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Research Article

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Use of Single Stranded Targeting DNA or Negative Selection does not Provide Additional Enrichment from a *GGTA1* Promoter Trap

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Abstract

Although several techniques have been developed to create gene knockouts in pigs, homologous recombination will continue to be required for site-specific genome modifications that are more sophisticated than gene disruption (base changes, domain exchanges, conditional knockouts). The objective of the present paper was to improve the efficiency of homologous recombination in porcine fetal fibroblasts, which would be used to produce gene knockout pigs by somatic cell nuclear transfer. A promoter-trap was used to enable selection of *GGTA1* targeted cells. Cells were transfected with either a single stranded or double stranded targeting vector, or a vector, with or without a negative selectable marker gene (diphtheria toxin-A). Although targeting efficiencies were numerically lower for single stranded targeting vectors, statistical differences could not be detected. Similarly, the use of a negative selectable marker (in *cis* or *trans*) provided numerically lower targeting efficiencies, statistical differences again could not be detected. Overall, the targeting efficiencies ranged from 1.5×10^{-5} to 2.5×10^{-6} targeting events per transfected cell. Given the results, it may be applicable to investigate multiple enrichment techniques for homologous recombination, given that every targeted locus is different.

Keywords

Gene targeting; Homologous recombination; Transgenic; Single-stranded DNA; Promoter-trap; Transfection efficiency; Swine

Introduction

Gene targeting, through the use of homologous recombination (HR), provides the ability to modify any endogenous gene in a predetermined and precise manner. This technology has proven to be robust in mouse embryonic stem cells (ESC). Due to the lack of established ESC in livestock, HR in livestock requires the use of somatic cells, instead of ESC [1]. 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 much lower than in mouse ESC [2,3]. The efficiency of gene targeting in mouse ESC averages approximately 1×10^{-6} targeting events per cell, when exposed to electroporation [4,5], while initial gene targeting studies in somatic cells demonstrated efficiencies two to three orders of magnitude lower [6,7]. Thus, gene targeting in somatic cells could be expected to be much less efficient than in ESC. In recent years, several groups have targeted genes in cultured porcine somatic cells, at efficiencies similar to those observed in mouse ESC, 9.3×10^{-5} to 8.3×10^{-7} targeting events per exposed cell [8-15]. Recent studies in other livestock species also suggest that the overall rate of HR in primary somatic cells is comparable to gene targeting in ESC [16]. Thus, the utility of HR to produce animals with genome modifications clearly extends to livestock.

Experimentally induced HR 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 [17]. 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, and makes 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. These targeting enrichment strategies reduce the number of random integration events that survive selection. To achieve enrichment based on a vector design, several strategies have been used: promoter-trap (mouse, [18]; pig, [8]), poly(A) trap (mouse, [19]; pig, [13]), and positive-negative selection (mouse, [20]; pig, [21]). It has also been observed that single-stranded DNA (ss-DNA) can serve as substrate for HR [22,23], but may not randomly integrate, as efficiently as double-stranded DNA (ds-DNA) [24]. It is, therefore, possible that ss-DNA may participate in homologous recombination at the similar rates to ds-DNA, while producing fewer random integrations events. Although the utility of ss-DNA as an enrichment strategy has not been thoroughly investigated, it has been used to produce gene-targeted pigs [14]. In the context of a promoter trap, this study examines the efficiency of gene-targeted colony production and recovery, using two distinct enrichment strategies: 1) transfection of single-stranded verses double-stranded DNA conformations, and 2) positive-negative selection.

The first enrichment strategy was based upon the observations of Lorson et al. [14], regarding the efficiency of targeting the porcine *SMN* gene through the use of a single-stranded targeting vector. In the report of Lorson et al. [14], targeting was not observed from a ds-DNA vector, but was observed after transfection with the same vector, after it had been denatured to a single stranded conformation. However, the overall targeting efficiency at the *SMN* locus was too low to statistically establish if the observed targeting frequencies were different between ss-DNA and ds-DNA. We chose to re-evaluate the use of ss-DNA for homologous recombination at the porcine alpha1,3-galactosyltransferase gene (*GGTA1*), because several groups have successfully targeted this locus in porcine cells, at rates that were expected to allow estimations of treatment effects [2,9,10,21,25]. These experiments were designed to evaluate, whether the conformation of the targeting vector DNA affects the ability to

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recover targeted colonies (linearized ss-DNA versus linearized ds-DNA). The hypothesis was that DNA conformation would alter the ratio of targeted-integration versus random-integration. This hypothesis was based on the expectation that ss-DNA may produce fewer random integration events [24], and thus, provide enrichment, or may produce more targeting events [14], and thus, increase targeting efficiency.

