ELSEVIER

A Study of the Glycoantigens of Neonatal Porcine Islet-Like Cell Clusters Using a Lectin Microarray

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ABSTRACT

Background. The pig pancreas is considered to be the most suitable source of islets for clinical xenotransplantation. Two types of islet transplantation are: adult pig islets and neonatal porcine islet-like cell clusters (NPCC). However, besides a-Gal expression, differences in glycosylation and xenoantigenicity between both types were not clear so fat to date. In this study, we performed lectin microarray analyses of NPCCs cultured for 1, 5, or 9 days.

Methods. We studied differences in gycoantigens among several kinds of wild-type NPCCs isolated from 1- to 3-day-old neonatal wild-type pigs (Large White/Landrace × Duroc) and cultured for 1, 5 and 9 days in Ham's 10 in the presence of nicotinamide, using a previously published technique. After sonication and centrifugation, supernatant proteins from each islet were labeled with Cy3, applied to a lectin array and scanned with an SC-Profiler for evaluation using an Array Pro Analyzer.

Results. The overall signals of NPCC at days 5 and 9, showed almost the same values to most lectins, whereas those on day 1 showed differences, suggesting that the NPCC on day 1 contain immature cells that gradually turn to mature NPCCs in culture.

THE INCREASING PROBLEM associated with the worldwide shortage of donor organs has led to renewed interest in xenotransplantation with clinical trials in New Zealand.[1] the pig pancreas is considered to be the most suitable source of islets for clinical xenotransplantation. Two types of sources for transplantations are: adult pig islets (APIs)[2] and neonatal porcine islet-like cell clusters (NPCCs).[3] However, in addition to α-Gal expression, the natures of glycosylation and xenoantigenicity of NPCC are unclear.[4] In this study, we performed lectin microarray analyses of NPCCs cultured for 1, 5, and 9 days.

MATERIALS AND METHODS

Preparation of NPCC

Using the technique described by Korbutt et al,[5] pancreata were isolated from 1- to 3-day-old neonatal pigs (Large White/Landrace × Duroc 1.5–2.0 kg bdy weight) of both sexes. The dissected pancreas was kept until processing in cold Hanks' balanced saline solution (HBSS; GIBCO Labs, Grand Island, NY, USA) supplemented with 0.25% bovine serum albumin (Nakalai Tesque, Kyoto, Japan), 10 mmol/L Hepes (Nakalai Tesque, Kyoto, Japan), and antibiotics. In a typical preparation, a pancreas minced into 1- to 2-mm^3 fragments was digested with collagenase type V (2.5 mg/mL; Sigma, St Louis, USA) at 37°C with gentle shaking. After filtration through nylon screen (500 μm), the tissue was washed in HBSS three times before cultured at 37°C in a humidified atmosphere with 5% CO_2 for 7 to 9 days in Ham's F10 medium (GIBCO Labs) containing 10 mmol/L glucose, 50 μmol/L isobutylemthylxanthine (Nakalai Tesque, Kyoto, Japan), 0.5% bovine serum albumin (Nakalai Tesque), 2 mmol/L L-glutamine (Nakalai Tesque), 10 mmol/L nicotinamide (Sigma), and antibiotics. The culture medium was changed on the first day after isolation and every other day thereafter.

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Preparation of the Samples

Each sample was washed with phosphate-buffered saline (PBS), and sonicated in PBST (PBS containing 1.0% Triton X-100). The whole cell lysate was frozen once at −80°C. After defreezing on ice, and centrifugation at 14,000 g for 5 minute, we collected the supernates for analysis.

