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# Oocyte Gal$\alpha$1,3Gal Epitopes Implicated in Sperm Adhesion to the Zona Pellucida Glycoprotein ZP3 Are Not Required for Fertilization in the Mouse*

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**The Gal$\alpha$1→3Gal structure is displayed on the zona pellucida glycoprotein ZP3 on murine oocytes. This trisaccharide has been implicated in sperm-zona pellucida adhesive events thought to be essential to fertilization in the mouse. To determine directly if this molecule is required for fertilization, we have generated mice that are deficient in a gene ($\alpha$1,3GT) encoding the UDP-Gal:$\beta$-D-Gal-$\alpha$1→3Gal-galactosyltransferase enzyme responsible for Gal$\alpha$1→3Gal synthesis and expression. These mice develop normally and exhibit no gross phenotypic abnormalities. The Gal$\alpha$1→3Gal epitope is absent from the vascular endothelium and other tissues in $\alpha$1,3GT (−/−) adult mice. By contrast, $\alpha$1,3GT (−/−) mice, like humans, develop naturally occurring anti-$\alpha$-galactoside antibodies normally absent in wild type mice. Female $\alpha$1,3GT (−/−) mice yield oocytes that are devoid of the Gal$\alpha$1→3Gal epitope; however, these mice are fully fertile. These observations indicate that the Gal$\alpha$1→3Gal moiety is not essential to sperm-oocyte interactions leading to fertilization or to essentially normal development. They further suggest that $\alpha$1,3GT (−/−) mice will find utility for exploring approaches to diminish anti-Gal-dependent hyperacute xenograft rejection, which presents a major barrier to the use of porcine and other non-primate organs for xenotransplantation in humans.**

Fertilization in mammals involves an adhesive interaction between sperm and the zona pellucida, a glycoprotein-containing shell that surrounds the oocyte. Sperm receptor activity of the murine oocyte resides in the zona pellucida glycoprotein ZP3 (1). Sperm recognition of murine ZP3 depends upon *O*-linked oligosaccharides displayed by ZP3 (Ref. 2; reviewed in Refs. 3–5). Treatment of purified egg ZP3 and ZP3-derived *O*-linked oligosaccharides with $\alpha$-galactosidase eliminates sperm receptor activity (6). These observations have been taken to imply that terminal $\alpha$-galactosides on ZP3 glycoconjugates are critical for sperm binding activity (6). This notion is supported by more recent observations demonstrating that structurally defined bi- and tetraantennary blood group I-related oligosaccharides containing terminal Gal$\alpha$1→3Gal moieties inhibit binding of sperm to eggs in a dose-dependent manner (7).

In the mouse, at least one UDP-Gal:$\beta$-D-Gal-$\alpha$1→3Gal-galactosyltransferase ($\alpha$1,3GT)[1] is responsible for the synthesis of terminal Gal$\alpha$1→3Gal$\beta$1→4GlcNAc trisaccharides from common lactosamine-terminated glycoconjugates (8, 9). Mice and other placental mammals express the Gal$\alpha$1→3Gal$\beta$1→4GlcNAc trisaccharide products of $\alpha$1,3GT on a variety of glycoproteins and in a variety of tissues (10, 11). Aside from the postulated role of Gal$\alpha$1→3Gal moiety in murine fertilization, the function(s) of this structure are not known.

By contrast, humans, apes, and Old World monkeys lack the ability to synthesize these oligosaccharide moieties, because the genetic homologues of the murine $\alpha$1,3GT locus are pseudogenes incapable of encoding a functional $\alpha$1,3GT (12, 13). Consequently, these latter species are reciprocally replete with immunoglobulins of all classes directed against terminal Gal$\alpha$1→3Gal epitopes (14, 15). These antibodies are presumed to arise through immunization by environmental antigens similar or identical to the Gal$\alpha$1→3Gal epitope (16). In humans these natural antibodies (termed anti-Gal) comprise approximately 1% of circulating IgG, as well as a significant fraction of circulating IgM class antibodies (14, 17). Anti-Gal antibodies are clinically important in the context of the proposed use of porcine and other non-primate mammalian organs to circumvent the shortage of human organs for transplantation purposes (reviewed in Refs. 18 and 19). Anti-Gal antibodies serve to initiate hyperacute rejection of xenografts derived from such mammalian species, via complement-mediated cytolytic events involving terminal Gal$\alpha$1→3Gal$\beta$1→4GlcNAc glycoconjugates expressed by the vascular endothelium of the xenotransplant (20, 21).

