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# Production of $\alpha$ 1,3-Galactosyltransferase- Deficient Pigs

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The enzyme  $\alpha$ 1,3-galactosyltransferase ( $\alpha$ 1,3GT or GGTA1) synthesizes  $\alpha$ 1,3-galactose ( $\alpha$ 1,3Gal) epitopes (Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-R), which are the major xenoantigens causing hyperacute rejection in pig-to-human xenotransplantation. Complete removal of  $\alpha$ 1,3Gal from pig organs is the critical step toward the success of xenotransplantation. We reported earlier the targeted disruption of one allele of the  $\alpha$ 1,3GT gene in cloned pigs. A selection procedure based on a bacterial toxin was used to select for cells in which the second allele of the gene was knocked out. Sequencing analysis demonstrated that knockout of the second allele of the  $\alpha$ 1,3GT gene was caused by a T-to-G single point mutation at the second base of exon 9, which resulted in inactivation of the  $\alpha$ 1,3GT protein. Four healthy  $\alpha$ 1,3GT double-knockout female piglets were produced by three consecutive rounds of cloning. The piglets carrying a point mutation in the  $\alpha$ 1,3GT gene hold significant value, as they would allow production of  $\alpha$ 1,3Gal-deficient pigs free of antibiotic-resistance genes and thus have the potential to make a safer product for human use.

The enzyme  $\alpha$ 1,3-galactosyltransferase ( $\alpha$ 1,3GT or GGTA1) synthesizes  $\alpha$ 1,3Gal epitopes (Gal $\alpha$ 1,3Gal $\beta$ 1,4GlcNAc-R) on the cell surface of almost all mammals with the exception of humans, apes, and Old World monkeys (1).  $\alpha$ 1,3Gal epitopes are the major xenoantigens causing hyperacute rejection (HAR) in pig-to-human xenotransplantation (2–4). Many reports have also indicated that

$\alpha$ 1,3Gal epitopes are involved in acute vascular rejection (AVR) of xenografts (4–6). Piglets with  $\alpha$ 1,3GT heterozygous knockout have been cloned by our group (7) and another team (8) in the last year. To produce homozygous  $\alpha$ 1,3GT knockout piglets by natural breeding, assuming both male and female heterozygous knockout pigs are available at the same time and are fertile, is feasible but takes up to 12 months. However, by using a second-round knockout and cloning strategy, we could save up to 6 months and all cloned piglets would be  $\alpha$ 1,3GT double knockout (DKO). We have selected and enriched for  $\alpha$ 1,3GT DKO cells by using a bacterial toxin, toxin A from *Clostridium difficile*, which binds with high affinity to  $\alpha$ 1,3Gal epitopes and produces a cytotoxic effect on cells that are  $\alpha$ 1,3Gal-positive (9). Toxin A uses  $\alpha$ 1,3Gal epitopes as a cell

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surface receptor and causes "rounding" and lifting of the  $\alpha$ 1,3Gal-positive cells from the surface of the growth vessel (10, 11).

