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Impact of gene duplication of *B4GALNT2* in pigs for xenotransplantation- technical & practical

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Abstract

The *B4GALNT2* gene has become an important target for xenotransplantation because its inactivation reduces the antigenicity of porcine tissues. The growing use of organ-source pig models has led to an increased demand for the rapid creation of these animals. However, the physiological role of this gene in pigs remains poorly understood. In 2015, after generating pigs lacking *B4GALNT2* expression, researchers observed a third allele for this gene. Subsequently, another gene, described as *B4GALNT2-like*, was found in the porcine genome. We have collected data over four years since the production of our first line of xenotransplantation pigs lacking *B4GALNT2* and *B4GALNT2-like* expression. In this study, we were able to show that pig cells expressing *B4GALNT2-like* also reacted to *Dolichos biflorus* Agglutinin (DBA) lectin, which recognizes GalNAc epitopes. Additionally, we demonstrated that *B4GALNT2/B4GALNT2-like* knockout embryos were able to be carried to term in females with the same genotype. We hypothesized that the presence of both genes in the porcine genome might have occurred due to duplication, inversion, and reinsertion of part of the *Phospho1-B4GALNT2* segment. Finally, the pig *B4GALNT2-like gene* showed greater similarity to the human, bovine, and murine *B4GALNT2* genes than the original pig *B4GALNT2*. These data clarify the importance of targeting both *B4GALNT2* and *B4GALNT2-like* for xenotransplantation studies and have improved our knowledge about their genomic structure and role in pig reproduction.

Keywords

pig; B4GALNT2; B4GALNT2-like; duplication; xenotransplantation; reproduction

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Author Contributions

CGL and KDW conceived and designed the experiments. KMW assisted with the methodology, provided husbandry for the pigs, revised and edited the manuscript. MSS assisted with animal tissue collection, coordinate pig breeding and establishment of pig cell lines. BKR contributed to the optimization of the DBA staining protocol, revised and edited the manuscript. KDW and RSP supervised the work, provided the funding, revised and edited the manuscript. CGL wrote the manuscript. All the authors read and approved the final manuscript.

Conflict of Interest

The authors have declared that no conflict of interest exists.

Introduction

The elimination of carbohydrate-based antigens, that can be targeted by natural human antibodies, has been crucial in overcoming the immunological barriers to xenotransplantation. Efforts to eliminate galactose α 1,3-galactose (Gal) in pigs, one of the major molecules triggering hyperacute rejections [1,2,3], resulted in extended graft survival after pig-to-nonhuman primate transplantation, but it was not sufficient to prevent antibody mediated rejection (AMR) [4].

In addition to Gal epitopes synthesized by the α1,3-galactosyltransferase (GGTA1), other xenoantigens have been found relevant for xenotransplantation. The porcine glycan Sd(a), produced by B4GALNT2 (β1,4 *N*-acetylgalactosaminyl transferase 2), has been shown to act as an antigen to both humans and primates [5]. *B4GALNT2* expression has been detected in pig endothelial cells and a wide range of tissues, including heart, liver, kidney, pancreas, lung, and spleen. The porcine *B4GALNT2* gene sequence has been cloned and characterized, revealing 76% and 70% amino acid identity with human and murine *B4GALNT2* genes, respectively [6]

To further investigate cross-species incompatibilities, Estrada et al. 2015 [7] created the first pigs lacking *B4GALNT2*. Interestingly, they identified three alleles for *B4GALNT2*, suggesting possible gene duplication, as the animals exhibited normal karyotypes. Subsequently, the presence of four copies for *B4GALNT2* was reported [8], and later, a gene with high homology to *B4GALNT2*, known as *B4GALNT2-like* (*B4GALNT2L*), was discovered [9]. Since then, *B4GALNT2*-like (LOC110255214 in Sus Scrofa11.1) has been increasingly described in the literature [10,11,12,13,14,15,16], but its transcriptional and translational activity remains poorly understood.