Lectin Microarray

The lectin microarray was prepared as previously described with minor modifications. The glycoprotein from each cell was incubated with 10 μg of Cy3-succimidyl ester (Amersham Biosciences, Tokyo, Japan) for 60 minutes at room temperature in the dark. The reaction product was loaded on a Sephadex-G25 spin column to remove excess fluorescent reagent. After centrifugation, the collected solution was adjusted to 200 μL with probing buffer (Tris-buffered saline cotnaining 1.0% Triton X-100 and 500 mmol/L glycine) before incubation for 2 hours at room temperature, in order to completely inactivate residual fluorescent reagent. To triplicate wells on a glass slide, we applied 60 μL of Cy3-labeled glycoprotein solution before incubation overnight at 20°C in a humid chamber. After the binding reaction was complete, we acquired a fluorescence image of the array using an evanescent-field fluorescence scanner, Glycostation Reader (Moritex, Yokohama, Japan) with analysis using the Array Pro analyzer version 4.5 (Media Cybernetics, bethesda, Md, USA). Acquired data were differentially analyzed using normalized signals obtained for all lectin, with the lectin showing the strongest signal intensity (max intensity) being assigned a value of 1.0.[6]

Table 1. Comparison in Net Intensity Among Samples From a New Bone Porcine

	Day 1	Day 5	Day 9
LTL	2014 ± 128	394 ± 57	784 ± 52
RCA	3620 ± 435	4799 ± 345	6332 ± 351
NPA	8376 ± 265	3415 ± 239	4624 ± 311
GNA	2971 ± 81	474 ± 114	1782 ± 143
ABA	6163 ± 156	6076 ± 82	8009 ± 223
UDA	22,800 ± 379	12,174 ± 500	19,378 ± 721

Each value indicates the signal intensity, with the average ± standard deviation of triplicate determinations. Data were analyzed as described in materials and methods. Reported specificity of each lectin: LTL (*Lotus tetragonolobus*), Fucα1–3(Galβ1–4)GlcNAc & Fucα1–2Galβ1–4GlcNAc; RCA (*Ricinus communis*)120, Galβ1–4GlcNAc; NPA (*Narcissus pseudonarcissus*), High-mannose and Manα1–6Man; GNA (*Galanthus nivalis*), high-mannose and Manα1–3Man; MBA (*Agaricus bisporus*), Galβ1–3GalNAc; UDA (*urtica dioica*), GlcNAcβ1–4GlcNAc and mixture of Man5 to Man9.

RESULTS

NPCC at day 1 were richer than the others in reactivity with the *Lotus tetragonolobus, Narcissus pseudonarcissus, Galanthus nivalis,* and *Urtica dioica,* implying the presence of high-mannose and fucose forms. The displayed less activity toward *Ricinus communis,* and *Agaricus bisporus,* which bind lactosamine and core 1 form. However, in general, the signal by day 5 and day 9 NPCC were almost the same toward many lectins, in contrast with the signals for day 1 NPCC (Table 1).

The intensity of most signals were between those of APIs (data not shown) and day 5 and 9 NPCC, suggesting that day 1 NPCC contains numerous immature islets and other cells that are gradually converted into NPCC during culture.

DISCUSSION

NPCCs reduced fucose/core fucose, and high-mannose forms, and upregulated lactosamine and core 1 forms in 5-day cultures. The results reported herein may contain useful information for future xeno-islet transplantation studies.

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REVIEW

Carbohydrate antigens

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Purpose of review
To summarize the current knowledge of carbohydrate antigens as related to xenotransplantation. The emphasis is on non-Gal carbohydrate antigens identified in many institutes. In addition, several topics such as glycosyltransferase-transgenic pigs, innate cell receptors and porcine endogenous retrovirus (PERV) will be discussed.

Recent findings
Studies related to iGb3 and neoantigens after knocking out GalT (GGTA1) were reviewed. Available data do not support the conclusion that GalT-KO remains iGb3 and/or that neoantigens are produced in the pigs. Concerning non-Gal antigen, in addition to the Hanganutziu-Deicher (H-D) antigen (NeuGc), Forrsman antigen, Galα1–3Lew^x, α-linked or β-linked GalNAc, β3 linked Gal, NeuAc, such as Neu5Acα2-3Galβ1–3GlcNAc, and Sid blood group (Sd^a)-like antigens are candidates. However, to date some of these remain controversial and others need further study to completely identify them. Regarding the H-D antigen, different from the α-Gal, it has a complicated expression system, but has cytotoxic effects toward pig cells. To modify other carbohydrate antigen apart from α-Gal, only the overexpression of GnT-III appears to have an effect on the suppression of the N-linked sugar of non-Gal antigen. Concerning innate cell receptors related to carbohydrates (ligands), the focus turned from natural killer (NK) receptor to others, such as monocytes. Finally, PERV contains a ligand with an N-linked sugar. Modification of the glycosylation pattern appears to be associated with regulating PERV infectivity.