The second enrichment strategy was based on positive-negative selection, in the context of a promoter-trap vector. Promoter traps can provide significant enrichment, since expression of the selectable marker requires the acquisition of an endogenous promoter. However, since we routinely observe random integration as the major source of resistance to selection, it seemed possible that further enrichment may be possible. In addition, we further investigated whether the negative selectable marker could be provided in *trans*. A *trans* strategy was considered because co-integration of two DNA fragment is very efficient during random integration [26,27], and a *trans* strategy requires less cumbersome plasmid construction. In fact, a *trans* strategy facilitates the use of positive-negative selection as an alternative strategy, or even as an afterthought. For these experiments, a diphtheria toxin-alpha expression cassette (DT-α) was included in *cis* with the targeting vector, or supplied in *trans* to provide the negative selection against random integration events. Upon homologous recombination through a double crossover event, DNA sequences that are outside of the targeting arms would not become integrated, and therefore, would not provide negative selection.

Herein, we report the production of *GGTA1* +/- porcine fibroblast cells through the use of two targeting enrichment strategies. Gene targeting was observed at 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 porcine fetal fibroblasts, male cells 104821 "Minnesota Miniature" breed (National Swine Resource and Research Center (NSRRC)). The targeting constructs are graphically represented in figure 1A-1E. A 6,641 bp *GGTA1* 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, between pBB4 and the target locus that may have been introduced by PCR errors was not determined. The pBB4 plasmid served as the template to isolate the *GGTA1* recombination arms. Plasmid pBB7 was generated to include a 5,740 bp *lox*P-IRES (internal ribosome entry site)-mNeo^R-*lox*P-CAG-hCD55-*att*B-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 *lox*P-IRES-mNeo^R-*lox*P was isolated from a chemically synthesized in-house plasmid (pKW2), that contained all four components. The neomycin resistance (Neo^R) gene is based on mammalian codon usage. A 1,962 bp PmeI/BsrBI restriction fragment

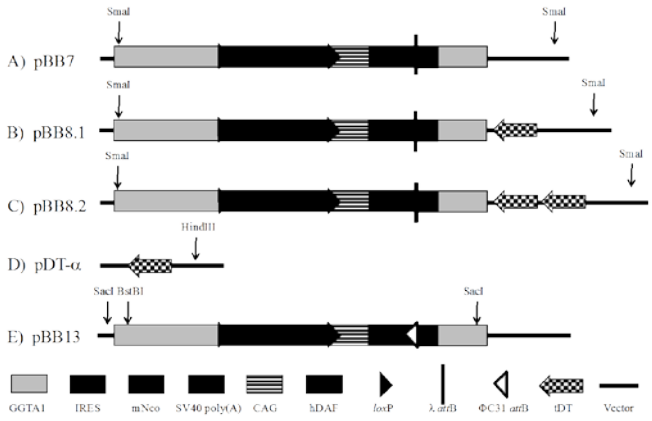

Figure 1: Schematic of the test plasmids. A, B, C and E are graphical representations of each targeting construct. Each construct included the corresponding porcine *GGTA1* genomic sequences, used for the 5` and 3` recombination arms in the knockout vectors. Each construct included a mammalian optimized 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, B, and C all contain a λ *att*B site located between the hCD55 cassette, and the SV40 poly (A) represented by a solid vertical bar. Construct E has a φC31 *att*B site located at the same location, represented by an open triangle. Constructs B and C contain one and two truncated diphtheria toxin-α (tDT) genes, respectively. Construct D is a representation of the pDT-α vector used as a co-transfected plasmid. pDT-α contains the coding sequence for diphtheria toxin-alpha cassette driven by the *Pol*II promoter, and contains a SV40 poly(A).