To directly address the role of Gal$\alpha$1→3Gal containing oligosaccharides in fertilization in the mouse, we have used a gene disruption approach in embryonic stem cells (22) to generate mice homozygous for a null $\alpha$1,3GT allele. $\alpha$1,3GT (−/−) mice are deficient in the expression of Gal$\alpha$1→3Gal epitopes on oocytes but are as fertile as their wild type litter mates, indicating that Gal$\alpha$1→3Gal epitopes are not essential to sperm-oocyte binding in this species. As with humans, apes, and Old World monkeys, $\alpha$1,3GT (−/−) mice maintain naturally occurring anti-Gal antibodies but are deficient in the expression of Gal$\alpha$1→3Gal epitopes on vascular endothelium and other tissues. These observations imply that the inactivated $\alpha$1,3GT gene represents the only functional murine $\alpha$1,3GT locus, and they suggest that the $\alpha$1,3GT (−/−) mouse may prove useful as a small animal model for studying approaches that can diminish anti-Gal-dependent hyperacute organ transplant rejection.

## EXPERIMENTAL PROCEDURES

*Generation of $\alpha$1,3GT (−/−) Mice*—A genomic clone of the $\alpha$1,3GT locus was isolated from the 129SV mouse strain and restricted with *Not*I-*Mlu*I, and the resulting 12-kilobase fragment was cloned into pGEM-5 (Stratagene). A neomycin resistance cassette, pgkNeo, was inserted into the *Sal*I site within the catalytic domain, and a 500-base pair *Bst*EII-*Not*I fragment was subsequently removed from a position

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[1] The abbreviations used are: $\alpha$1,3GT, UDP-Gal:$\beta$-D-Gal-$\alpha$1→3Gal-galactosyltransferase; ES, embryonic stem cell(s); BSA, bovine serum albumin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSIB4, *B. simplicifolia* isolectin B4.

~900 base pairs 3′ of the pgkNeo insertion. A thymidine kinase cassette (pgkTK) (23) was then inserted into the targeting vector at a position corresponding to the *Bst*EII site. The Neo insertion disrupts the largest coding exon in the α1,3GT gene (8); fusion of pgkNeo sequences to this exon yields premature termination codons in all three exonic reading frames. D3 embryonic stem cells (ES) (24) were electroporated and selected by standard methods (25). Homologous recombination of the targeting vector with the native allele was detected in individual ES clones by a nested polymerase chain reaction strategy (Fig. 1*a*, *solid arrowheads*). Polymerase chain reaction-positive ES clones were expanded and were subjected to extensive restriction analysis by Southern blotting of genomic DNA. ES clones containing a single, homologously integrated targeted allele were used to generate chimeric mice via blastocyst injection, as described previously (26). Progeny from two ES lines (1G7 and 1F3) were used for subsequent experiments.

*Determination of Mouse Fertility*—Chimeric males resulting from ES cell injections were mated with F1(C57BL/6J × DBA/2J) females, to yield heterozygous (α1,3GT +/−) progeny. The F1(129SV × C57BL/6J × DBA/2J) α1,3GT (+/−) littermates were bred to yield F2 α1,3GT (+/+), (+/−), and (−/−) progeny. F2 α1,3GT (−/−) females were housed with proven fertile wild type males and control wild type females. F2 α1,3GT (−/−) males were housed with F2 α1,3GT (−/−) female litter mates and wild type control females. Litter sizes were determined based on the number of pups found on the day of birth.

*Immunological Assays*—Lungs were removed from α1,3GT (+/+) and α1,3GT (−/−) littermates, fixed in 4% paraformaldehyde, and embedded in paraffin, and 4 μM sections were made. Sections were pretreated with 50 mM $NH_4Cl$ and were then treated either with 2 units/ml green coffee bean α-galactosidase (Sigma) or with buffer alone. Endogenous peroxidase was quenched with 0.3% $H_2O_2$ in methanol. Sections were preblocked with 1% crystalline BSA (Sigma) in PBS, and were then incubated for 2 h at room temperature with 20 μg/ml antigen affinity-purified anti-Gal (22), in binding buffer (1% BSA, PBS, and 0.05% Tween 20; Bio-Rad). Slides were washed with Tween/PBS and were then incubated for 1 h at room temperature in binding buffer containing 1:100 biotinylated anti-human IgG and IgM (Vector), followed by peroxidase ABC staining (Vector). Rabbit erythrocyte agglutination assays were performed as described previously (14), with the exception that sera were also preincubated with 10 mg of either Synsorb H (Fucα1→2Galβ1→4GlcNAc) or Synsorb 115 (Galα1→3Galβ1→4GlcNAc) beads (Chembiomed, Edmonton, Alberta, Canada).