Summary
A considerable amount of data related to carbohydrate antigens is now available. At the same time, however, discrepancies between studies complicate this issue. Further studies will be needed to completely understand this complicated area of interest.

Keywords
carbohydrate antigens, nongal, xenotransplantation

INTRODUCTION

Xenograft rejections are strongly related to species-specific glycoantigens. The α-Gal epitope (Gal α1–3Galβ1–4 GlcNAc-R) [1], which is biosynthesized by the action of α1,3 galactosyltransferase (α1,3GT) [2–4], is closely associated with hyperacute rejection (HAR) in pig-to-human xenotransplantation. Before α1,3GT was knocked out using nuclear transfer technique, in attempts to reduce or eliminate the α-Gal epitope from pig tissues, a variety of strategies have been pursued, such as enzyme competition by α1,3GT with other glycosyltransferases. Some of the strategies continue to pursue downregulating the non-α-Gal epitopes, include the Hanganutziu-Deicher (H-D) epitope and other non-Gal epitopes. Concerning non-Gal epitopes, many studies about glycoproteins and glycolipids are continued to find them. Some studies presented herein identified some block. However, we do not yet know everything about non-Gal glycoantigens. On the contrary, the analysis of the innate immune cell receptors on the basis of the importance of glycosyl epitopes is also under study. In addition, the down-regulation in the infectivity of porcine endogenous retrovirus (PERV) to human cells by modification of glycosyltransferases is another important topic.

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KEY POINTS

- Before α-Gal-KO pigs were established using homologous recombination, several strategies, such as enzyme competition and/or the control of sugar processing by glycosyltransferases, had provided some very interesting insights into the downregulation of xenogenetic epitopes.
- Increasing evidence has accumulated to suggest that NK cells play an important role in pig-to-human xenogenic cytotoxicity.
- The reduction in high mannose type N-linked sugars on pig cell surface glycoproteins by knockdown or KO of pig dolichyl-phosphate mannosyltransferase represented a novel and potentially useful strategy for reducing porcine endogenous retrovirus transmission to humans.

ISOGLOBOTRIAOSYLCERAMIDE

After Gal-knockout (KO) pigs were produced in the several institutes by knocking out the α1,3galactosyltransferase (GGTA1), another transferase, the GGTA2 remains in pigs. The gene can produce α-Gal antigens on a Lactosyl core, thereby forming isoglobotriaosylceramide (iGb3; Galα3Galβ4GlcNAcβ1Ceramide) [5]. Therefore, several researchers reported this possibility in Gal (GGTA1)-KO pigs [6–8]. On the contrary, Diswall *et al.* [9,10,11▪], using thin layer chromatography, characterized glycolipids from GalT-KO pig tissues with respect to the expression of Gal epitopes. They failed to detect the Galα1–3Gal determinant from the tissues of GalT-KO pigs from their continuous reports. That is, on the basis of their findings, iGb3 was absent and only fucosylated iGb3 was found. Likewise, Kiernan *et al.* [12] also failed to detect the expression of GGTA2 in GalT-KO pig cells in their study that used gene libraries of monkeys that had been immunized by GalT-KO cells. They denied the expression of iGb3 synthetase in GalT-KO pig cells.

Moreover, Yung *et al.* [13] summarized many studies related to the expression of α-Gal, including iGb3 in their review, and concluded that the available data do not support a relevant role for iGb3 in antibody-mediated pig-to-human xenotransplantation.

The issue of the existence of this GGTA2 gene and its function in pigs became less controversial. It may not be the major reason for xenograft rejection.