Pigs carrying knockout alleles for *GGTA1*, *B4GALNT2* and *B4GALNT2-like*, and the human transgene *hCD55* (human decay accelerating factor), known as NSRRC:0086 line, have been created in the National Swine Research and Resource Center and phenotypically characterized over a period of 4 years. Here, we investigated whether *B4GALNT2-like* could also be involved in the biosynthesis of the Sd(a) antigen. Additionally, we shared our observations regarding the annotation of the *B4GALNT2* gene and the creation of these animals by breeding instead of cloning. This publication will clarify the importance of targeting both *B4GALNT2* and *B4GALNT2-like* for xenotransplantation studies and provide valuable insights about the *B4GALNT2* gene structure and its impact during reproduction in pigs.

Material and Methods

All animal care and experiments were conducted in accordance with approved protocols and standard operating procedures by the Animal Care and Use Committee of the University of Missouri.

Generation of the NSRRC:0086 line

B4GALNT2 and *B4GALNT2-like* modifications were introduced into a *GGTA1* KO background expressing hCD55 to generate quadruple-modified (4GM) pigs by using the CRISPR/Cas9 system. *GGTA1* KO targeted (Tg)-hCD55 fetal fibroblast cells (2×10^5 cells) were co-transfected via electroporation with gRNA/Cas9 plasmids targeting both *B4GALNT2* and *B4GALNT2-like* genes (gRNA oligonucleotide 1 forward: 5' - CACCGTGAGGATCGACAGACATCTA −3', reverse: 5'- AAACTAGATGTCTGTCGATCCTCAC- 3'; gRNA oligonucleotide 2 forward [7]: 5' CACCGTGTATCGAGGAACACGCTT-3', reverse: 5'-AAACAAGCGTGTTCCTCGATACAC-3'). Two hundred cells were seeded per 100 mm dish for colony formation (~200 cells/plate). Following culture for 10 days, individual colonies were collected for genotyping (1/3 of the cells) and the remaining (2/3) were expanded to be cryopreserved for long-term preservation [17].

Primers were designed to amplify homologous regions between *B4GALNT2* and *B4GALNT2-like*. These assays encompassed unique nucleotide signatures, allowing us to identify all four alleles. PCR amplicons were cloned and Sanger sequenced, and colonies showing bi-allelic modifications for *B4GALNT2* and *B4GALNT2-like* were used for somatic cell nuclear transfer (SCNT) [18]. Six embryo transfer were performed, resulting in two pregnant recipient gilts that gave rise to female and male cloned founders (NSRRC:0086 line). For phenotypic analysis, liver and kidney sections from NSRRC:0086 and a wild-type (WT) pig were stained with isolectin B4 (IB4)-FITC (Sigma-Aldrich), which binds to Gal residues, and rhodamine labeled *Dolichos biflorus* agglutinin (DBA) (Vector Laboratories), which recognizes GalNAc epitopes as presented in the Sd(a) glycan. PCR diagnostic assays to confirm *GGTA1* homozygous deletion and *hCD55* integration were performed (data not shown). Additionally, we isolated primary porcine kidney (PKC) cells as previously described [19] from two of the euthanized NSRRC:0086 piglets and from a WT piglet to confirm the results observed in the *ex vivo* experiment.

A breeding colony was established by crossing NSRRC:0086 founder (F0) clones with *B4GALNT2/B4GALNT2-like* KO pigs (R614 line, produced via zygote injection) and non-cloned *GGTA1 KO/hCD55* pigs. Genotyping and phenotyping confirmed successful transmission of genetic modifications to F1 progeny. To generate *GGTA1/B4GALNT2/B4GALNT2-like* KO piglets, F1 individuals were bred with each other and with F0 founders. Subsequently, F2 pigs with the desired modifications were bred with WT pigs to enhance genetic diversity.

Plasmid construction

Plasmids expressing the most common transcript of *B4GALNT2* (Accession No: NM_001244330) or *B4GALNT2-like* (Accession No: XM_021067117.1) were generated commercially (GenScript) by inserting synthesized coding sequence between the XhoI/XbaI restriction sites of pcDNA3.1/Hygro (+) (GenScript, SC1317).