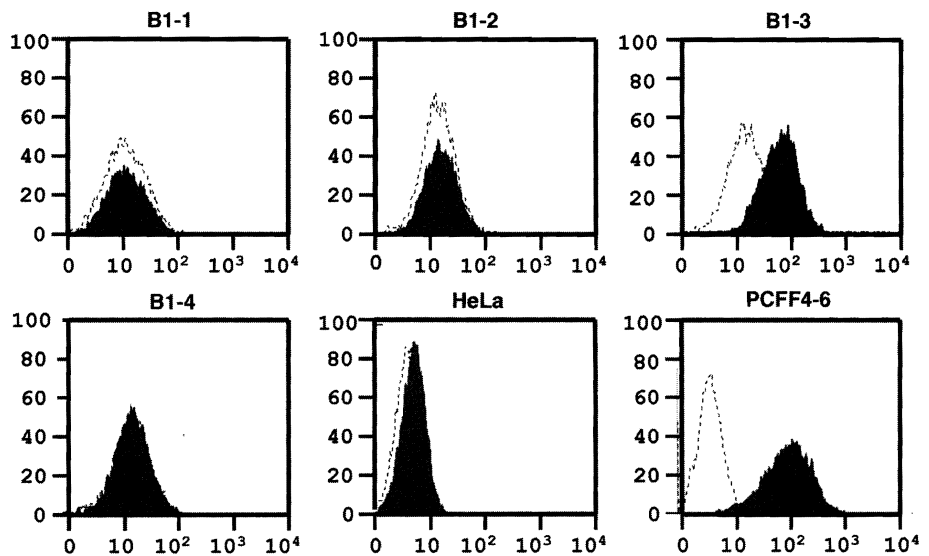
Heterozygous  $\alpha$ 1,3GT knockout fetal fibroblasts, 657A-I11 1-6 cells, were isolated from a day-32 pregnancy as described in (7). To avoid using a second antibiotic-resistance gene as a selection marker, we constructed an ATG (start codon)-targeting  $\alpha$ 1,3GT knockout vector, pPL680 (12), which also contains a *neo* gene, to knock out the second allele of the  $\alpha$ 1,3GT gene. 657A-I11 1-6 cells were transfected by electroporation with pPL680 and selected for the  $\alpha$ 1,3Gal-negative phenotype with purified *C. difficile* toxin A (13). One colony (680B1) was isolated and expanded after toxin A selection. When the 680B1 cells were stained with a fluorescein-labeled  $\alpha$ 1,3Gal-specific lectin, GS-IB4, about 80% of the cells were found to be  $\alpha$ 1,3Gal-negative. The fact that fewer than 100% of the cells in the colony were negative with GS-IB4 staining indicated that this colony contained a mixture of  $\alpha$ 1,3Gal-negative and -positive cells. We used 680B1 cells for somatic cell nuclear transfer (cloning) as described in (7). We transferred embryos to five recipient gilts, and three initial pregnancies were established, of which only one went beyond day 35 of gestation.

To determine whether all the fetuses cloned from 680B1 cells were  $\alpha$ 1,3GT DKO, we terminated the remaining pregnancy at day 39 and recovered four normal-sized fetuses. Fibroblast cell lines (680B1-1 to B1-4) were isolated from each of these four fetuses, and fluorescence-activated cell sorting (FACS) analysis with GS-IB4 staining showed that B1-1, B1-2, and B1-4 cells were  $\alpha$ 1,3Gal-negative, whereas B1-3 cells were positive for  $\alpha$ 1,3Gal (Fig. 1). Normal human serum (NHS) contains preformed antibodies to  $\alpha$ 1,3Gal and complement proteins, which together cause rapid lysis of cells that are  $\alpha$ 1,3Gal-positive. A complement lysis assay on these cells showed that B1-1, B1-2, and B1-4 cells were resistant to lysis by NHS, but B1-3 cells were lysed by NHS at the same rate (about 40% of cells lysed) as control wild-type pig cells (Fig. 2). Analysis of genomic DNA from these fetal cells by polymerase chain reaction (PCR) and Southern blot analysis indicated that none of the three  $\alpha$ 1,3Gal-negative cell lines had the expected restriction fragment pattern predicted for targeted disruption of the second  $\alpha$ 1,3GT allele with the pPL680 knockout vector. Instead, these fetal cells appeared to have the same allele pattern (one targeted allele and one wild-type allele) as their parent 657A-I11 1-6 cells, which contained only one disrupted  $\alpha$ 1,3GT allele. Northern blot analysis of the four cell lines (B1-1 to B1-4) showed that they expressed two mRNAs of similar size to those seen in the 657A-I11 1-6 cells (fig. S1).

The 3.8-kb band corresponds in size to the wild-type  $\alpha$ 1,3GT transcript and the shorter 2.5-kb band is the same size expected for the truncated transcript of the first knockout allele (fig. S1). Because of the nature of the toxin A selection method,  $\alpha$ 1,3Gal-negative cells are selected, regardless of whether inactivation of the second  $\alpha$ 1,3GT allele was caused by targeted disruption via the pPL680 vector or by any other mechanism. The fact that one normal-sized allele was observed (instead of two shorter knockout alleles) in-