*Detection of α-Galactosides on Oocytes*—Four-week-old female wild type and α1,3GT (−/−) littermates produced by F1 heterozygous crosses were superovulated, and oocytes were isolated from the oviducts in M2 medium supplemented with 300 μg/ml hyaluronidase (Sigma). Oocytes were transferred through serial changes of buffer containing PBS, 0.4% BSA, and were then incubated for 1 h at 37 °C in PBS, 0.4% BSA, supplemented with *Bandeiraea simplicifolia* isolectin B4 (BSIB4)-FITC (10 μg/ml), with or without 20 mM methyl α-galactopyranoside (Sigma). Oocytes were washed, placed in flat-bottomed slide wells, and photographed using confocal microscopy.

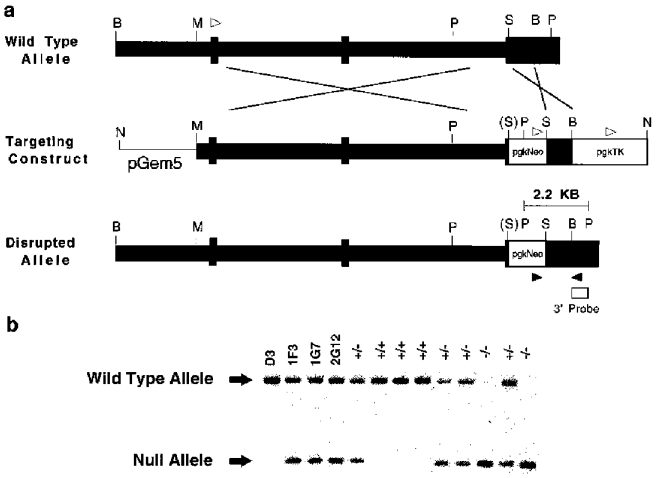


FIG. 1. **Targeted disruption of the murine α1,3GT gene.** *a*, restriction map of the α1,3GT allele, the targeting vector, and the disrupted allele. *Black rectangles* denote α1,3GT locus exons (*thickest portions*) and introns (*thinnest portions*). A neomycin resistance cassette (pgkNeo) was used to disrupt the α1,3GT catalytic domain (found in the largest α1,3GT exon), resulting in a frameshift and premature stop codons relative to both the Neo and α1,3GT translational reading frames. The targeting vector contains 11 kilobases of 5′ genomic DNA and 0.9 kilobases of 3′ sequence flanking the neomycin resistance cassette used to disrupt the α1,3GT catalytic domain exon (largest exon). Restrictions sites are indicated by abbreviations (*B*, *Bst*EII; *N*, *Not*I; *M*, *Mlu*I; *P*, *Pst*I; *S*; *Sal*I). Restriction sites destroyed during vector construction procedures are in *parentheses*. *Open arrows* (▷) denote the transcriptional orientations of the α1,3GT locus, the pgkNeo segment, and the pgkTK segment. *Solid arrows* (▶) indicate positions corresponding to polymerase chain reaction primers used to screen targeted ES lines. The position of the *Bst*EII-*Pst*I segment used in Southern blot analyses described in *b* is indicated below the schematic of the targeted allele (*3′ Probe*). *KB*, kilobase(s). *b*, disruption of the α1,3GT locus. Homologous recombination-mediated replacement of the wild type α1,3GT alleles by the disrupted allele was confirmed by Southern blot analysis. Genomic DNA isolated from the parental ES line, and from three ES clones that gave rise to germ line transmission, is compared to tail genomic DNA from the progeny of F1 heterozygous crosses of littermates. The DNA was digested with *Pst*I and probed with a DNA fragment flanking the 3′ end of the genomic sequence of the targeting vector (3′ probe; see *panel a*).

