toxin cassette
(tDT) isolated from an in-house plasmid pX5 with PvuI and was inserted into the unique PvuI site located in the backbone of plasmid pBB7 to make pBB8.1. 3) During the assembly of pBB8.1 a single clone was isolated that contained two concatamerized copies of the tDT cassettes and was named pBB8.2. pBB7, pBB13, pBB8.1, and pBB8.2 were linearized with either SmaI, SacI or BstBI for the various electroporations. To generate single-stranded DNA the linearized template was boiled for 3 minutes and placed on ice.
**Figure 5.** Schematic of the test plasmids. A-D, Graphical representations of each targeting construct. Each construct included the corresponding porcine GGTA-1 genomic sequences used for the 5’ and 3’ recombination arms in the knockout vectors. Each construct included a mammalian optimized neomycin resistance cassette utilizing an IRES (internal ribosome entry site), which functions as a translation initial site for the neomycin protein, and a simian virus 40 (SV40) poly (A). Each construct also contains a human decay accelerating factor (hCD55) cassette driven by the CAG promoter (cytomegalovirus early enhancer element and chicken beta-actin promoter). Constructs A, C, and D all contain a lambda attB site located between the hCD55 cassette and the SV40 poly (A) represented by a solid vertical bar. Construct B has a φC31 attB site located at the same location represented by an open triangle. Constructs D and E contain a truncated diphtheria toxin-α (tDT) gene located at a PvuI site flanking the 3’ region of the 3’ targeting arm containing either one or two tDT genes respectively. E, is a representation of the pDT-α construct used as a co-transfected plasmid. pDT-α contains the coding sequence for diphtheria toxin-alpha cassette driven by the PolII promoter and contains a SV40 poly(A).
Passage three 104821 cells were thawed, then cultured for 72 hours in 1X Dulbecco’s modified Eagle’s medium [DMEM] with 1 g/L glucose, L-glutamine and sodium pyruvate (Cellgro) with 12% fetal bovine serum (FBS) at 38.5°C in 5% CO₂, 5% O₂, and 90% balanced air in 100% humidity. Transfection was accomplished by a modification of the method described by Ross et al (2010). Fibroblasts were harvested by trypsinization and were re-suspended at a concentration of 1 X 10⁶ cells/mL in a modified electroporation media (25% OptiMEM (Invitrogen) + 75% buffered KCl (135mM KCl; 15 mM Tris; 11.3 mM BES; 3.7 mM PIPES, pH 7.3). 200 µl of the cell suspension (200,00 cells) and 1 µg linearized plasmid DNA were placed in an electroporation cuvette (2mM) and the cells were electroporated using 250 volts X 3 pulses for 1 ms/pulse using a square wave generator (BTX Electro-cell Manipulator 200, San Diego, CA). Electroporated cells were cultured in DMEM, L-alanyl-L-glutamine (Cellgro, 100 mM), and 15% FBS in twenty 100 mm tissue culture plates (~10,000 cells/plate). Twenty-four hours after plating, cells were administered G418 (Cellgro, 400mg/L) and cultured for 13 days. G418 resistant (G418⁵) colonies were then harvested using cloning cylinders and screened by PCR. Two thirds of harvested cells were transferred to a single well of a 24-well tissue culture plate for expansion under the same G418⁵ media conditions and one third of cells isolated were used for PCR analysis of targeted clones. Targeted colonies were expanded, frozen, and were used for SCNT.
**PCR Analysis of Neomycin-Resistant Colonies**

Approximately 1,000 cells (1/3 of a colony) were re-suspended in 5 µl of lysis buffer I (LBI) (40mM Tris, pH 8.9; 0.9% Triton X-100; 0.9% Nonidet P-40; 0.4 mg/ml proteinase K), incubated at 65°C for 15 minutes to disrupt the cells, and then heated to 95°C for 10 minutes to inactivate the proteinase K. Two primer sets were used for PCR analysis using the TaKaRa LA system (Takara Bio Inc., Japan, Code No. RR02AG). The first primer pair flanked the upstream homology arm and resulted in a band of 5.5 kb from a targeted locus. One µl of cell lysate was used as template in a 25 µl reaction volume with the following parameters: 94°C for 1 min, followed by 30 cycles of 30 s at 94°C, 30 s at 62°C, and 2.75 min increasing 6s/cycle at 68°C, with a final extension of 5 min at 72°C. The upstream assay primers were: GLR_L1 5’GGAGAGGAGAATGGTGTCACAGGGCCA and GSL_R2 5’CCAAGCGGGCTTCGGCCAGTAACCTTAG.