Cell culture and Transfection

To evaluate the potential involvement of *B4GALNT2-like* in the synthesis of the Sd(a) antigen, cells were transfected with plasmids expressing either *B4GALNT2* or *B4GALNT2-like*. WT and the NSRRC:0086 line, which did not stain for DBA, served as negative control. WT porcine kidney cells were used as a positive control, as DBA binding had been successfully confirmed previously.

Porcine tail, kidney, and fetal fibroblasts from WT and NSRRC:0086 lines, isolated as previously described [19,20,21], were cultured in Dulbecco Modified Eagle medium (DMEM; Gibco, Cat #11885084) supplemented with 0.5% Glutamax (Gibco, Cat #35050061) and 12% FBS (Gibco, Grand Island, NY). These cells were incubated at 38.5°C in a humidified atmosphere of 5% O_2 and 5% CO_2 and transfected with 2 μg of *B4GALNT2* or *B4GALNT2-like* pcDNA3.1/Hygro (+)-expressing plasmid. WT and NSRRC:0086 tail and fetal fibroblast cells were transfected via electroporation [17], while WT and NSRRC:0086 PKC were transfected by using Jet-OPTIMUS transfection reagent (Polyplus #101000025) and seeded in a 6 well-plate. Non-transfected WT fibroblast cells and NSRRC:0086 line served as negative controls. WT PKC were used as positive controls. Forty-eight hours post-transfection, the cells were incubated in the dark with DBA (1:400; Vector Laboratories) or PBS for 20 minutes at 4°C.

Fluorescence DBA lectin staining in porcine uterine tissues

Uterine cross sections from WT and *B4GALNT2/B4GALNT2-like* KO (derived from NSRRC:0086 × R614 breeding) females were fixed in 10% buffered formalin for 24–48 h and paraffin embedded using standard procedures. For fluorescence localization, sections were deparaffinized in xylene and rehydrated to water through a graded alcohol series. Sections were submerged into citrate solution and then blocked in SuperBlock™ (Thermo Scientific) solution. Sections were incubated with DBA lectin–FITC conjugate (30 μg/mL) in PBS solution overnight at 4C. Sections were lightly stained with hematoxylin and overlaid with a coverslip and antifade mounting reagent (VectaSheild, Vector Co, USA).

Cell Imaging to detect DBA staining

Cells and slides were examined under a fluorescence stereo microscope (M165FC, Leica Microsystems AG, Heerbrugg, Switzerland) to confirm binding to Rhodamine or FITC-labeled *Dolichos biflorus* agglutinin (DBA) lectin. All digital fluorescence and brightfield images were recorded by using the Leica LAS X Software.

Structural examination of the B4GALNT2 and B4GALNT2-like flanking regions in the current pig genome assembly

By using the annotated pig genome assembly *Sus scrofa* 11.1 from the NCBI database, we compared the *B4GALNT2* and *B4GALNT2-like* regions across four species. We identified conserved flanking sequences, which corresponded to the *IGF2BP1-ZNF652* interval, and then searched for this region in the human (GRCh38.p14), mouse (GRCm39), and bovine (ARS-UCD2.0) genomes. Genomic view images from the NCBI database were included in the Supplementary Data (Figure S4–S7).

To determine the conservation of the *B4GALNT2* gene across species, nucleotide and protein sequences for porcine, human, bovine, and murine orthologs were retrieved from the NCBI RefSeq database. Multiple sequence alignment (MSA) was performed using Clustal Omega (v1.2.4) via the EMBL-EBI web portal using default parameters. Percent identity scores were subsequently calculated based on the resulting pairwise identity matrix to quantify the genetic relatedness between the porcine B4GALNT2 isoforms and their mammalian counterparts.

Results

NSRRC:0086 and R614 pigs lack the xenoantigens Gal and Sd(a).