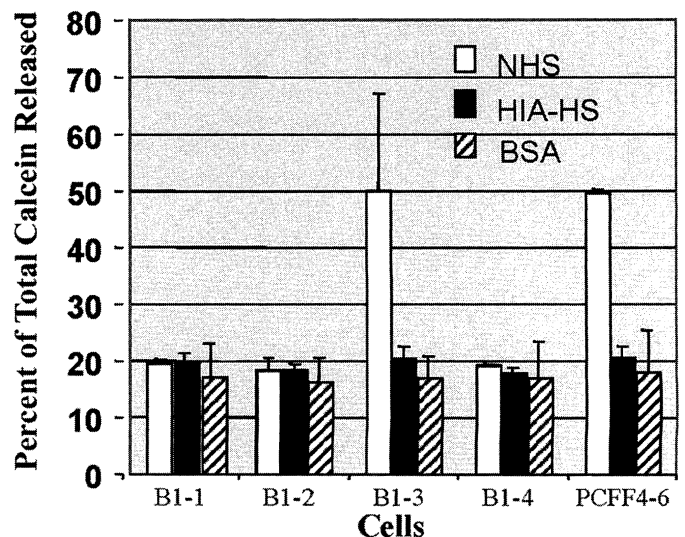
dicated that knockout of the second  $\alpha$ 1,3GT allele was due to mechanisms other than targeted homologous recombination-mediated disruption, promoter dysfunction, or mRNA missplicing and instability.

To identify the nature of the inactivation event for the second allele, we subcloned and sequenced  $\alpha$ 1,3GT cDNAs from all four cell lines (B1-1, B1-2, B1-3, and B1-4). Sequencing results revealed that there was a T-to-G transversion at the second base pair of exon 9 in the nontargeted  $\alpha$ 1,3GT allele of B1-1,



**Fig. 1.** Flow cytometry analysis of 680B1-1 to B1-4 cells with GS-IB4 lectin staining. Horizontal and vertical axes denote intensity of fluorescence and number of events, respectively. Dotted line represents unstained cells analyzed by a fluorescence-activated cell sorter (Becton-Dickenson, Franklin, NJ). Shadow represents cells stained with fluorescein isothiocyanate-labeled GS-IB4 lectin (EY Laboratories, Inc., San Mateo, CA). B1-1, B1-2, B1-3, and B1-4 are fetal fibroblasts derived from four day-39 fetuses. HeLa cells, a human cell line, were used as the negative control and PCFF4-6 cells, which were the parent cells for heterozygous and DKO of the  $\alpha$ 1,3GT gene, were used as the positive control.

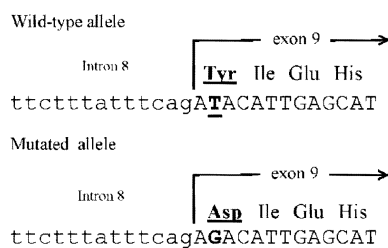
**Fig. 2.** Complement lysis assay for DKO fetal fibroblasts and wild-type pig cells. Results are the average of three individual assays. Open box represents NHS. Solid box represents heat-inactivated human serum (HIA-HS) as the negative control, and hatched box represents bovine serum albumin (BSA) as the reagent control. About 20% calcein release (no cells lysed) is the base value for the negative control (HIA-HS) and the reagent control (BSA). For quality control and reproducibility purposes, we did not use fresh human serum for the assay, which usually gives about 90% calcein release. About 50% calcein release (about 40% of cells lysed) is typical with commercial serum (frozen and lyophilized) from Sigma.



B1-2, and B1-4 cells, but not in B1-3 cells or in the first knockout allele of all four cell lines. This T-to-G transversion in the  $\alpha 1,3\text{GT}$  coding region caused a single amino acid change from tyrosine to aspartic acid in the  $\alpha 1,3\text{GT}$  protein (Fig. 3). Although this mutation has not been observed in the inactivated  $\alpha 1,3\text{GT}$  gene of humans or higher primates (14), it is likely that the change of tyrosine, a hydrophobic amino acid, to aspartic acid, a hydrophilic amino acid, could disrupt  $\alpha 1,3\text{GT}$  function. Crystal structure analysis of bovine  $\alpha 1,3\text{GT}$  protein supports this speculation and shows that this tyrosine is at the center of the catalytic domain of bovine  $\alpha 1,3\text{GT}$  protein and is involved in uridine 5'-diphosphate-Gal binding (15, 16).