The second primer pair flanked the downstream homology arm and produced a band of 3.8 kb from a targeted locus. One µl of cell lysate was used as template in a 25 µl reaction volume with the following parameters: 94°C for 1 min, followed by 30 cycles of 30 s at 94°C, 30 s at 62°C, and 2.5 min increasing 5 s/cycle at 68°C, with a final extension of 5 min at 72°C. The downstream assay primers were: GSR_L1 5’AGTGCCGTCCAGGGTTACAGAAGAGAACC and GLR_R1 5’GAGTAGGAGGCCCAGGGAAACAGTAGAG.
Nuclear Transfer and Embryo Transfer

Oocytes were purchased from ART Inc. (Madison, WI). SCNT and fused oocyte culture conditions were carried out as previously described by Zhao et al. (2010) with modifications to the manipulation medium that consisted of Hepes-buffered TCM-199, 0.3% BSA, and 7.5 mg/ml of cytochalasin B (CB). The medium for injection was the same medium without CB. Recipients on the first day of estrus (D0) or the first day after standing estrus (D1) were used. Embryo transfer was performed surgically as previously described (Lai and Prather 2003). Recipients were checked for pregnancy by ultrasound (Day 25-30).

Fetal Collection and Southern Blot Analysis

On day 35 of pregnancy, a surrogate was euthanized and hysterectomized to individually collect fetuses into a 50 mL conical bottom centrifuge tubes. The fetuses were washed twice by multiple tube inversions in Dulbecco’s PBS (Invitrogen) to remove excess debris, once in 70% EtOH to disinfect, and the last time in Dulbecco’s PBS to remove excess EtOH. The head and viscera were removed from the fetus and used to isolate genomic DNA. The tissue was suspended in 700 µl of lysis buffer II (LBII) (50mM Tris, pH8; 100 mM EDTA; 0.5% SDS; 35 µl 10mg/ml solution of proteinase K), and incubated at 55°C overnight. Genomic DNA was isolated following organic extractions of phenol and chloroform and the DNA precipitated with ethanol. A fetus (0903-2) was analyzed by Southern blotting (Southern 1975) by standard procedures. For Southern blotting, 8 µg of genomic DNA was digested with HindIII and separated on a 0.6% agarose gel. Following electrophoresis the DNA was transferred to a positively
charged nylon membrane (Roche Applied Science). A 691 bp and a 657 bp DNA probe corresponding to exon 9 of the GGTA-1 gene just outside the 3’ end of the targeting construct were produced by PCR using primer sets; 691 bp probe: Gal3L1

5’AAACAGCTTTTCAATCCCTTTT and 3probeR2
5’AGCCACAATCCATGACCAGACCA and 657 bp probe: 3probeL3
5’GTTCCAGGCCAGATATCGATCCA and 3probeR3
5’ACCTGGCTGTCCATATGTATGTTGT. The probe was labeled using [α-32P]dCTP (PerkinElmer) by random oligopriming (Stratagene, Prime-It II). The membrane was subsequently hybridized overnight at 42°C with the radiolabeled probe in a hybridization solution (Roche Applied Science), washed with increasing levels of stringency, and exposed to Lumi-Film (Roche Applied Science).