NEOANTIGEN

As a problem in the GalT-KO pig, a neoantigen might be produced, and would be expected to induce new immunological responses in humans. Gustafsson *et al.* reported an increase in *Maackia amurensis* (MAL)-reactive and *Erythrina cristagalli* (ECA)-reactive epitopes, Neu5Acα2,3Galβ1,4GlcNAc and Galβ1,4GlcNAc, in the aortic pig endothelial cell of GalT-KO pigs, compared with the wild-type pig, suggesting that the precursor chain for the Galα1,3Gal, lactosamine units, is exposed in these cells. Neither Lew^x [Galβ1,4(Fucα1,3) GalNAcβ-] nor sialyl-Lew^x structures were detected in any of the pig EC studied [14]. On the contrary, Diswall *et al.*, in their studies of glycolipid antigens in pig tissues, found increased levels of uncapped LacNAc precursor, fucosylated blood group H type2, the P1 antigen (Galα4nLc4), the X2 antigen (by β1,3GalNAcT). However, these epitopes are not believed to mediate rejection because these are present on human cells. They concluded that GalT-KO pigs did not produce new compensatory glycolipid compounds that react with human serum antibodies [9,10,11▪].

In our previous study, although no unique changes were detected in *LTL* (*Lotus tetragonolobus*) and *AAL* (*Aleuria aurantia*) that were relevant to Lew^x and sialyl-Lew^x, MAL and *ECA* were found to be upregulated in GalT-KO pigs, compared with the wild-type pig. However, the expression in human cells appears to be higher than that observed for the GalT-KO pig. On the contrary, concerning LacNAc, Milland *et al.* [15] reported a substantial increase in LacNAc levels in GalT-KO mice and that this exposed epitope could bind natural antibody (NA). However, in our previous study of glycoprotein, the net intensities of *ECA* and *RCA120* (*Ricinus communis*), binding to Lac and LacNAc, remained unchanged among the ECs. In addition, in human fibroblasts, a clear net intensity was observed for *ECA* and *RCA120*, contrary to α-LacNAc NA production in humans [16▪].

Taken together, these data do not support the Gal-KO that produce neo antigen in the pigs.

NON-GAL ANTIGEN

Even after GalT-KO pigs were produced most antigens, including the so-called non-Gal antigen, were still present, and this fact represents a significant obstacle to successful xenotransplantation.

In 1993, Oriol *et al.* [17] suggested that pig has a blood group AO system, then the pig blood type A antigen may trigger an immune response in human O or B recipients.

Before the α-Gal was knocked out, Bouhours *et al.* [18] in their analysis of glycosphingolipids from the pig kidney, reported that the Galα1,3Lew^x is a new epitope capable of being recognized by the human natural antibody. Using a similar method of

analysis, Hallberg *et al.* [19] also indicated that Galα1–3Lew^x and Galα1–3nLc4(neolactotetraosylceramide) were important targets for human natural antibody. In addition, recently, Kim *et al.* [20] reported that NeuGc-Gal-GlcNAc and Galα1–3Lew^x were novel antigens, as evidenced by a structural analysis of N-glycans from the miniature pig kidney, using matrix-assisted laser desorption/ionization time-of-flight/mass spectrometry and MS/MS. However, the proposed antigens appear to be related to the α-Gal and H-D antigens.

Similarly, Blixt *et al.* [21] reported on the carbohydrate specificities of sera obtained from the clinical patients in whom pig islet-like clusters (ICC) had been intraportally injected, using a printed covalent glycan array with 200 structurally defined glycans. Apart from α-Gal and H-D antigens, the patients had Abs against terminal α-linked GalNAc, β3-linked Gal especially Galβ1,3GlcNAc even if terminally sulfated or sialylated, β-GlcNAc except if β1,3-linked and oligomannosyl compounds, and Galα1–3Lew^x. Concerning the expression of the Forssman antigen in pigs, they mentioned the possibility that it is related to the terminal GalNAc. However, Diswell *et al.* in a recent study, denied this.