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 *GGTA1* locus, to build pBB6. The hCD55, lambda *att*B, and the SV40 poly (A) components were isolated from cDNA clone MGC: 5192 IMAGE: 3460621 (Open Biosystems #3460621). After modifications to 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 (Addgene plasmid 13776; [28]) 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 AattII and EcoRI restriction sites that had been digested, and then treated with T4 DNA polymerase (New England Biolabs), to produce blunt ends. The resulting plasmid was named pBB3. A 3,804 bp PvuI/SpeI restriction fragment containing the CAG-hCD55-*att*B-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 *att*B site with a φC31 *att*B site, to produce pBB13. These two plasmids, pBB7 and pBB13 differ by a total of 70 bp. 2) A truncated diphtheria toxin-alpha cassette (tDT) was isolated from 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 concatemerized 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 transfections. To generate single-stranded DNA, the linearized template was boiled for 3 minutes and placed on ice.

Cell culture, transfection, and selection

Passage three 104821 cells were thawed, then cultured for 72 hours in Dulbecco's modified Eagle's medium (DMEM), with 2.77 mM glucose, 1.99 mM L-glutamine, 0.5 mM sodium pyruvate (Cellgro), and 12% fetal bovine serum (FBS) at 38.5°C in 5% CO_2, 5% O_2, and 90% balanced air in 100% humidity. Transfection was accomplished by a modification of the method described by Ross et al. [29]. Fibroblasts were harvested by trypsinization, and were re-suspended at a density of 1×10^6 cells/mL in a modified electroporation media (25% OptiMEM (Invitrogen)+75% buffered KCl (135 mM 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 a 2 mM electroporation cuvette, and the cells were electroporated using 250 volts×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, supplemented with 1 mM L-alanyl-L-glutamine (Cellgro), and 15% FBS in twenty 100 mm tissue culture plates (~ 10,000 cells/plate). Twenty-four hours after culture, cells were administered G418 (Cellgro, 400 mg/L), and cultured for 13 more days. G418 resistant ($G418^R$) 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 $G418^R$ media, and one third of cells isolated were used for PCR analysis of targeted clones. Positive colonies were expanded, frozen, and were used for SCNT to produce transgenic fetuses and pigs.

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) (40 mM 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 L 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′CCAAGCGGCTTCGGCCAGTAACCTTAG.

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′AGTGCCGTCCAGGTTACAGAAGAGAACC and GLR_R1 5′GAGTAGGAGGCCCAGGGAAACAGTAGAG.

Somatic cell 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. [30], 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 or the first day after standing estrus were used. Embryo transfer was performed surgically, as previously described [31]. 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, and the remaining tissue was to establish fibroblast cultures. For DNA isolation, the tissue was suspended in 700 µL of lysis buffer II (LBII) (50 mM Tris, pH 8; 100 mM EDTA; 0.5% SDS; 35 µL 10 mg/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 [32], 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 *GGTA1* gene, just outside the 3′ end of the targeting construct, were produced by PCR using primer sets; 691 bp probe: Gal3L1 5′AAACAGCTTTTCAATCCCTTTC and 3probeR2 5′AGCCACAATCCATGACCAGACCA, and 657 bp probe: 3probeL3 5′GTTCCCAGGCCAGATATCAGATCCA and 3probeR3 5′ACCTGGCTGTCCATATGTATGGTGT. The probe was labeled using [α-^{32}P]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 objective of the current study was to evaluate linearized ss- or ds-DNA vector conformations for homologous recombination. Based upon recent success of HR with a single-stranded targeting vector [14], experiments were initiated using both ss-DNA and ds-DNA conformations, separately. The targeting construct pBB13 was either linearized with BstBI, or was released from the plasmid with SacI (Figure 1E). Since each enzyme generated a similar total length of homology to the endogenous gene, the targeting rate for each conformation was expected to be similar. The left and right targeting arms for the BstBI digest were 4211 bp and 1821 bp, respectively. The left and right targeting arms for the SacI digest were 4755 bp and 1206 bp, respectively. $G418^R$ colonies were initially screened using the upstream assay (primer pair GLR_L1 and GSL_R2). This assay produces a 5.5 kb amplimer from a targeted allele. Potential targeted colonies were confirmed with the downstream assay. The downstream assay (primer pair GSR_L1 and GLR_R1) produces a 3.8 kb amplimer from a targeted allele. Primer binding sites are shown in figure 2. The results from a total of twelve transfections (3 replicates of 4 treatments) of pBB13 linearized with either BstBI or SacI in ss- or ds- conformations, are summarized in table 1. Representative results from colony screening are shown in figure 3. Colonies resulting from