Results

DNA Conformations, ss-DNA and ds-DNA

The goal was to evaluate the relative efficiency of electroporation using linearized ss-DNA or linearized ds-DNA. Based upon recent success of HR with a single-stranded targeting vector (Lorson et al., 2011) experiments were initiated using both ss-DNA and ds-DNA conformations, separately. The targeting construct pBB13 was linearized with either the restriction enzyme BstBI (linearized the plasmid so that the upstream and downstream homology arms contained 4,211 bp and 1,821 bp respectively for a total
length of 6,032 bp) or SacI (linearized the plasmid and releases the vector backbone so that the upstream and downstream homology arms contained 4,755 bp and 1,206 bp, respectively for a total length of homology of 6,026 bp). Either linearization method generated a similar total length of homology to the endogenous gene so the predicted total colony numbers that were G418\(^R\) and are targeted were expected to be similar. G418\(^R\) colonies were initially screened using the upstream assay (primer pair GLR_L1 and GSL_R2). This assay produces a 5.5 kb amplicon if a targeting event occurred (Figure 6). Targeted colonies were confirmed with the downstream assay. The downstream assay (primer pair GSR_L1 and GLR_R1) produces a 3.8 kb targeted amplimer (Figure 6). Representative results from screening G418\(^R\) colonies, fetuses, and live piglets demonstrate targeting of the GGTA-1 locus (Figure 7). The results from a total of twelve transfections (3 replicates) of pBB13 linearized with either BstBI or SacI in ss- or ds- conformations suggest that there is a trend for enrichment of G418\(^R\) targeted colonies (Table 2). Data also suggest a different targeting rate for pBB13 depending on the enzyme used to release the insert (Table 2). Colonies resulting from the transfection of BstBI-linearized ss-DNA or ds-DNA were targeted at a rate of 14.8% or 20.6%, respectively. Colonies resulting from the transfection of SacI-linearized ss-DNA or ds-DNA were targeted at a rate of 0% or 10.3%, respectively. Based upon these results, for this targeting sequence, we have elected to perform all additional experiments using ds-DNA.
**Figure 6.** Graphical representation of potential plasmid integration sites. The two integration possibilities using this type of vector yield either a site-specific or random integration. The use of promoter trap in the vector requires that integration occur near an endogenous promoter for proper transcription of the neomycin resistance gene. This allows for both integration possibilities, but with the use of the truncated diphtheria toxin-α (tDT) as a negative selectable marker the random integration possibilities should be reduced. Short arrows along with their respective names indicate the names and positions of the primers used for upstream and downstream PCR assays. For the upstream PCR assay, the primers were GLR_L1 and GLR_R2 for the forward and reverse primers, respectively. For the downstream PCR assay, the primers were GSR_L1 and GLR_R1 for the forward and reverse primers, respectively. The short bars indicate the location of the probe used for Southern blot analysis. The predicted size of Southern hybridization bands with HindIII digestion, for both the endogenous GGTA-1 locus and the GGTA-1 targeted locus, is as indicated.
Figure 7. Upstream and Downstream PCR Confirmation Assays. A representative PCR screening from fetal and piglet DNA. The upstream assay was generated using GLR_L1 as the forward primer and GSL_R2 as the reverse primer. The resulting 5.5 kb PCR product can only be generated in the event of a targeting event since the GLR_L1 primer is flanking the 5´ targeting arm and the GSL_R2 primer is located within the IRES sequence. In this figure, samples 4, 5, 12, 16, 19, 20, 21, and 24 are confirmed targeted by the upstream PCR. The downstream assay was used as a confirming diagnostic on upstream assay positive colonies. The downstream PCR assay was generated using GSR_L1 as the forward primer and GLR_R1 as the reverse primer. The resulting 3.8 kb PCR product can only be generated when targeted since the GSR_L1 primer is located within the hCD55 sequence and the GLR_R1 primer is flanking the 3´ targeting arm. Samples 4, 5, 12, 16, 19, 20, 23, and 24 are confirmed targeted by both PCR assays.
Sample 9 does not have a PCR product in the downstream PCR assay and denoted as a random integrant. The controls used in the assays; negative controls: H2O used as template and wild-type genomic DNA; positive controls: previously targeted colonies. The standard used was the λ genome digested with BstEII.
Table 1. Targeting results for pBB13 using ss-DNA and ds-DNA conformations.

<table>
<thead>
<tr>
<th>Knockout Vector</th>
<th>No. of G418(^R) Colonies</th>
<th>No. of PCR(^+) Colonies (%)</th>
<th>Targeting Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total#</td>
<td>Average¹ SD</td>
<td></td>
</tr>
<tr>
<td>pBB13 ss-BstBI</td>
<td>27</td>
<td>9 +/- 5.3</td>
<td>4 (14.8%) 1.3 +/- 2.3</td>
</tr>
<tr>
<td>pBB13 ds-BstBI</td>
<td>34</td>
<td>11.3 +/- 6.7</td>
<td>7 (20.6%) 2.3 +/- 3.2</td>
</tr>
<tr>
<td>pBB13 ss-SacI</td>
<td>4</td>
<td>1.3 +/- 0.6</td>
<td>0 (0.0%) 0 +/- 0</td>
</tr>
<tr>
<td>pBB13 ds-SacI</td>
<td>29</td>
<td>9.7 +/- 2.5</td>
<td>3 (10.3%) 1 +/- 1</td>
</tr>
</tbody>
</table>

* Student T Test was performed to compare treatment means (G418\(^R\) & PCR\(^+\))
# Chi Square Test was performed to test the proportional data of PCR\(^+\) to G418\(^R\)
Effect of Negative Selection

