Direct and Rapid Modification of a Porcine Xenoantigen Gene (GGTA1)

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The ability to modify animal genomes rapidly at a specific locus would be valuable both for research purposes and in the development of animals suitable for xenotransplantation. In a proof-of-concept study, we used a unique, homology-dependent strand transferase protein called drosophila recombination-associated protein (DRAP) and DNA oligonucleotides to modify the porcine gene encoding alpha 1,3 galactosyl transferase (GGTA1). This gene is responsible for generating xenotransplantation antigens resulting in hyperacute rejection. Pronuclear injection of DRAP and mutant oligonucleotides yielded piglets with heritable, modified alleles of GGTA1 in a direct, rapid and efficient manner. Cells derived from these piglets had markedly reduced alpha 1,3 galactosyl sugar epitopes. The simplicity of this method should permit rapid sequential or simultaneous modification of the various genes encoding or producing antigens that impose limits on xenotransplantation as they are discovered.

Keywords: Genome modification, Xenotransplantation, GGTA1, DRAP method.

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One limitation to using pigs for xenotransplantation in humans has been the strong immune response to porcine antigens. The most immunogenic epitopes, generated by alpha 1,3 galactosyl transferase (GGTA1; E.C. 2.4.1.151), are responsible for hyperacute rejection (1, 2). GGTA1-null pigs were developed through the selection of rare GGTA1-null somatic cells generated by homologous recombination followed by complex nuclear transfer methods (3, 4). The removal of the GGTA1-dependent antigens has uncovered other strong antigens that still impose barriers to xenotransplantation (5, 6). The genes responsible for these antigens will need to be modified.

The approach that was successful for GGTA1 will be impractical if multiple, independent selection methods are required. To overcome these limitations we developed a method utilizing a protein called Drosophila recombination-associated protein (DRAP) with homology-dependent strand transferase and topoisomerase activities (7, 8). In a proof-of-concept study, we generated piglets with heritable, modified GGTA1 alleles through pronuclear coinjection of DRAP and DNA oligonucleotides.

Recombinant DRAP (60 ng/ul), with an N-terminal (His6), was expressed in bacteria and purified by Ni-NTA, Phosphocellulose and Superose chromatography (8).

Oligonucleotides with 5’-OH ends (Invitrogen; HPLC-purified) were used for microinjection with DRAP singly, annealed as duplexes, or in non-complementary combinations: Oligonucleotides were suspended in sterile microinjection buffer (10 mM Tris-HCl, 10 mM NaCl, 1.0 mM MgCl2, and 0.1 mM EDTA, pH 7.5) to a final concentration of 75 ng/µl. For microinjection of single oligonucleotide–DRAP complexes into zygotes, 1 µl of 5°C, 3°C, 5°C2, or 3°C2 oligonucleotides (75ng/µl) and 1 µl of DRAP (1.5 mg/ml) were diluted into 98 µl microinjection buffer and incubated on ice for 30 min prior to injection (9).

Ear notch-derived fibroblasts were collected from 100-mm culture dishes maintained at less than 60% confluence. For controls, a porcine fibroblast cell line from a commercial breed (+/+) and SK-N-DZ human neuroblastoma (−/−) cell line were used. Cells were trypsinized for two to three min, transferred to media, pelleted, washed 1× with phosphate-buffered saline solution, pelleted and resuspended in 4.0% paraformaldehyde and incubated at room temperature for 10 min. Fixed cells were pelleted, washed two times with 0.4% BSA, and divided into two aliquots for fluorescein isothiocyanate conjugated isoelectin 4 (FITC-IB4) labeling and for unlabeled, autofluorescence measurements. Cells for labeling were resuspended in 0.5 µg/ml FITC-IB4, incubated in the dark at 37°C for 30 min., and washed two times with 0.4% body surface area (BSA). Both labeled and unlabeled cells were re-suspended in 400 µl 0.4% BSA prior to flow cytometry. The raw adjusted geometric mean (AGM) fluorescence was calculated for each cell line by dividing the geometric mean fluorescence of the labeled

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nuclear injection of DRAP and oligonucleotides showed no
untoward effects on the establishment of IVF pregnancies or
on litter size (9). From 15 successful pregnancies a total of 96
live-born piglets were delivered (Table 1).

Fibroblasts were obtained for culture from collagen-
digested ear notches of each offspring. The amount of alpha
1,3 galactosyl sugar residues on the fibroblast cell surface
was determined by staining with FITC-labeled isoelecin B4
(FITC-IB4) and quantified by flow cytometry of 10,000 cells.
The AGM for each experimental cell line was normalized to
the AGM obtained for the positive control line to correct for
any run-to-run variability during each of the 15 separate flow
studies carried out over a period of 18 months (Table 1).