PCR diagnostic assays and Sanger sequencing confirmed the absence of WT alleles in the genetically modified lines (Figure S1). Consistent with these genotypic results, no fluorescence signal was detected when organs or primary kidney cells (PKCs) from founder pigs were stained with Rhodamine-labeled *DBA* lectin to detect B4GALNT2 and B4GALNT2-like activity (Figure S2), or with FITC-conjugated isolectin B4 to detect GGTA1 expression Additionally, uterine tissues from a *B4GALNT2/B4GALNT2*-like null female did not stain for DBA when compared to a WT female (Figure S3).

Quadruple-modified (4GM) piglets were generated by breeding

Male and female founder cloned lines (NSRRC:0086) were bred; however, they were unable to establish pregnancies. Thus, we developed a breeding plan by using different combinations of genotypes, as shown in Figure 1. We produced 13 independent pregnancies that reached full term, resulting in viable null offspring. These *B4GALNT2/B4GALNT2-like* knockout piglets were derived from founder lines NSRRC:0086 and R614. The consistent birth and survival of these animals provide definitive evidence that the loss of these enzymes does not impair porcine fetal development or parturition.

As summarized in Figure 1, three specific breeding strategies generated a total of 14 *B4GALNT2/B4GALNT2-like* null piglets. Notably, most of these offspring also carried knockout alleles for the *GGTA1* gene and the human transgene *hCD55*. To our knowledge this is the first report showing the creation of a domestic line of *GGTA*1$^{-/-}$, *B4GALNT2* $^{-/-}$, *B4GALNT2-like*$^{-/-}$, *Tg-hCD55* piglets by breeding.

Validation of B4GALNT2-like function by immunofluorescence

Edited pig cells lacking *B4GALNT2* and *B4GALNT2-like* expression were stained with Rhodamine-DBA lectin after transfection with either pcDNA3.1/*B4GALNT2* or pcDNA3.1/*B4GALNT2-like* expressing vectors (Figure 2). Similarly, WT tail and fetus cell lines that previously did not stain for DBA fluoresced red after transfection and Rhodamine-DBA staining (Figure 2). Non-transfected (Figure 2) or PBS-treated (data not shown) fetal and tail lines did not react with DBA lectin. Transfected and non-transfected WT PKC cells were positive for DBA staining (Figure 2).

Comparison of the IGF2BP1- ZNF652 interval among different species

We found that the IGF2BP1-ZNF652 interval is conserved among the four species (Figure 3A and 3C). However, in the pig genome we observed a duplication of the genes *Phospho1, ABI3, GNGT2* and *B4GALNT2* (Figure 3C). We propose that a chromosomal rearrangement occurred involving three distinct events: duplication, inversion, and reinsertion. Specifically, we hypothesize that this rearrangement was triggered by a double-strand break (DSB) repaired via a template-switching mechanism involving the homologous chromosome (Figures 3B and 3C).

Interestingly, the porcine tRNA region appears to be the site of reinsertion. Since the tRNA was not duplicated and now resides between the rearranged segments, we suspect that the duplicated genes replaced a portion of the original tRNA region (Figure 3C).

Multiple sequence alignment revealed that, at nucleotide level, *B4GALNT2-like* exhibits greater similarity to bovine, murine, and human *B4GALNT2* orthologs than does the annotated porcine *B4GALNT2* (Tables S1 and S2). Consequently, we hypothesize that *B4GALNT2-like* represents the ancestral locus, while the current porcine *B4GALNT2* is the duplicated copy (Figure 3D).

Discussion

Recently, genetically modified pig organs have been used in studies with decedents [22,23,24] and living patients [25,26] and this field is moving forward to clinical trials. *GGTA1/B4GALNT2/B4GALNT2-like* knockout modifications have often served as base modifications for creating pig-to-primate models. *GGTA*1 KO pigs have been studied since 2002, and extensive literature is available. However, information regarding the role of *B4GALNT2*, and the recently discovered *B4GALNT2-like* gene remains limited.