To determine the effect of a negative selectable marker in the context of a promoter-trap and targeting enrichment, four treatment groups were established: 1) a promoter-trap targeting vector that does not contain a negative selectable marker (pBB7), 2) a targeting vector that contains one copy of the negative selectable marker tDT (pBB8.1), 3) a targeting vector that contains two copies of tDT (pBB8.2), and 4) co-transfection of a targeting vector (pBB7) with a plasmid containing the DT gene (pDT-\(\alpha\)). By adding a negative selectable marker, the frequency of isolating a targeting event should increase because the number of random integrants should be reduced. The targeting constructs pBB7, pBB8.1, and pBB8.2 were linearized with the restriction enzyme SmaI and pDT\(\alpha\) was linearized with HindIII. G418\(^R\) colonies were screened using upstream PCR and downstream PCR assays (Figure 6). Only through successful targeting at the GGTA-1 locus would the upstream PCR product of 5.5 kb and the downstream PCR product of 3.8 kb be generated (Figure 7). Table 3 shows the results from a total of four sets of transfections for each vector type. Colonies resulting from the transfection pBB7 were targeted at a rate of 12.2% while transfection of pBB8.1 and pBB8.2, which contain one or two copies of the DT-\(\alpha\) negative selectable marker, targeted at a rate of 11.4% and 4.5%, respectively. Colonies resulting from the co-transfection of pBB7 and pDT\(\alpha\) were targeted at a rate of 5.0%. When comparing the four treatment groups of knockout vectors based upon a Student-T test there is no statistical difference in the total number of colonies picked or the number of targeted colonies. To test the proportional data (PCR\(^+\) to G418\(^R\)), a Chi Square Test was performed and there was no statistical difference when comparing the four treatment
groups of knockout vectors. Thus, based upon our results we have chosen to perform all additional experiments using constructs that do not contain a negative selectable marker.
Table 2. Comparison of targeting efficiency using promoter-trap, promoter-trap/PNS, and promoter-trap co-transfection with negative selection strategies.

<table>
<thead>
<tr>
<th>Knockout Vector</th>
<th>No. of G418&lt;sup&gt;R&lt;/sup&gt; Colonies</th>
<th>No. of PCR&lt;sup&gt;+&lt;/sup&gt; Colonies (%)</th>
<th>Targeting Efficiency</th>
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<tr>
<td></td>
<td>Total&lt;sup&gt;#&lt;/sup&gt;</td>
<td>Average&lt;sup&gt;*&lt;/sup&gt;</td>
<td>SD</td>
</tr>
<tr>
<td>pBB7 SmaI</td>
<td>49</td>
<td>12.3 +/- 5.1</td>
<td>6 (12.2%)</td>
</tr>
<tr>
<td>pBB8.1 SmaI</td>
<td>35</td>
<td>8.8 +/- 3.8</td>
<td>4 (11.4%)</td>
</tr>
<tr>
<td>pBB8.2 SmaI</td>
<td>44</td>
<td>11 +/- 4.1</td>
<td>2 (4.5%)</td>
</tr>
<tr>
<td>pBB7 + pDTα</td>
<td>40</td>
<td>10 +/- 2.9</td>
<td>2 (5.0%)</td>
</tr>
</tbody>
</table>

* Student T Test was performed to compare treatment means (G418<sup>R</sup> & PCR<sup>+</sup>)
# Chi Square Test was performed to test the proportional data of PCR<sup>+</sup> to G418<sup>R</sup>
Cells from all positive clones were expanded and cryopreserved. To confirm that the targeted colonies could generate live offspring via SCNT, cells from Clone B.1.8 (pBB7 targeted allele) were used for four rounds of SCNT (Table 4). We transferred between 224 and 261 SCNT embryos to each of the four recipient gilts (day 0 or 1 of estrus cycle). Three of the four recipients were confirmed pregnant. Of those pregnancies, one pregnancy was terminated at day 35 to collect fetuses, the other two pregnancies developed to term. Eight and nine B.1.8 SCNT male fetuses and piglets, respectively, were produced from this project. Eight fetuses were from nuclear transfer 1 (NT1), six stillborn piglets were from NT4, and nine piglets from NT3. Of the nine piglets from NT3, one was stillborn and the other eight piglets survived, shown in Figure 8. In all, eight animals were born alive and appeared normal.
Table 4. Cloned offspring produced following SCNT.

<table>
<thead>
<tr>
<th>Nuclear Transfer</th>
<th>Number of reconstructed embryos transferred</th>
<th>Surrogate number</th>
<th>Outcome</th>
<th>Number born alive</th>
<th>Additional comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT1</td>
<td>228</td>
<td>O903</td>
<td>Pregnant</td>
<td>N/A</td>
<td>8 fetuses collected</td>
</tr>
<tr>
<td>NT2</td>
<td>245</td>
<td>O891</td>
<td>Return</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>NT3</td>
<td>261</td>
<td>O911</td>
<td>Pregnant</td>
<td>8</td>
<td>1 stillborn</td>
</tr>
<tr>
<td>NT4</td>
<td>224</td>
<td>O859</td>
<td>Pregnant</td>
<td>0</td>
<td>6 stillborn</td>
</tr>
</tbody>
</table>

* All nuclear transfers were performed using the clone B.1.8 and the fusion/activation methods were performed electrically.
Figure 8. GGTA-1 +/- knockout piglets at 3 days of age. Piglets from colony clone B.1.8, allele targeted with pBB7, were born December 13, 2010.
**GGTA-1 +/- Feti and Piglets**