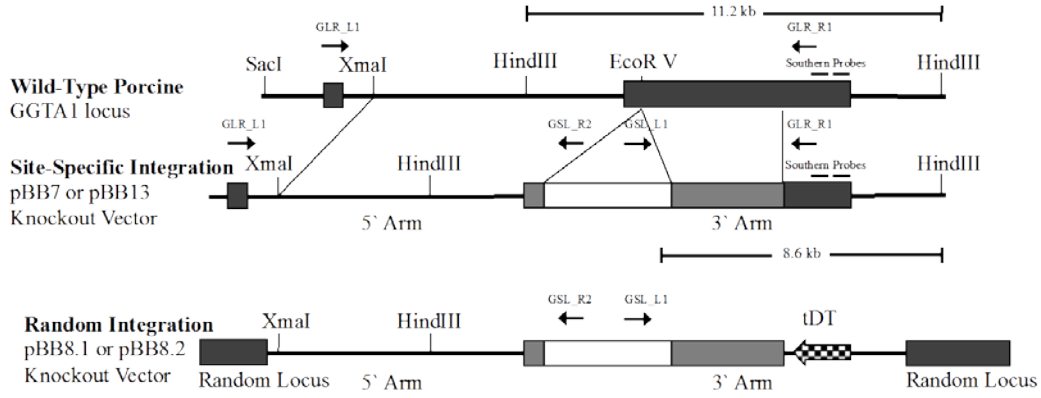

Figure 2: Graphical representation of potential plasmid integration sites. The two integration possibilities using this type of vector yield either a site-specific or random integration. The use of promoter trap in the vector requires that integration occur near an endogenous promoter, for proper transcription 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 GSL_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 *GGTA1* locus and the *GGTA1* targeted locus, is as indicated.

Table 1: Targeting results for ss-DNA and ds-DNA conformations, pBB13.

Knockout vector	No. of G418^R colonies			No. of PCR$^+$ colonies (%)			Targeting efficiency
	Total	Average	SD	Total	Average	SD	
pBB13 ss-BstBI	27	9	± 5.3	4 (14.8%)	1.3	± 2.3	6.7×10^{-6}
pBB13 ds-BstBI	34	11.3	± 6.7	7 (20.6%)	2.3	± 3.2	1.6×10^{-5}
pBB13 ss-SacI	4	1.3	± 0.6	0 (0%)	0	± 0	0
pBB13 ds-SacI	29	9.7	± 2.5	3 (10.3%)	1	± 1	5.0×10^{-6}

Count data were analyzed by Student's T-test. Due to the absence of targeting in ss-DNA that had been digested with SacI, statistical analysis of this treatment could not be performed. The proportional data were analyzed by pairwise Chi-squared analysis. No treatments could be demonstrated to be statistically significant. The number of PCR$^+$ colonies is represented both by a number and a percentage; the percentage is representing the frequency of the targeting event per G418^R colony. The targeting efficiency is calculating the targeting events per transfected cell. Abbreviations: Number (No.); Standard Deviation (SD).

the transfection of BstBI-linearized ss-DNA or ds-DNA were targeted at a frequency (number of targeted colonies/total number G418^R colonies) of 14.8% and 20.6%, respectively. Colonies resulting from the transfection of SacI-linearized ss-DNA or ds-DNA were targeted at a frequency (number of targeted colonies/total number G418^R colonies) of 0% and 10.3%, respectively. Count data were analyzed by Student's T-test. Due to the absence of targeting from ss-DNA that had been digested with SacI, statistical analysis of this treatment could not be performed. The proportional data were analyzed by pairwise Chi-squared analysis. No treatment effects could be demonstrated to be statistically significant.