In this study, we provide functional evidence supporting the findings of Zhang et al. (2023) [27] that B4GALNT2-like is not a pseudogene, but rather an active enzyme involved in the synthesis of the Sda xenoantigen. Transfected WT and NSRRC:0086 fibroblast cells reacted to DBA lectin, confirming the potential enzymatic role of B4GALNT2-like, as demonstrated by Zhang *et al.* [27]. This result highlights the importance of targeting both genes, as B4GALNT2-like may be immunogenic in humans. Similar to observations by Söllner JH *et al.* [28], we could not detect B4GALNT2 expression with DBA in WT pig fibroblasts.

We also collected data regarding the breeding of quadruple-modified (4GM) pigs, as the *B4GALNT2* gene has been described to regulate reproduction in different species [29,30,31,32]. In pigs, we showed that females lacking *B4GALNT2* and *B4GALNT2-like* expression were able to become pregnant and carry pregnancies to term. This leads us to the conclusion that *B4GALNT2/B4GALNT2-like* null embryos were able to attach to the uterine surface and establish pregnancy. In mice, blocking B4GALNT2 *in vivo* and *in vitro* impaired embryo implantation. Additionally, *B4GALNT2* expression in the uterus was shown to be modulated by progesterone and estrogens [29]. DBA binding has been detected in porcine uterine epithelia during the luteal phase of the estrous cycle and glandular epithelium (GE) formation, suggesting a role for n-acetyl-D-galactosamine

molecules during the peri-implantation period [33]. We believe that Sd(a) antigen could be involved in pig embryo attachment, but it is not essential for that to occur, or the lack of *B4GALNT2/B4GALNT2-like* expression triggered a compensation mechanism. These differences observed between pig