To demonstrate the zygosity of the GGTA-1 knockout allele in the feti and piglets, we performed PCR (Figure 7) and Southern blot analysis using DNA isolated from brain or tail snips, respectively, using the same primers used to identify the original targeted clones. Genomic DNA was isolated from a GGTA-1 +/- fetus (0903-2) and used for Southern blot analysis. In an attempt to optimize conditions for Southern blot analysis we generated multiple 3’ GGTA-1 probes, with only two probes proving to be useful. Genomic DNA was digested with HindIII. An 11,173 bp HindIII band is expected for the non-targeted wild-type allele and an 8,598 bp HindIII band is expected for the disrupted GGTA-1 locus due to the targeted insertion of pBB7. The targeted locus will produce a smaller band than the wild-type locus due to the presence of a HindIII restriction site being located within the 5,740 bp \( \text{loxP-IREs-mNeo}^R-\text{loxP-CAG-}\text{hCD55-attB-SV40 poly (A)} \) cassette (Figures 6 and 9). PCR and Southern blot analysis demonstrated fetus 0903-2 contained a disrupted GGTA-1 allele in a heterozygous state.
Figure 9. Southern blot analysis of GGTA-1 +/-. Representative analysis of DNA from day 35 fetus 0903-2. Lanes 1 to 3 are HindIII-digested genomic DNA from, 1: 104821 wild type cell line, 2: 0903-2 GGTA-1 +/- fetus, and 3: 104821 with the equivalent of 2 molecules per porcine genome of a control plasmid which contained probe sequences. A 691 bp and 657 bp PCR DNA fragment isolated from genomic DNA served as the probes. The 11.2 kb band represents the endogenous GGTA-1 gene, the 8.6 kb band represents the disrupted GGTA-1 locus, and the 7.2 kb band represents 2 molecules of the probe control vector. All lanes were from the same Southern blot, to remove unrelated data lane 3 was moved next to lanes 1 and 2.
Discussion

Gene targeting techniques permit manipulations to livestock genomes and allows for the analysis of gene function in the context of the whole animal. Such targeted animals can have human applications due to the similarities in size and physiology between some livestock species and humans. It has become possible to engineer specific genetic alterations ranging from subtle mutations to tissue-specific inducible gene targeting.

In the generation of a gene targeted animal the integrating exogenous DNA occurs through one of two reactions, non-homologous recombination (random integration) and homologous recombination (site-specific integration). Random integration introduces incoming sequences with no control over insertion site, potentially resulting in variable gene expression and insertional mutagenesis. Gene targeting through the mechanism of HR enables a precise modification to the targeted gene, but affords a frequency much lower than that of random integration (Capecchi et al, 1989). As a result, the creation of genetically manipulated animals can be labor and time intensive.

With the application of SCNT routinely producing genetically modified animals, the practicality of generating live animals from genetically modified somatic cells has been proven both plausible and efficient. However, the limited lifespan and fairly low HR frequencies of somatic cells have necessitated the development of various techniques to optimize gene targeting frequency of somatic cells in livestock species to that of murine ESC. Many groups are looking to optimize the various processes involved in the
incorporation of the exogenous DNA into the genome of the cell as a means to increase the gene targeting efficiency.

To test the feasibility of increasing targeting efficiency in porcine somatic cells we evaluated two different enrichment methods and chose to target the porcine GGTA-1 gene because of the amount of data available and the knowledge that this gene has been efficiently targeted (Denning et al., 2001; Dai et al., 2002; Harrison et al., 2002; Lai et al., 2002; Jin et al., 2003; Takahagi et al., 2005). In the first enrichment study the targeting efficiency was evaluated utilizing either ss-DNA or ds-DNA conformations of the targeting vector. In the second study, enrichment using a promoter trap with or without the addition of positive/negative selection was evaluated. According to Lorson et al. (2011) enrichment was experienced when the application of the targeting vector was single-stranded rather than double-stranded; however, they were only able to achieve a targeting event with the use of ss-DNA but not when using ds-DNA. Based upon these results, the experiment performed herein was designed to evaluate whether or not enrichment could be achieved using a promoter-trap vector in the conformation of ss-DNA compared to that of ds-DNA. Here it is demonstrated that there is no relevant improvement in targeting efficiency when comparing the targeting efficiencies of ss-DNA and ds-DNA. Subsequent transfections to be performed with the vector targeting the porcine GGTA-1 locus will be in ds-DNA conformation.

In the second study we investigated whether the addition of negative selection in the context of a promoter-trap. In order to determine a baseline targeting efficiency a promoter-trap vector was solely used. The data generated suggests that the addition of a negative selectable marker in the vector or as a co-transfectant do not provide further
enrichment of targeting events. Therefore, it is concluded using this specific targeting vector for the sequence being targeting (GGTA-1), there is no advantage to add the negative selectable marker in the context of a promoter-trap.