The bimodal distribution of the normalized AGMs for
all of the experimental cell lines (Fig. 1) spanned a broad
range relative to replicates of the positive control cell line.
Each oligonucleotide produced animals yielding fibroblast
lines exhibiting reduced FITC-IB4 staining. The AGM values
were comparable for both males and females. Mean AGM
values for six out of seven oligonucleotides tested were signifi-
cantly reduced (Table 1; Student’s t test; P<0.02 or lower).
None of the experimental cell lines had a reduction in FITC-
IB4 staining comparable to the human null cell line.

Six F1 animals, developed from injections of DRAP and
duplex oligonucleotide possessing 5’ single stranded exten-
sions and having among the lowest normalized AGMs, were
bred. Three independent litters yielded a total of 22 healthy F2
piglets in one breeding cycle. Cultured ear notch fibroblasts of
the F2 piglets were used to quantify FITC-IB4 staining of
alpha 1,3 galactosyl epitopes. Furthermore, new fibroblast
cultures were established from each of the F1 pigs to examine
the stability of the reduced FITC-IB4 staining phenotype ob-
served in the neonatal period. The reduction in FITC-IB4
staining is stable over the course of more than one year (not
shown) and the reduced expression of alpha 1,3 galactosyl
epitopes is heritable (Fig. 1).

A majority of the F1 piglet cell lines had a normalized
AGM less than half of the mean value for the positive control
line. That is, less than what might be expected from the com-
plete ablation of one active allele. This might occur if the
aneles were compound heterozygotes with the activity of
the gene products from each allele being diminished relative
to the activity of the wild-type allele. Heterozygososity was con-
firmed by sequencing.

A 1.2 Kbp genomic fragment, corresponding to the

### TABLE 1. Genomic modifications in pigs

<table>
<thead>
<tr>
<th>DNA oligonucleotides</th>
<th>5’ Coding 1</th>
<th>3’ Noncoding 1</th>
<th>5’ Coding 2</th>
<th>3’ Noncoding 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(5’-ATACATTGAGCATTAATTGGAGAGTTCTTA-3’)</td>
<td>(5’-TATATGATAATCCCATGATTTCTGGGGAT-3’)</td>
<td>(5’-TCCTGGCCCATCCAGCCTAGGTCGACCT-3’)</td>
<td>(5’-GCAGAAGAGAGTGCTCCACCTAGTGCTGGATG-3’)</td>
</tr>
</tbody>
</table>

This table summarizes the success of using DRAP and oligonucleotide-directed mutagenesis in the pig. DRAP injections had no untoward effects on litter size or phenotypic features in the pigs. The coinjection procedure yielded animals with efficiencies comparable to standard IVF. DRAP plus oligonucleotide injections were successful in increasing the amount of GGTA1-dependent alpha 1,3 galactosyl residues on porcine fibroblasts as determined by quantitative and specific binding of FITC-labeled isoelecin B4 (FITC-IB4) to these epitopes during flow cytometry. The normalized AGM was significantly reduced for six of seven oligonucleotides tested.

Semen for in vitro fertilization (IVF) was obtained from three Yorkshire males. Oocytes were collected from ovaries of undefined commercial breeds and matured in vitro. Approximately 50 zygotes 21 to 22 hr after insemination were visualized for the presence of two pronuclei whereupon DRAP and DNA were injected into the male pronucleus. Embryos at various stages of development were surgically transferred into uteri of asynchronous recipients.Recipient females (parity 0 or 1) were selected that exhibited first standing estrus 24 hr prior to oocyte insemination. Embryos that were cultured less than 48 hrs (1–2 cell stage) were placed in the ampullar region of the oviduct. Embryos cultured 48 hr or more (≥4 cell stage) were placed in the tip of the uterine horn. Typically, 30–70 injected embryos were placed in the oviduct. The pronuclear injection of DRAP and oligonucleotides showed no