and mice could also be attributed to their distinct implantation process. Pig embryo attachment to the uterine luminal epithelia is noninvasive (epitheliochorial placentation), whereas in mice, implantation is achieved by invasive growth of trophoblast cells through the epithelial layer of the endometrium (hemochorial placentation) [34].

Next, special attention was paid to the porcine *B4GALNT2* genomic region since we observed the presence of a highly conserved region among mammalian species. We postulated that duplication, inversion and reinsertion of part of the *Phospho1-B4GALNT2* segment might have occurred. These mutations could play key roles in organism evolution; however, little is known about these structural divergences. We also reported that at the nucleotide levels, pig *B4GALNT2-like* showed greater similarity to the human, bovine, and murine *B4GALNT2* genes than the original pig *B4GALNT2*. Further studies are needed to confirm if the pig *B4GALNT2* is appropriately annotated in the reference genome.

Collectively our data provide important information about the pig *B4GALNT2*/*B4GALNT2-like* genes, which can positively impact in the process of creation of organ-source pigs. Recently, the Food and Drug Administration approved the first clinical trials that will require the large-scale breeding and raising of these animals. Thus, understanding the possible effects of the lack of function of *B4GALNT2* and *B4GALNT2-like* in a genetically modified pig will help to overcome future barriers in the xenotransplantation field, which is currently becoming a viable life-saving option.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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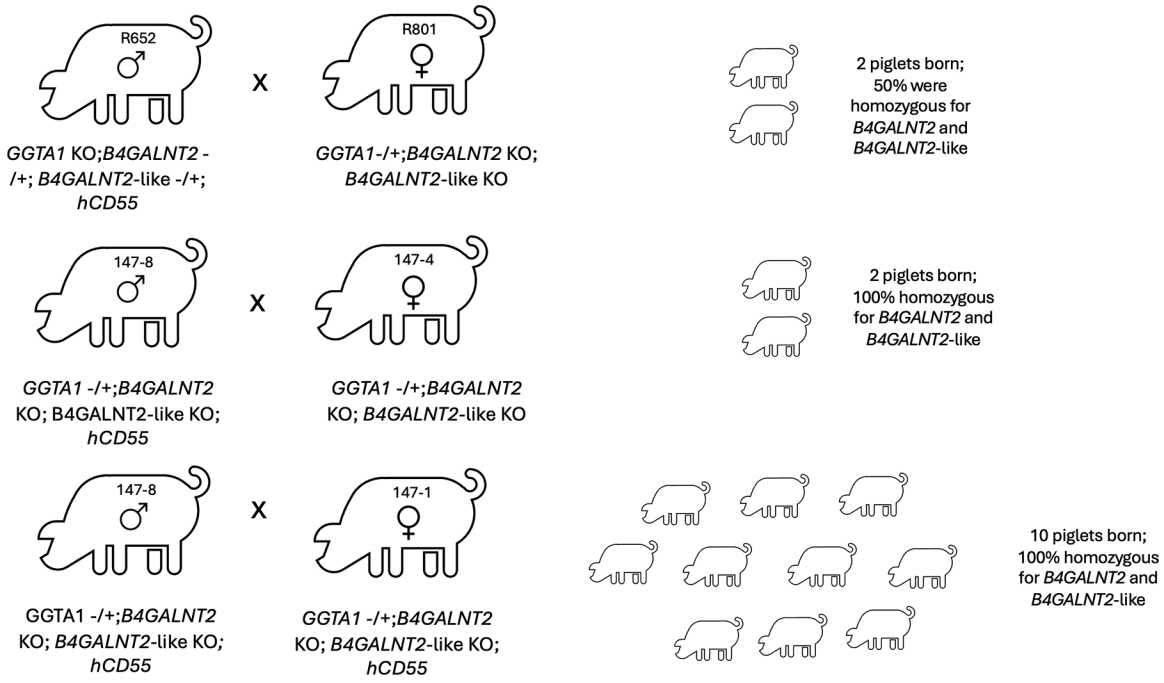