Effect of negative selection

To determine the effect of a negative selectable marker in the context of a promoter-trap, four treatment groups were evaluated: 1) transfection of a promoter-trap targeting vector that does not contain a negative selectable marker (pBB7), 2) transfection of a targeting vector that contains one copy of the negative selectable marker tDT (pBB8.1), 3) transfection of a targeting vector that contains two copies of tDT (pBB8.2), and 4) co-transfection of a targeting vector (pBB7), with a separate plasmid containing the DT gene (pDT-α). The targeting constructs pBB7, pBB8.1, and pBB8.2 were linearized with SmaI, and pDT-α was linearized with HindIII. G418^R colonies were screened as described above and representative results are shown in figure 3. Table 2 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 frequency (number of targeted colonies/total number G418^R colonies) of 12.2%, while transfection of pBB8.1 and pBB8.2, which contain one or two copies of the DT-α negative selectable marker, were targeted at a frequency of 11.4% and 4.5%, respectively. Colonies resulting from the co-transfection of pBB7 and pDTα were targeted at a frequency of 5.0%. No statistical difference in the total number of colonies recovered, or the number of targeted colonies, could be demonstrated. Similarly, no statistical differences in the proportion of colonies that were targeted could be demonstrated.

Production of GGTA1 +/- porcine fetuses and pigs by nuclear transfer

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 3). We transferred between 224 and 261 SCNT embryos to each of the four surrogate gilts (day 0 or 1 of estrus cycle). Three of the four surrogates established pregnancies. One pregnancy was terminated at day 35 to collect fetuses, the other two pregnancies developed to term. Eight fetuses were produced from nuclear transfer 1 (NT1), six stillborn piglets were from NT4,

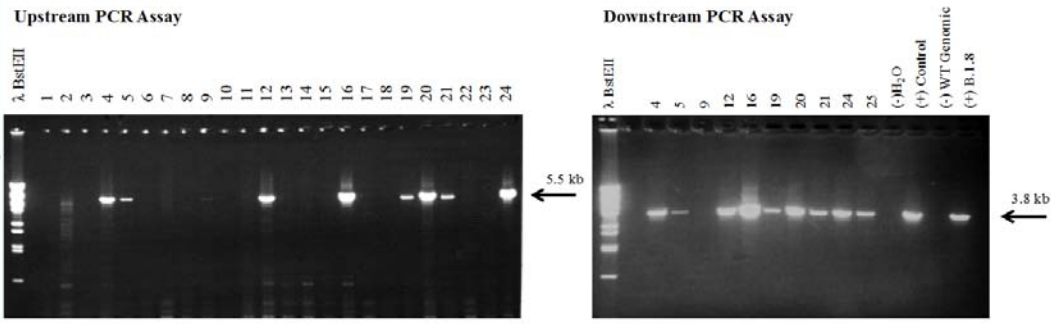

Figure 3: Representative PCR screening from colony screening efforts. The result of a single PCR product for each assay was the same for screening colonies, fetuses, and piglets. The upstream assay was generated using GLR_L1 as the forward primer and GSL_R2 as the reverse primer. The resulting 5.5 kb 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, 21, 24, and 25 (not shown in Upstream PCR Assay) 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: H_20 used as template and wild-type genomic DNA; positive controls: previously targeted colonies. The standard was the λ genome digested with BstEII.

Table 2: Comparison of targeting efficiency strategies using promoter-trap, promoter-trap/PNS, and promoter-trap co-transfected with negative selection.