In conclusion, we were able to achieve targeting results of the porcine GGTA-1 gene that ranged from $1.5 \times 10^{-5}$ to $2.5 \times 10^{-6}$, which compares to $1 \times 10^{-6}$ targeting efficiencies in mouse ESC. As the data presented shows the performance of gene targeting in porcine somatic cells is equal to the performance of gene targeting in mouse ESC. In designing a targeting vector, it should remain known that not every targeting site targets at the same efficiency. With that in mind, the combination and/or rotation of targeting strategies should be implored to determine the most efficient method for each specific targeting location.
CHAPTER IV

EXPRESSION OF A HUMAN CD55 TRANSGENE INSERTED TO DISRUPT THE GGTA-1 LOCUS

Abstract

The production of α1,3-Galactosyltransferase (GGTA-1) knockout pigs that have additional genes located at the site of disruption is described in Chapter III. The major advantage of targeted insertion of a transgene(s) within a particular locus is to provide a genetic linkage for multiple genetic modifications and the disrupted gene, in the instance of GGTA-1. Genetic linkage of multiple genetic manipulations provides a logistical advantage during propagation of the resulting engineered genotype. However, the utility of this strategy is completely dependent upon appropriate expression of the transgene from within the disrupted locus. Here, expression of human CD55 (hCD55) from a transgene designed for ubiquitous expression has been inserted into the GGTA-1 locus and evaluated for transgene expression. These studies demonstrate that the GGTA1 locus provides a “safe harbor” integration site for the hCD55 transgene. Human CD55 expression was observed in all tissues examined. Additionally, the GGTA-1 integration site appears to facilitate a non-variegated expression pattern.
Introduction

Due to the lack of viable human donor organs, the possibility of utilizing animal organs to overcome this shortage has been considered as a possible solution. Over the past decade, the development of technologies that would allow pigs to meet the shortage of viable human donor organs has greatly expanded. With the ability to genetically engineer pigs, the disruption of the α1,3-Galactosyltransferase (GGTA-1) gene has been accomplished by several groups (Denning et al., 2001; Dai et al., 2002; Harrison et al., 2002; Lai et al., 2002; Jin et al., 2003; Takahagi et al., 2005). The GGTA-1 gene encodes the enzyme responsible for deposition of α1,3-linked terminal galactose residues, the major xenoantigen (Galili et al., 1988; and Cooper et al., 1993). Humans have preformed antibodies against the GGTA-1 epitope, which initiate hyperacute rejection of porcine cells that are naturally abundant in GGTA-1 terminal residues (Lambrigts et al., 1998). Thus, the absence of GGTA-1 expression appears to be one of the elements essential for successful xenotransplantation. Additional study regarding other immune barriers will be required for porcine organs to become suitable for human therapeutic use (Ramsoondar et al., 2003; and Fujimura et al., 2004; Diaz-Roman et al., 2006).

As reviewed by Tai et al. (2007), the use of several transgenes in addition to GGTA-1 knockout is likely essential for xenotransplantation to become a feasible. Current strategies to reduce rejection include the production of GGTA-1 negative pigs with the addition of random insertions of transgenes encoding human proteins that would combat other barriers to organ survival (i.e., coagulation, innate immune responses, and complement cascade). However, random insertion of transgenes can produce unpredictable expression levels and expression patterns. Additionally, sequential
addition of transgenes at random locations does not genetically link each new transgene to the previous insertions; therefore at subsequent breeding, each transgene will segregate independently. If these complex genotypes prove to be required, the breeding schemes that would be needed to produce piglets with the desired, complete set of transgenes become prohibitive. For example, a pig that contains two genetic manipulations in a hemizygous state will produce the desired genotype at a rate of only 25%. If this genetic manipulation has to be in the homozygous state to be useful (i.e. Knockouts), the required breeding and genotyping becomes very cumbersome. The ability to genetically link multiple genetic modifications by transgene stacking would improve the efficiency of maintenance and propagation of a “xenopig”.

We have constructed transgenic swine expressing the human decay accelerating factor (hCD55) gene located within the porcine GGTA-1 locus. Human decay accelerating factor is glycoprotein that inhibits the complement cascade, thus a modification to try and block immune barriers that would cause rejection. Our initial study may suggest that the GGTA-1 locus could be a safe-harbor integration site for transgenes that have non-variegated expression.
Materials and Methods