## RESULTS AND DISCUSSION

A targeted disruption of the murine UDP-Gal:β-D-Galα1→3Gal-galactosyltransferase (α1,3GT) gene in embryonic stem cells (ES) was completed as shown in Fig. 1*a*. F1 heterozygous (α1,3GT (+/−)) littermates were intercrossed to yield viable progeny with genotype frequencies (22% (−/−), 50% (+/−), and 28% (+/+)) corresponding to a Mendelian inheritance pattern, indicating that homozygosity for the null α1,3GT allele is compatible with essentially normal intrauterine development. Mice that are homozygous for the null allele do not differ in size or appearance from their wild type litter mates. The major organs of the α1,3GT (−/−) animals are grossly and histologically normal, as are the levels of a variety of serum analytes. Total and differential blood leukocyte counts, red cell counts, and platelet counts are not significantly different between the α1,3GT (−/−) mice and wild type control mice.

We used human Galα1→3Gal antibodies to confirm that the α1,3GT (−/−) mice are deficient in Galα1→3Gal expression. In humans, at least 1% of the circulating IgG class antibodies, and substantial amounts of circulating IgM class antibodies, are directed against terminal Galα1→3Galβ1→4GlcNAc-containing oligosaccharides (14, 17). By contrast, human tissues are essentially devoid of Galα1→3Gal epitopes, because a functional α1,3GT locus is apparently not present in humans (12, 13). In humans, "naturally occurring" polyclonal antibodies directed against the Galα1→3Gal epitope (27) (termed "anti-Gal" antibodies) are presumed to occur as a consequence of continuous immunization by gastrointestinal flora containing glycoconjugates with terminal α-galactoside structures (16). As noted previously (10), human anti-Gal antibody detects α-galactosidase-susceptible Galα1→3Gal epitopes on a variety of wild type murine cells, including vascular endothelium (Fig. 2, *a* and *b*). By contrast, the vascular endothelium of α1,3GT (−/−) mice is devoid of detectable terminal α-galactosides (Fig. 2*c*). α1,3GT activity, normally present in murine spleen cells, is not detectable in α1,3GT (−/−) splenocytes (data not shown).

The reciprocal relationship between the absence of Galα1→3Gal epitopes and the presence of anti-Gal antibody observed in humans and some other primates (12) is recapitulated in the α1,3GT (−/−) mice. Sera from α1,3GT (−/−) mice directly agglutinate Galα1→3Gal-positive (14, 28) rabbit erythrocytes, whereas sera from α1,3GT (+/+) mice, as expected, do not and thus are devoid of anti-Gal antibody activity. The rabbit erythrocyte hemagglutinating activity present in α1,3GT (−/−) sera can be removed by preincubation of the sera with immobilized synthetic Galα1→3Galβ1→4GlcNAc structures, whereas removal of hemagglutinating activity does not