Knockout vector	No. of G418^R colonies			No. of PCR$^+$ colonies (%)			Targeting efficiency
	Total	Average	SD	Total	Average	SD	
pBB7 SmaI	49	12.3	± 5.1	6 (12.2%)	1.5	± 1.3	7.5×10^{-6}
pBB8.1 SmaI	35	8.8	± 3.8	4 (11.4%)	1.0	± 0.8	5.0×10^{-6}
pBB8.2 SmaI	44	11.0	± 4.1	2 (4.5%)	0.5	± 0.6	2.5×10^{-6}
pBB7 SmaI+pDTα HindIII	40	10.0	± 2.9	2 (5.0%)	0.5	± 1.0	2.5×10^{-6}

Count data were analyzed by Student's T-test. The proportional data were analyzed by pairwise Chi-squared analysis. No treatments could be demonstrated to be statistically significant. The number of PCR$^+$ colonies is represented both by a number and a percentage; the percentage is representing the frequency of the targeting event per G418^R colony. The targeting efficiency is calculating the targeting events per transfected cell. Abbreviations: Number (No.); Standard Deviation (SD).

Table 3: Cloned offspring produced following SCNT.

Nuclear transfer	Number of reconstructed embryos transferred	Surrogate number	Outcome	Number born alive	Additional comments
NT1	228	O903	Pregnant	N/A	8 Fetuses collected
NT2	245	O891	Return	N/A	N/A
NT3	261	O911	Pregnant	8	1 Stillborn
NT4	224	O859	Pregnant	0	6 Stillborn

*All nuclear transfers were performed using the clone B.1.8, and the fusion/activation methods were performed electrically.

and nine piglets from NT3. Of the nine piglets from NT3, one was stillborn, and the other eight piglets survived, a portion of the litter is shown in figure 4. In all, eight animals were born alive and appeared normal. Targeting was confirmed in all fetuses and piglets by both PCR assays (Figure 5).

GGTA1 +/- feti and piglets

To validate targeting and demonstrate the zygosity of the *GGTA1* knockout allele, fetus 0903-2 was evaluated by Southern blot (Figure 6). Genomic DNA was digested with HindIII. An 11,173 bp HindIII band is expected for the non-targeted allele, and an 8,598 bp HindIII band is expected for the targeted *GGTA1* locus. The targeted allele will produce a smaller band than the wild-type allele, due to the presence of a HindIII restriction site located within the 5,740 bp *lox*P-IRES-mNeo^R-*lox*P-CAG-hCD55-*att*B-SV40 poly (A) cassette. The location of the fragments used as probes is shown in figure 2. PCR and Southern blot analysis demonstrated that fetus 0903-2 contained a disrupted *GGTA1* allele in a heterozygous state.

Discussion

Gene targeting techniques permit manipulation of livestock genomes and facilitate analysis of gene function in the context of the whole animal. It has become possible to engineer specific genetic

Figure 4: *GGTA1* +/- knockout piglets at 3 days of age. Piglets were cloned from colony clone B.1.8, allele targeted with pBB7, and born on December 13, 2010.