Characterization of hCD55 Expression in 0903-2 Cells

Heterozygous α1,3-GT knockout cells (0903-2 fetal fibroblasts) were produced by gene targeting using a vector that harbors a neomycin resistance cassette and a hCD55 transgene (pBB7) as described in Chapter III. To characterize hCD55 expression fetal fibroblasts from the transgenic fetus, 0903-2, targeted with the construct pBB7 and normal male fetal fibroblast cells, 104821 “Minnesota Miniature” breed (National Swine Resource and Research Center [NSRRC]), were grown on a 24-well plate in complete media (CM), Dulbecco’s modified Eagle’s medium [DMEM] with 1g/L glucose, L-glutamine and sodium pyruvate (Cellgro, No. 10-014-CV) with 12% fetal bovine serum (FBS), at 38.5°C in 5% CO₂, 5% O₂, and 90% air in 100% humidity. Cells were incubated with antibody, FITC Mouse Anti-Human CD55 (555693, BD Pharmingen), at a dilution of 1 to 500 in CM for 90 min at 38.5°C. Photographs were captured on a Nikon eclipse 800 microscope using a FITC filter with a Nikon Digital Sight DS-Qi1Mc camera operated by NIS-Elements BR3.0 imaging software.

Immunohistochemistry

Xenotransplantation organs (heart, kidney, liver, lung, and pancreas) from transgenic and wild type piglets were harvested, formalin fixed, paraffin embedded, sectioned and mounted. Tissue sections on slides underwent the sequential process of de-paraffinization, rehydration and antigen retrieval steps (RADIL histology lab, University of Missouri-Columbia, MO). The sections on slides were reacted with FITC Mouse
Anti-Human CD55 in PBS for 1 hour at room temperature. Photographs were taken at 20 X magnifications under FITC filter by Leica microscope and Leica DFC480 camera operated by Leica Application Software Version 2.8.0 imaging software.

Results

Cell Surface Expression of Human CD55 from the pBB7 Targeted Allele

During the production of 0903 series fibroblasts some pregnancies were allowed to develop to term. The resulting animals and harvested fetal cells harbor a hCD55 transgene within the disrupted GGTA-1 locus that resulted from HR with pBB7. Here, we evaluated expression of hCD55 from both 0903 series fetal fibroblasts and the counterpart piglets. Surface expression of hCD55 was visible on live 0903-2 cells (see Figure 10). Fixed tissues from euthanized piglets also displayed robust hCD55 expression that was associated with cell membranes. (See Figure 11)

Figure 11 demonstrates that hCD55 was properly expressed in tissues of heart, liver, lung, kidney, and pancreas from the transgenic piglet and not expressed in the same tissues of a wild-type piglet. Together with the result of immunocytochemical staining (Figure 10), these results suggest that the transgene is properly expressed on the cell surface and in the xeno-related organs of the transgenic piglet.
Figure 10. Cell surface expression of hCD55. Immunocytochemistry of fetal fibroblast cells with FITC labeled Mouse Anti-Human CD55 antibody. (a) 104821: Wild-Type porcine cells under bright light; (b) 0903-2: +/- GGTA-1, pBB7 targeted porcine cells under bright light; (c) 104821: Wild-Type porcine cells using a FITC filter; (d) 0903-2: +/- GGTA-1, pBB7 targeted porcine cells under FITC filter. The wild-type cells clearly don’t have any hCD55 expression and the transgenic cell line has diffuse staining.
Pane A

HEART

Wildtype Animal 15-6

Transgenic Animal 177-4 (+/- GGTA-1; 1 copy hCD55)
Pane B

KIDNEY

Wildtype Animal 15-6

Transgenic Animal 177-4 (+/- GGTA-1; 1 copy hCD55)
LIVER

Wildtype Animal 15-6

Transgenic Animal 177-3 (+/- GGTA-1; 1 copy hCD55)
Pane D

**LUNG**

Wildtype Animal 15-6

Transgenic Animal 177-4 (+/- GGTA-1; 1 copy hCD55)
Pane E

**PANCREAS**

Wildtype Animal 15-6

Transgenic Animal 177-4 (+/- GGTA-1; 1 copy hCD55)
Figure 11. Surface expression of hCD55 on xeno-organs. Immunohistochemistry of heart, kidney, liver, lung, and pancreas tissues for a wild-type pig (15-6) and a +/- GGTA-1 pigs (177-3 and 177-4) using a FITC-conjugated anti-Human CD55 antibody. Pane A: Heart Tissue; Pane B: Kidney Tissue; Pane C: Liver Tissue; Pane D: Lung Tissue; Pane E: Pancreas Tissue. The +/- GGTA-1 pigs show diffuse-ubiquitous hCD55 expression on all transgenic tissues with no expression on wild-type tissues.
Discussion

Through a novel genetic engineering strategy, we have shown that it is possible to link multiple genes (neomycin resistance cassette and hCD55) that would innately guarantee the co-inheritance of multiple genetic modifications. This strategy is only valuable if the linked transgene(s) is appropriately expressed when integrated at the targeted locus. As a practical proof-of-principle, we elected to evaluate transgene linking to a disrupted locus within the context of xenotransplantation. It has become clear in the xenotransplantation community that in addition to GGTA-1 knockout, xenopigs will require multiple transgenes in order to combat multiple human immune barriers. In addition, the ability to have such transgenes genetically linked could be extremely useful in building the initial xenopig, as well as for propagation of the genetic lineage.