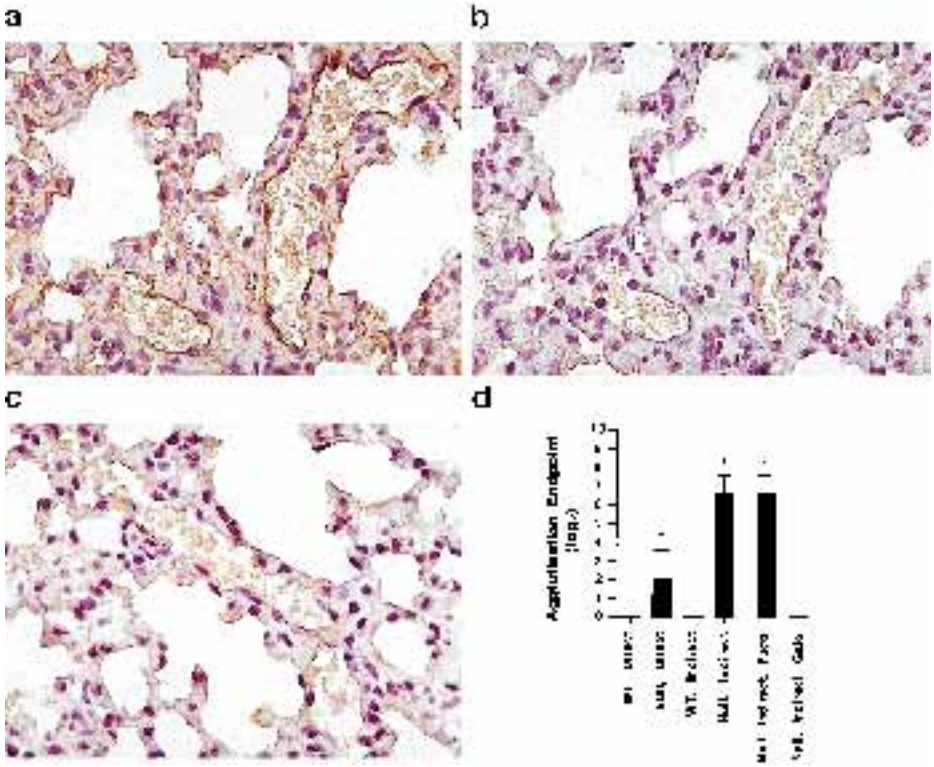


FIG. 2. **α1,3GT (−/−) mice are deficient in vascular endothelial cell Galα1→3Gal epitopes and generate naturally occurring anti-Gal antibodies.** Vessels of the lung were stained with human anti-Gal antibodies. The characteristic strong staining of α1,3GT (+/+) lung vessels is shown in *a*. Pretreatment of sections containing α1,3GT (+/+) lung vessels with α-galactosidase eliminates reactivity (*b*) and, as shown previously (29), confirms the specificity of the anti-Gal antibody for terminal α-galactosides. In contrast to α1,3GT (+/+) lung vessels, the vessels of α1,3GT (−/−) mice do not stain with the anti-Gal antibody (*c*) (original magnification, × 480). *d*, anti-Gal titers in sera α1,3GT (−/−) mice (*Null*) were compared α1,3GT (+/+) (*WT*) mice by direct (*Direct*) hemagglutination of rabbit erythrocytes (4) and indirect agglutination (*Indirect*) using an anti-mouse IgG reagent. Indirect hemagglutination was also completed using α1,3GT (−/−) sera that had been preincubated with the immobilized synthetic trisaccharide Galα1→3Galβ1→4GlcNAc (*Galα*) or Fucα1→2Galβ1→4GlcNAc (*Fucα*) glycoconjugates (*, $p < 0.001$ direct; $p < 0.0001$ indirect).

occur when the sera are preabsorbed with Fucα1→2Galβ1→4GlcNAc structures (Fig. 2*d*). Sera prepared from α1,3GT (−/−) mice contain antibodies that also bind to murine laminin, a glycoprotein containing terminal α-galactosides (29), but do not bind α-galactosidase-treated laminin. By contrast, sera from α1,3GT (+/+) mice do not bind to murine laminin (data not shown). These observations indicate that the α1,3GT (−/−) mice maintain naturally occurring anti-Gal antibodies and indicate that these mice are therefore essentially deficient in the expression of terminal Galα1→3Gal moieties.

Studies *in vitro* indicate that terminal α-galactosides displayed by *O*-linked glycans on the mouse zona pellucida glycoprotein ZP3 are required for the binding of sperm to the oocyte (2–4). These glycoconjugates are easily demonstrated on the zona pellucida of wild type oocytes (Fig. 3*b*), using a lectin (BSIB4) that specifically recognizes these molecules (30). By contrast, oocytes obtained from α1,3GT (−/−) females do not stain with this lectin (Fig. 3*e*). The same result was also observed by staining oocytes with human anti-Gal (data not shown). The loss of the ability to detect oocyte α-galactosides is not due to a blocking effect of maternal anti-Gal immunoglobulins bound to the oocyte, since anti-mouse immunoglobulins did not interact with these oocytes (data not shown). These observations directly demonstrate that the α1,3GT locus determines oocyte expression of terminal α-galactosides.

Table I summarizes breeding studies completed to determine if fertility is affected by absence of zona pellucida terminal α-galactosides consequent to nullizygosity at the α1,3GT locus. In matings between α1,3GT (−/−) females and fertile wild type males of the same genetic background, we observed fertility rates and litter sizes equivalent to those observed in control matings involving α1,3GT (−/+) and α1,3GT (+/+) females. These observations demonstrate that absence of zona pellucida terminal α-galactosides is compatible with normal fecundity and indicate that terminal α-galactosides do not represent an essential component of the mouse oocyte sperm receptor(s). This conclusion leaves open the possibility that Galβ1→4GlcNAc-terminated blood group I-related oligosaccharides capable of blocking sperm-egg binding (7) are instead responsible for sperm-egg adhesion during fertilization. Absence of an essential role for terminal Galα1→3Gal structure in fertilization is also consistent with an alternative hypothesis that murine sperm-egg adhesion during fertilization is accomplished through an interaction between terminal *N*-acetylglucosamine moieties on the oocyte and surface-localized β(1,4)galactosyltransferase on murine spermatids (31).