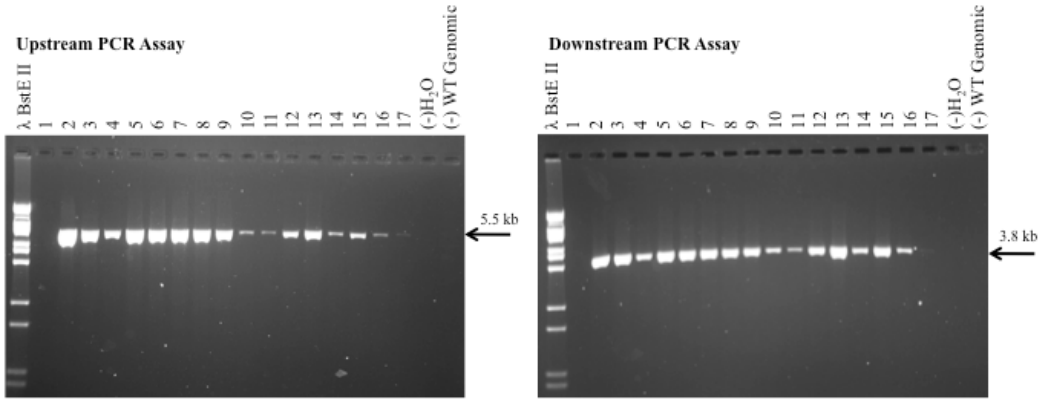

Figure 5: PCR screening of *GGTA1* +/- fetuses and piglets. The downstream (3.8 kb PCR product) and upstream (5.5 kb PCR product) PCR assays, are as previously described. The standard is the λ genome digested with BstEII. The samples represented are: (1) empty lane, (2) fetus 0903-1, (3) fetus 0903-2, (4) fetus 0903-3, (5) fetus 0903-4, (6) fetus 0903-5, (7) fetus 0903-6, (8) fetus 0903-7, (9) fetus 0903-8, (10) piglet 177-1, (11) piglet 177-2, (12) piglet 177-3, (13) piglet 177-4, (14) piglet 177-5, (15) piglet 177-6, (16) piglet 177-7, (17) piglet 177-8, (-) control sample H_2O, (-) control wild type cell line genomic DNA. The samples are the same for both PCR assays.

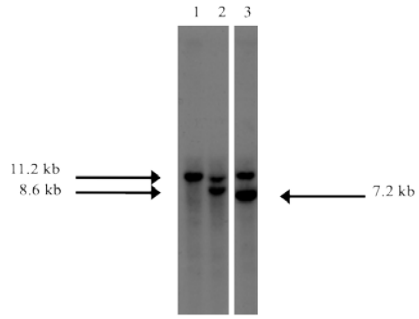

Figure 6: Southern blot analysis of *GGTA1* +/-. Representative analysis of DNA from day 35 fetus #0903-2. Lanes 1 to 3 are HindIII-digested genomic DNA from 1: wild type cell line (104821), 2: *GGTA1* +/- fetus (#0903-2), and 3: wild type cell line (104821), with the equivalent of 2 molecules per porcine 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 *GGTA1* gene, the 8.6 kb band represents the disrupted *GGTA1* locus, and the 7.2 sskb 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.

alterations, including, insertions, replacements, deletions and subtle modifications. With increased use of pigs to model human disease, the ability to generate gene modifications in the pig will be a requirement.

To examine the efficiency of gene-targeted colony production and recovery, we evaluated two different enrichment methods and chose to target the porcine *GGTA1* gene, because of the amount of data available and the knowledge that this gene has been efficiently targeted [2,8-10,21,25]. In the first enrichment study, the targeting efficiency was evaluated using either ss-DNA or ds-DNA conformations of the targeting vector. Although, ss-DNA appears to have been required to observe targeting at *SMN1* [14], in this report, there was not a benefit with the use of ss-DNA. There may have been a negative impact. Numerically, the use of ds-DNA generated more targeted colonies. We have concluded this may be due to the high targeting rate at this locus, and the ss-DNA did not provide any enrichment, beyond the already high targeting rate.

In the second study, enrichment using a promoter trap, with or without the addition of positive/negative selection, was evaluated. Although, the addition of DT-α numerically yielded fewer targeted colonies, treatment differences could not be statistically demonstrated in either *cis* or *trans* conformations. However, targeted colony number was inversely proportional to the DT-α copy number in *cis*. This observation may be due to lethal transient expression of the negative selectable marker [33].

In conclusion, we were able to achieve gene-targeting efficiency rates of porcine *GGTA1* that ranged from 1.5×10^{-5} to 2.5×10^{-6}, which is similar to rates observed for other genes in mouse ESC (1×10^{-6}) [4,5], and within the range observed in pig in other reports (9.3×10^{-5} to 8.3×10^{-7}) [8-15]. Variation was so large that differences were difficult to demonstrate. For the direct comparison of targeting efficiency between mouse ESC and pig fibroblasts, it is granted that a much larger dataset would need to be generated. This dataset ideally would include multiple targeted genes. However, the data presented shows that it is clear that gene targeting in pigs can sometimes is just as efficient as in mouse ESC.

We would recommend for future gene HR efforts that a variety of vector modifications, as part of a standard procedure, be explored. Each gene will have its own unique targeting rate. In addition, it may be that the effect of vector design or enrichment strategy varies between different genes. Therefore, a varied approach to targeting strategy may be useful. For example, a rotation of restriction endonucleases to create varying lengths of the linearized vector, and/or DNA conformation, may be explored. Perhaps for routine targeting projects, multiple DNA fragments and conformations can be pooled in single transfections, to increase the robustness of a standard protocol.

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