We have demonstrated the ubiquitous, non-variegated expression of a human CD55 transgene that disrupts the GGTA-1 gene. Using the vector pBB7, that contains hCD55 under the control of the ubiquitous CAG promoter, we observed in both fetal fibroblast cells and xeno-related tissues high level of ubiquitous, non-variegated expression. Since the transgene used here utilizes a ubiquitous promoter placed in a locus that is nearly ubiquitously expressed, it remains possible that some promoters may not fully recapitulate the predicted expression pattern at this locus. Based upon previous studies (Cozzi et al., 1997; Diaz-Roman et al., 2006; Lee et al., 2011) transgene-delivered hCD55 inhibits the human complement cascade and could represent a potential strategy for future clinical xenotransplantation. It should also be noted, that hCD55 expression was comparable to all tissues examined, and these results partially make sense. The
GGTA-1 gene is expressed ubiquitously in all cells/tissues except in the pancreas. The pancreas appears to express GGTA-1 at low levels. A predicted expression level of hCD55 would be that compared to the expression of GGTA-1 in each cell type/tissue. However, this does not remain true for the pancreas since GGTA-1 expression is comparable in the pancreas to all other tissues as well.

Work performed by Friedrich and Soriano (1991, 1999) as well as many other groups have shown the murine ROSA26 locus to be a safe harbor for generalized transgene expression. Based on the results of hCD55 expression targeted to GGTA-1 and the high efficiency of targeting this locus, we propose GGTA-1 locus as a safe-harbor in the porcine genome, at least for ubiquitous expression.

From the hCD55 transgene placement described here, the logistics of propagating multi-transgene pigs appears feasible. Future work would include the production of homozygous GGTA-1 knockout pigs that would contain two copies of hCD55 (one on each targeted allele). Once these pigs are generated, the characterization of the hCD55 transgene and GGTA-1 ablation will be evaluated. Based upon the targeting data presented here and the resulting hCD55 transgene expression, we are looking forward to generating homozygous knockout animals and to evaluate the levels of hCD55 transgene (two copies) expression.
CHAPTER V

SUMMARY

The aim on this thesis was to incorporate several issues related to the production of transgenic animals. We wanted to evaluate two strategies that could potentially increase the targeting efficiency and frequency in porcine somatic cells, with the end goal of rendering a knockout cell. In addition, we wanted to evaluate the functionality of a human transgene when inserted into the porcine GGTA-1 gene.

To evaluate the strategies to increase the frequency and efficiency of gene targeting, we chose to target the porcine GGTA-1 gene. We chose to target this gene due to the fact that several groups have achieved targeting at this locus. For targeting enrichment strategies we had two goals in mind: 1) to increase the overall targeting efficiency and 2) to increase the targeting frequency by reducing the number of background colonies that are produced by random integration. In recent studies, it is implied that the introduction of ss-DNA increased the incidence of HR. The first experiment was to evaluate the targeting efficiency of transfecting with ss-DNA and ds-DNA. Our results suggested there was no advantage of using one conformation of DNA over the other conformation in that both produced targeting events. However, the use of ds-DNA produced targeting events at a higher frequency.

The second form of enrichment was to target using a negative-selectable marker in our vector in the context of a promoter-trap. The purpose of this experiment was to increase the targeting frequency by decreasing the number of background colonies with the negative selectable marker killing random integrants and only living colonies would
be produced from homologous recombination, thus increasing the targeting frequency. We evaluated the effectiveness of a negative selectable maker with the marker being the diphtheria toxin-α (DT-α). We compared a promoter-trap vector, to a promoter-trap vector that contained either one or two copies of a truncated DT-α gene, as well as a co-transfection of the promoter-trap vector with a DT-α expressing plasmid. Our results suggested there was no advantage to add a negative selectable marker in either one copy, two copies, or as a co-transfectant. The targeting efficiency and targeting frequency was not significantly different between treatment groups suggesting that the addition of a negative selectable marker did not contribute to a higher targeting efficiency or frequency.

To evaluate the ability to add a functional transgene into the porcine GGTA-1 gene we added a human transgene (hCD55) into the targeting construct. In doing so, if HR occurs, the transgene will be located within the GGTA-1 gene. After producing targeted colonies, we generated feti and piglets. The feti and piglets were evaluated for transgene expression with an antibody. It was confirmed the transgene, is ubiquitously expressed in a non-variegated pattern.