To further confirm that the mutated cDNA cannot make functional  $\alpha 1,3\text{GT}$  protein, we cloned  $\alpha 1,3\text{GT}$  cDNAs from the nontargeted allele of B1-1 to B1-4 cells and wild-type pig cells into an expression vector and transfected them into human HeLa cells, which normally do not express  $\alpha 1,3\text{GT}$  protein. HeLa cells transfected with cDNA expression vectors from B1-1, B1-2, and B1-4 cells were negative for GS-IB4 lectin staining, indicating that the transfected pig cDNA from these cells did not make functional  $\alpha 1,3\text{GT}$  protein. In contrast, HeLa cells transfected with the cDNA from B1-3 cells and wild-type pig cells were positive with GS-IB4 staining. These results verified that the point mutation in cDNA from the second allele of the  $\alpha 1,3\text{GT}$  gene in B1-1, B1-2, and B1-4 cells resulted in synthesis of a defective  $\alpha 1,3\text{GT}$  protein. Although toxin A selection was repeated several times on 657A-111 1-6 cells, with or without pPL680 vector transfection, no additional toxin A-resistant colonies were detected.

We performed somatic cell nuclear trans-



**Fig. 3.** Sequencing analysis of the  $\alpha 1,3\text{GT}$  gene from wild-type pig cells and DKO porcine fetal fibroblasts. Upper and lower alignment show nucleotide sequence of the  $\alpha 1,3\text{GT}$  intron 8–exon 9 boundary from wild-type pigs and the second allele of the DKO pig fetuses (B1-1, B1-2, and B1-4), respectively. Small letters and capital letters denote intron and exon sequences, respectively. Underlined capital letters indicate the nucleotide where the point mutation occurred. Amino acids deduced from the correspondent mutated and wild-type DNA sequence are underlined. No other mutations were found in the coding region of the  $\alpha 1,3\text{GT}$  gene from the second allele of the DKO pig fetuses in our genomic and reverse transcriptase-PCR libraries.

fer (cloning) with all three DKO cell lines as described in (7). We transferred cloned embryos into 16 estrus-synchronized recipient gilts. Ten initial pregnancies were established, only two of which went to term. Two pregnancies were lost before day 30, five were lost between day 30 and day 40, and one was lost by day 60. The first five female  $\alpha 1,3\text{GT}$  DKO piglets (761-1 to 761-5), cloned from 680B1-2 cells, were born on 25 July 2002. One piglet (761-1) died shortly after birth, and necropsy revealed an enlarged tongue and unusually large kidneys. We have observed this phenotype in a few other  $\alpha 1,3\text{Gal}$ -positive cloned pigs, and it appears to be a function of the cloning process (incomplete reprogramming) and not the  $\alpha 1,3\text{GT}$  gene knockout per se. The other four DKO piglets were of normal size and healthy. Aorta endothelial cells and muscle and tail fibroblasts isolated from the dead piglet (761-1) were negative with GS-IB4 lectin staining. FACS analysis of muscle fibroblasts from piglet 761-1 also showed a negative result for GS-IB4 binding. Neonatal tail fibroblasts isolated from the four healthy piglets, when analyzed by FACS with GS-IB4, were all negative (fig. S2). Tissue sections of liver, kidney, spleen, skin, intestine, muscle, brain, heart, pancreas, lung, aorta, tongue, umbilicus, and tail obtained from piglet 761-1 were all negative with GS-IB4 staining, indicating a complete lack of detectable cell surface  $\alpha 1,3\text{Gal}$  epitopes. The GS-IB4 staining results for liver sections from a newborn wild-type piglet and from piglet 761-1 are shown in fig. S3. Southern blot and sequencing analysis of DNA samples from all five piglets confirmed the targeted disruption of the first allele of the  $\alpha 1,3\text{GT}$  gene and the T-to-G point mutation in the second base of exon 9 in the second allele of the  $\alpha 1,3\text{GT}$  gene. It has been reported that  $\alpha 1,3\text{GT}$  DKO mice developed cataracts at 4 to 6 weeks of age (17). Physical examination of the four piglets at 7 weeks of age did not reveal any abnormalities or cataracts. We will continue to monitor the piglets for the presence of cataracts.