Figure 1. Summary of breeding plan to generate quadruple-modified (4GM) pigs.

A total of fourteen *B4GALNT2*, *B4GALNT2 -like* knockout piglets were born, most of which also carried knockout alleles for *GGTA1* and the human transgene *hCD55*. Females lacking *B4GALNT2/B4GALNT2-like* expression were able to carry *B4GALNT2/B4GALNT2-like* KO embryos to term. The crosses R652 × R801 and 147–8 × 147–1 resulted in two *GGTA1/B4GALNT2/B4GALNT2-like* full KO piglets expressing the *hCD55* transgene. KO: knockout; −/+: heterozygous modification. Pigs R652, R801, 147–8, and 147–4 were generated through selective breeding of established founder lines. Specifically, R652 was derived from crosses between the NSRRC:0086 line and non-cloned GGTA1 KO/hCD55 pigs. Furthermore, R801, 147–8, and 147–4 were produced from crosses between the NSRRC:0086 and R614 lines.

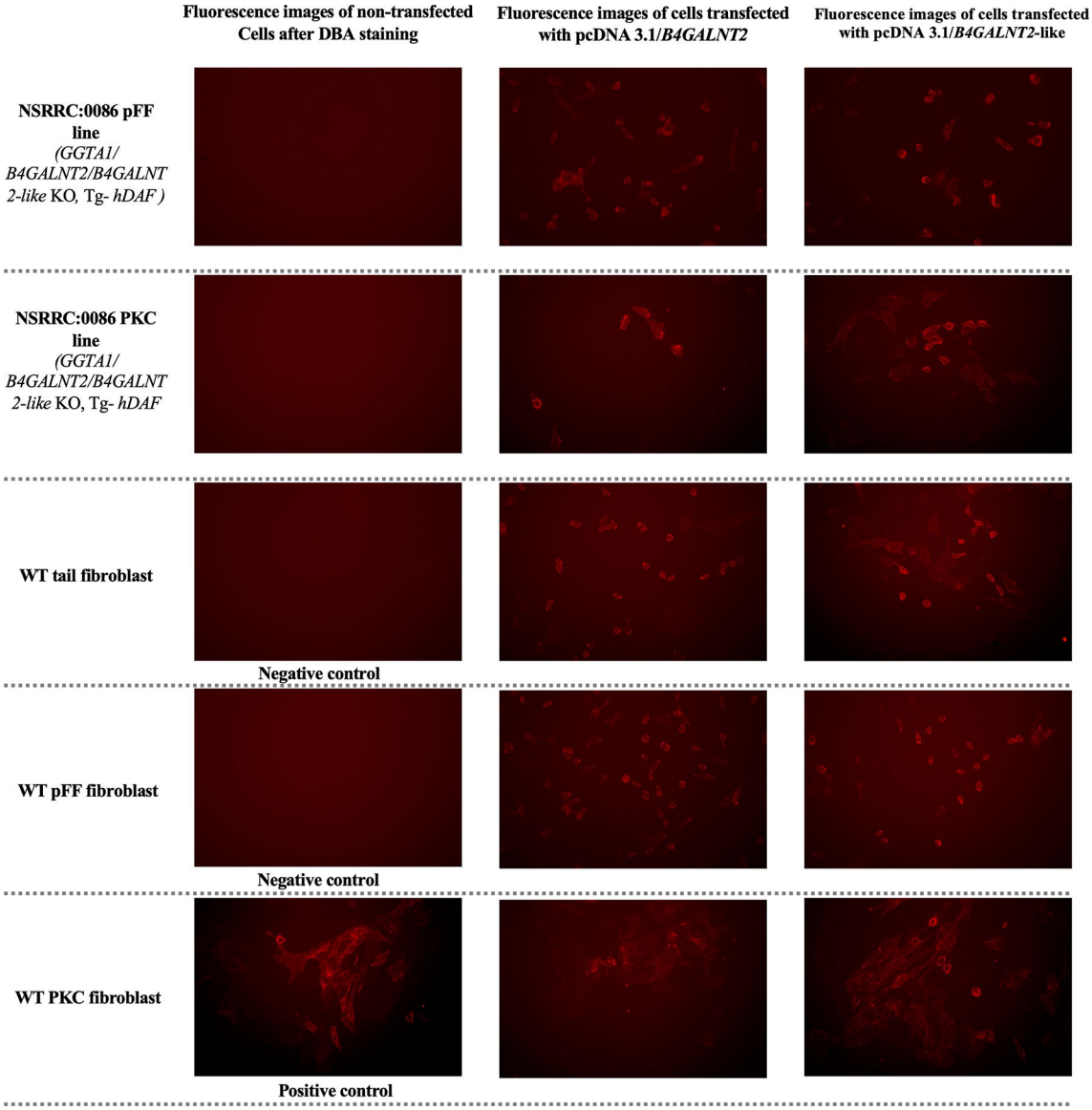

Figure 2. DBA staining images of pig cells transfected with pcDNA3.1/*B4GALNT2* or pcDNA3.1/ *B4GALNT2-like* expressing vectors.

Cells that previously did not stain for DBA (WT tail fibroblast and pFF) became DBA positive after transfected with vectors expressing *B4GALNT2* or *B4GALNT2*-like. pFF: porcine fetal fibroblast; WT: wild-type; DBA: *Dolichos biflorus* Agglutinin; +: positive for DBA staining; -: negative for DBA staining.