In humans, naturally occurring anti-Gal antibodies of the type found in the α1,3GT (−/−) mice present a major obstacle to the use of porcine and other non-primate organs for human xenotransplantation. These antibodies bind to terminal α-galactosides on vascular endothelial cells of these mammals (17, 18) and mediate hyperacute xenograft rejection (19, 21) through complement-dependent endothelial cell cytotoxicity. Early attempts to completely block these interactions *in vivo* met with limited success (21). More recent work involving transgene-directed overexpression of complement inhibitors in the xenograft has shown substantial promise as a means to mitigate anti-Gal-dependent hyperacute xenograft rejection (32). Currently, Old World monkeys, which are naturally deficient in the Galα1→3Gal epitope but reciprocally replete with

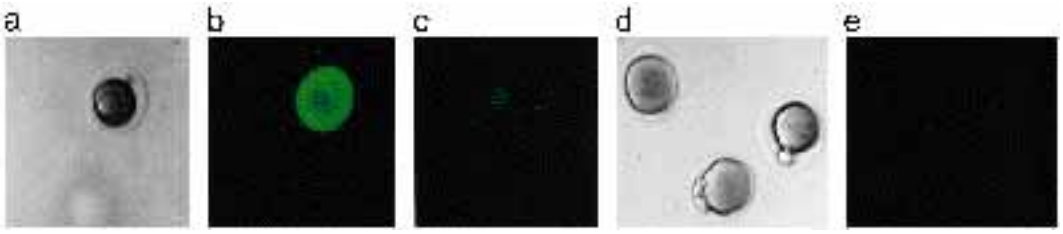


FIG. 3. **Oocytes from α1,3GT (−/−) mice are deficient in Galα1→3Gal epitopes.** Live wild type mouse oocytes (*a–c*) and α1,3GT (−/−) oocytes (*d* and *e*) were stained with fluoresceinated *B. simplicifolia* isolectin B4 (BSIB4-FITC), a terminal α-galactoside-specific lectin (15). *a* and *d* show phase contrast images corresponding to confocal fluorescence images seen in *b* and *e*, respectively. Wild type oocytes (*b*) show strong BSIB4-FITC binding of both the zona pellucida and the oocyte. The specific interaction of BSIB4-FITC with α-galactosides is shown by loss of staining in the presence of methyl α-galactopyranoside (15) (*c*). By contrast, α1,3GT (−/−) oocytes (e) lack detectable BSIB4-FITC binding. Ten oocytes from both α1,3GT (+/+) and α1,3GT (−/−) females were examined in three separate experiments (original magnification, × 100).

TABLE I

*Comparison of the fecundity of α1,3GT null mice with wild type mice*

Chimeras that transmitted the inactive allele were derived from ES lines 1G7 and 1F3. Male chimeras were mated with female F1(C57BI/6J × DBA/2J) mice to yield F1(129SV × C57BI/6J × DBA/2J) offspring. The percentages of different genotypes in heterozygous F1 crosses were 28%, 50%, and 22%, for wild type, heterozygous, and null, respectively. Null crosses were performed with male and female littermates. Wild type crosses with null mice were performed with proven fertile wild type animals of the same F2(129SV × C57BI/6J × DBA/2J) genetic background. Numbers in parentheses indicate the number of pups born.

| Mating | Average litter size |
|---|---|
| Maternal transmission (−/−) × (+/+) | 7 ± 2 (24) |
| Paternal transmission (−/−) × (+/+) | 5 ± 2 (48) |
| Heterozygous cross (+/−) × (+/−) | 7 ± 1 (160) |
| Null cross (−/−) × (−/−) | 5 ± 2 (47) |

circulating anti-Gal antibodies, represent the only available experimental animal recipient for such studies; cost and logistical considerations associated with the care of these large animals can represent a substantial impediment to experimental progress in this area. The α1,3GT (−/−) mice we describe here may represent a useful alternative small animal for this work, since it can be anticipated that the naturally occurring anti-Gal antibodies in an α1,3GT (−/−) murine graft recipient will lead to hyperacute graft rejection of a transplanted organ taken from an α1,3GT (+/+), Galα1→3Gal-positive, but otherwise syngeneic donor mouse. The extensive experience with organ transplants in mice (33), the well defined histocompatibility loci in this species (34), and highly developed systems for murine transgenesis represent additional advantages of this system for the study of anti-Gal-dependent hyperacute organ transplant rejection. Studies are currently in progress to study hyperacute transplant rejection utilizing these α1,3GT (−/−) mice.

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