We performed an *in vivo* immunogenicity test with  $\alpha 1,3\text{GT}$ -knockout mice. We injected islet-like cell clusters (ICCs) isolated from the pancreas of piglet 761-1 intraperitoneally into  $\alpha 1,3\text{GT}$  knockout mice. We used ICCs from a neonatal wild-type piglet as a control. As shown in fig. S4, no increase in the titer of immunoglobulin M (IgM) to  $\alpha 1,3\text{Gal}$  was observed in  $\alpha 1,3\text{GT}$  knockout mice after injection with ICCs from the  $\alpha 1,3\text{GT}$  DKO piglet, in contrast to significant IgM titer increases observed in those mice injected with wild-type piglet ICCs. This result clearly demonstrates that the DKO piglet cells do not make any  $\alpha 1,3\text{Gal}$  epitopes.

Thus, we have successfully produced four  $\alpha 1,3\text{GT}$ -deficient piglets by a toxin A–medi-

ated selection method. Although our intent was to knock out the second allele of the  $\alpha 1,3\text{GT}$  gene by homologous recombination, this did not occur. Instead, because we used this powerful selection method, which allows us to isolate any event that results in loss of  $\alpha 1,3\text{GT}$  activity, we discovered a mutation in the second allele of the  $\alpha 1,3\text{GT}$  gene. Had we used standard selection methods with puromycin or hygromycin, we would not have found the mutation. Although the rate of spontaneous mutation in the pig genome is very low [about  $4 \times 10^{-8}$  for a spontaneous mutation per replication (18) in a mammalian gene similar in size to the  $\alpha 1,3\text{GT}$  gene], toxin A selection still enabled us to detect this crucial mutation. Clearly inactivation of the  $\alpha 1,3\text{GT}$  protein by this point mutation is a better outcome than by gene targeting with the pPL680 vector. It provides the opportunity to produce  $\alpha 1,3\text{GT}$ -deficient pigs without any antibiotic-resistance genes or other foreign DNA sequences, which should facilitate regulatory approval and, potentially, make a safer product for human use. It is certain that this point mutation will be maintained in the genome of these DKO pigs and their offspring, just as the few critical point mutations in the  $\alpha 1,3\text{GT}$  gene of humans and higher primates have been maintained over 20 million years (14). This genomic stability is not only due to the rarity of a reverse mutation event [about  $5 \times 10^{-11}$  per replication (18) for mammals] but, more importantly, the strong selection pressure against  $\alpha 1,3\text{Gal}$ -positive cells by the presence of antibodies to  $\alpha 1,3\text{Gal}$  in  $\alpha 1,3\text{Gal}$ -negative animals. Our results have demonstrated that removal of  $\alpha 1,3\text{Gal}$  epitopes on pig cells did not preclude development *in utero*, even though pig cells express up to 500 times the number of  $\alpha 1,3\text{Gal}$  epitopes as do mouse cells (4, 19). In addition, three consecutive rounds of cloning with rederived fetal cells did not appear to have a major detrimental effect on the overall development or health of the cloned pigs in this study. Analysis of tissues and organs from these  $\alpha 1,3\text{GT}$  DKO pigs in nonhuman primate models should provide clear indications of the involvement of  $\alpha 1,3\text{Gal}$  in HAR, AVR, and chronic rejection.

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- pPL680 was made from three parts: a 1.8-kb PCR-

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- generated fragment of  $\alpha 1,3GT$  intron 2 to exon 4 ended at the start codon ATG as the 5' arm; Neo/pA coding region fused into the ATG of the 5' arm in-frame; a 9.6-kb PCR-generated fragment of  $\alpha 1,3GT$  exon 4 to exon 7 as the 3' arm.
13. 657A-111 1-6 cells were electroporated with pPL680 and cultured for 5 days. Cells were exposed to *C. difficile* toxin A (2  $\mu\text{g}/\text{ml}$ ) (Techlab, Blacksburg, VA) in growth medium for 2 hours. Toxin A medium was then replaced with fresh growth medium. Medium was changed 7 and 11 days posttransfection to remove the detached cells. One colony (680B1) was harvested 13 days posttransfection for expansion and cryopreservation.
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### Supporting Online Material

[www.sciencemag.org/cgi/content/full/1078942/DC1](http://www.sciencemag.org/cgi/content/full/1078942/DC1)

Materials and Methods

Figs. S1 to S4

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