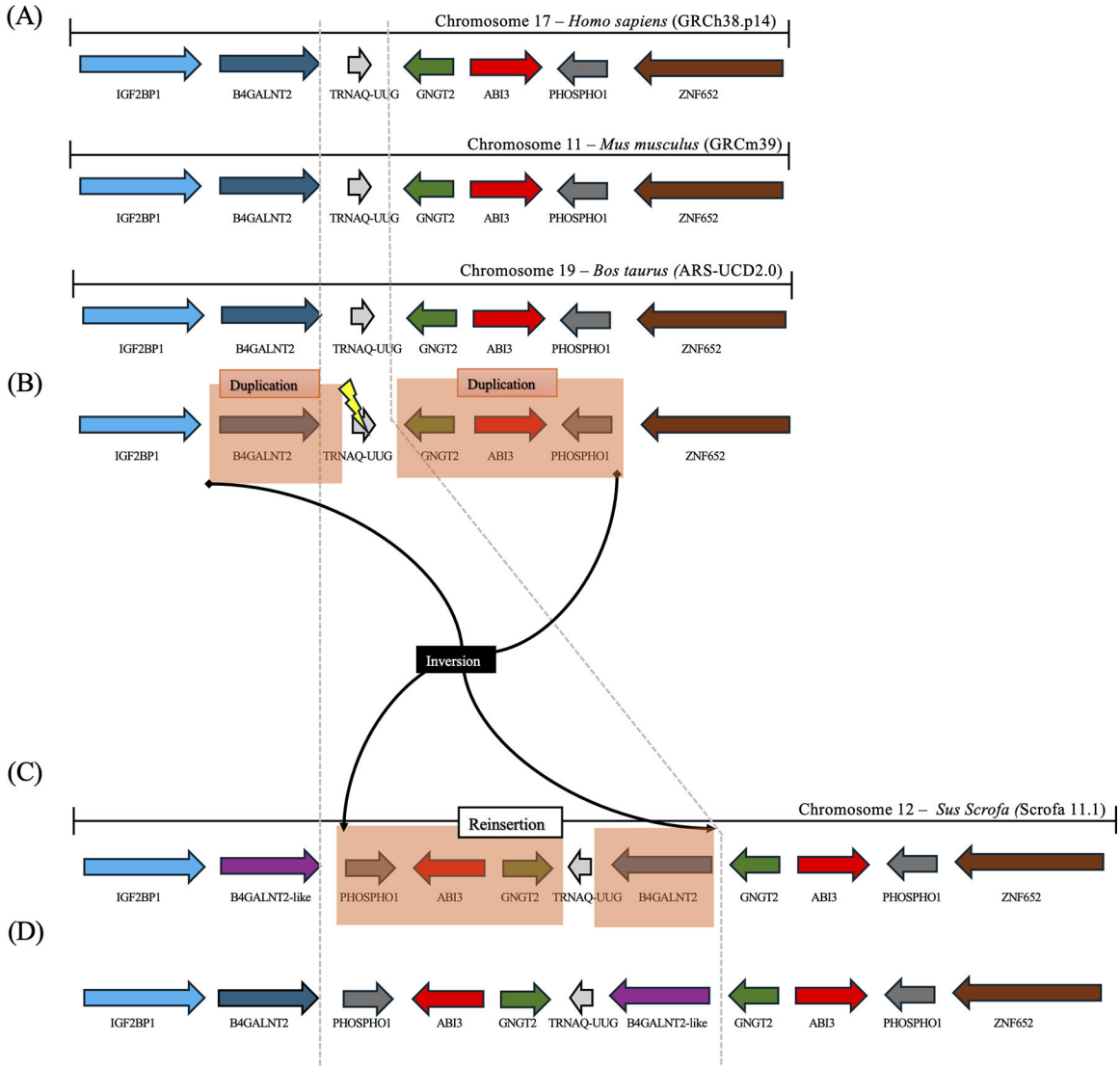

Figure 3. Structural examination of the *B4GALNT2* locus in the porcine genome.

(A) Comparative genomic organization of the *B4GALNT2* flanking region in human (*Homo sapiens*), mouse (*Mus musculus*), and cow (*Bos taurus*), based on NCBI database annotation. **(B)** Proposed mechanism for the porcine-specific rearrangement: a double-strand break (DSB) at the *TRNAQ-UUG* region (indicated by the yellow lightning bolt), followed by a segmental duplication and inversion event. **(C)** Genomic view of the *B4GALNT2* locus in the porcine (*Sus Scrofa*) genome, based on the NCBI database. Insertion of the duplicated segments (orange boxes) into the tRNA region, resulting in the expanded porcine locus. **(D)** Proposed model for the porcine *IGF2BP1–ZNF652* interval organization. The tRNA region appears to be the site of reinsertion, as the tRNA was not duplicated but resides between the rearranged segments; we hypothesize that the duplicated genes replaced a portion of the original tRNA region. Based on structural, nucleotide, and protein analyses, we suggest that *B4GALNT2-like* represents the ancestral gene, while *B4GALNT2* is the duplicated copy. Colored arrows denote transcriptional orientation and gene identity.