### TABLE 2. Modifications in porcine GGTA1 activity

<table>
<thead>
<tr>
<th>Step</th>
<th>Coding 1</th>
<th>Coding 2</th>
<th>Coding 1 + Noncoding 1</th>
<th>Coding 2 + Noncoding 1</th>
<th>Coding 2 + Noncoding 2 (duplex)</th>
<th>Noncoding 2</th>
<th>Noncoding 1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo transfers</td>
<td>9</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>8</td>
<td>1</td>
<td>9</td>
<td>30</td>
</tr>
<tr>
<td>Pregnancies</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Deliveries</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Live born piglets</td>
<td>9</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>36</td>
<td>13</td>
<td>23</td>
<td>96</td>
</tr>
<tr>
<td>Fractional</td>
<td>0.827</td>
<td>0.668</td>
<td>0.538</td>
<td>0.654</td>
<td>0.669</td>
<td>0.775</td>
<td>0.870</td>
<td></td>
</tr>
<tr>
<td>IB4</td>
<td>0.151</td>
<td>0.093</td>
<td>0.106</td>
<td>0.116</td>
<td>0.226</td>
<td>0.086</td>
<td>0.279</td>
<td></td>
</tr>
<tr>
<td>Staining (mean SD</td>
<td>0.024</td>
<td>0.003</td>
<td>1E-05</td>
<td>1E-04</td>
<td>0.003</td>
<td>0.75</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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GGTA1 coding portion of exon 9 (694 bp) along with several hundred bp of flanking sequence, was amplified by high-fidelity polymerase chain reaction (PCR) and cloned from 25 cell lines having the most reduced FITC-IB4 staining as well as the reference control cell line. This analysis identified several single nucleotide polymorphisms (cSNPs) in the GGTA1 sequence as well as frequent and consistent nucleotide substitutions in the experimental lines.

Mutations occurred downstream from the 3’ end(s) of the mutagenic oligonucleotide(s). The different oligonucleotides produced very similar mutations within the gene and there appear to be preferred sequences for mutagenesis. The most prevalent mutation occurred at AGA/TCT followed by GTG/CAC triplets—although mutations did not occur at every such triplet—and other trinucleotides were modified (Fig. 1).

Biallelic mutations occur. This is illustrated in the sequence data from pig 676—the animal with the most reduced FITC-IB4 staining—generated from an injection of DRAP and a duplex oligonucleotide. Each allele shows unique substitutions as well as three common, defined, substitutions and a fourth, indeterminant nucleotide (n). The latter is likely to be the common C→T cSNP that was identified. There are several silent mutations as well as mutations leading to non-conservative amino acid substitutions (Fig. 2).

DRAP is encoded by a variant (T312G; ins603G) of a cDNA identified subsequently during the Drosophila genome project (NM_168011) having both strand transferase and topoisomerase activity. DRAP contains phosphorylserine binding and pleckstrin homology domains associated with protein-protein interactions and/or signal transduction (12). Neither domain predicts a role in homology-dependent strand transfer. However, DRAP has residues, appropriately spaced, consistent with the Asp-Asp-Glu (DDE) catalytic domain found in various site-specific recombinases and viral integrases (13).

We hypothesized that if DRAP and a mutant oligonucleotide were introduced into a cell nucleus, homology-dependent strand transfer would occur (7, 14). The DNA intermediate would need to be resolved, possibly by the topoisomerase activity inherent in DRAP (8), and result in a recombinogenic DNA strand break (15). Subsequent modifications in the gene could be brought about by the actions of endogenous DNA repair enzymes. No evidence of general genotoxicity such as altered fertility, cancer or anatomic abnormalities in F1 or F2 animals was seen.

The predominant mutations induced by DRAP and oligonucleotides in pigs are G/C→A/T transitions and T/A→G/C transversions. This bears a striking similarity to the single nucleotide mutations that arise during the somatic hypermutation phases of immunoglobulin gene maturation and cellular defense against retroviral infection (16, 17).

Mutations in AGA or GTG triplets define a repertoire of 36 possible codon changes that can potentially induce 17

**FIGURE 1.** Phenotypic and genotypic changes. (A) Fibroblast cell lines were established from ear notches taken from each piglet. The amount of alpha 1,3 galactosyl sugar found on the cell surface was measured by flow cytometry using FITC-labeled isolectin B4. A fibroblast cell line derived from a Yorkshire male pig served as a positive control and a human cell line (SK-N-DZ) served as a negative control for each experiment. The raw AGMs were normalized to the value of the positive control line (1.0) for each experiment. The distribution of normalized AGMs is displayed for each replicate determination from the two control lines used throughout the study, the 96 individual experimental lines (F1 generation) and the 22 F2 generation piglets from three independent litters. (B, C) Single nucleotide changes occur predominantly at the middle nucleotide within two specific trinucleotide sequences: AGA/TCT or GTG/ACA. This is quite similar to the final maturation step in immunoglobulin genes known as somatic hypermutation.
silent, 3 conservative, and 16 nonconservative substitutions involving numerous amino acids. The extent to which the changes affect total gene product activity in a cell depend upon the specific modifications in each allele and the degree to which each is expressed. We anticipate that ongoing studies will lead to a more complete understanding of this mutagenic mechanism and refinements in the method, allowing for more precise mutagenesis or homologous recombination.

At present, the DRAP method offers two advantages over approaches producing a null allele—that of speed and the ability to create gene products with variable activity. Furthermore, as there are no selection steps it should be possible to modify several genes simultaneously or in succession. This will be important in the production of pigs for xenotransplantation.

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REFERENCES