In our previous study, apart from the Forssman, the terminal GalNAc (related to the Tn-antigen: GalNAcα-O-Ser/Thr) was clearly expressed on glycoproteins from pig ECs and fibroblasts, but fortunately, its level was reduced in cells from the GalT-KO pig [16▪]. In addition, not only this Tn antigen but the T (Thomsen-Friedenreich; Galβ1,3GalNAcα-O-Ser/Thr) and sialyl-Tn antigen (NeuAcα2,6GalNAcα-O-Ser/Thr) are also thought to be important as mentioned by Breimer *et al.* [22]. However, concerning the expression of them on pig cells, Zhu *et al.* [23] concluded that terminal α-linked or β-linked Gal residues and α-linked GalNAc residues, such as the non-Gal Ab are not present, as evidenced by the treatment of pig red calls with α-galactosidase or β-galactosidase. Using a pig-to-baboon transplant or presensitization model, Buhler *et al.* [24] also ruled out the possibility that common oligosaccharides, such as α-linked or β-linked Gal and α-GalNAc, are targets for non-Gal antibody. The discrepancy between their results and ours may be due to differences in the experimental methods and the cells used, but the terminal GalNAc can be considered to remain as a non-Gal antigen.

In our previous study concerning the antigenicity of adult pig islets, neuraminidase sensitive sialic acid antigens, other than the H-D antigen are related to the binding of non-α-Gal Ab [25]. Neu5Acα2-3Galβ1–3GlcNAc, which was reported on by Blixt *et al.* may also be included.

In addition, quite recently after their protein study [26], Byrne *et al.* reported that Sid blood group (Sd^a)/CAD (produced porcine β1,4N-acetylgalactosaminyl transferase 2 gene) [27] like antigen may represent a new antigen, using a porcine DNA expression library system and primate serum. This carbohydrate(s) is displayed on the human embryonic kidney cells with pig B4GALNT2. However, human and primate have the B4GALNT2, further study is then needed to define the structure of this carbohydrate(s) [28▪▪].

Taken together, in spite of that Diswell *et al.* indicated glycolipids from Gal-KO pigs contains many epitopes that react with human serum apart from the iGb3, Forrsman antigen and H-D antigens, only a few carbohydrates can be nominated as non-Gal antigens so far.

The Hanganutziu-Deicher antigen

H-D antigen (NeuGc) is widely distributed in mammalian species including monkeys and apes, but is not found in humans and birds. The expression of NeuGc is controlled by the enzymatic activity of cytidine monophospho-N-acetylneuraminic acid hydroxylase (CMAH). Two different groups reported that human CMAH is caused by the deletion of exon 6 in the genomic DNA [29,30], and lack of an alternative pathway for the synthesis was also reported [31].

Concerning anti-H-D antibody production in humans, Zhu, *et al.* [33] in 2002, after many twists and turns [32], indicated that the majority of human anti-non-Gal antibodies are specific for the H-D antigen. These experiments involved the use of flow cytometry and pig red blood cells that had been preincubated with sialidase. In 2003, Magnusson *et al.* [34] reported that, in a patient who had been extracorporeally perfused with a pig kidney, the staining of both α-Gal terminal structures, as well as, the H-D reactive gangliosides, NeuGc-GM3, and NeuGc-GD3, was increased. Another study, using ELISA, also showed the existence of anti-H-D IgM and IgG in all naive human sera [35]. In addition, quite recently, Basnet *et al.* [36] using a mice strain lacking both the α-Gal and H-D antigen suggested that it is unlikely that the anti-H-D antibody is involved in HAR, but that the H-D antigen may elicit a significant humoral response and could play a role in a delayed form of rejection. In the case of NeuGc, different from the α-Gal, it can provide the antigenic complexity, such as the variety of positions (C4, C7, C8and/or C9) and the linkages (α2,3Gal, α 2,6Gal(NAc) or α2,8Sia). Moreover, it can be metabolically incorporated into human cells

through consumption of NeuGc-rich foods [37,38▪▪].

On the contrary, several strategies for reducing the H-D antigen levels in pig cells are under consideration and include knocking down and knocking out the CMAH gene. Song *et al.* [39] have previously described the knockdown of CMAH expression in pig kidney cell line, PK15. The siRNA for pig CMAH in the porcine endothelial cell line also reduced the expressions of the mRNA and the H-D antigen substantially. The further reduction in the xenoantigenicity of the α-Gal-KO porcine endothelial cells and fibroblasts from *in vivo* by the siRNA for pig CMAH was confirmed (author's unpublished data).

OTHER METHODS

Before α-Gal-KO pigs were established using homologous recombination, several strategies, such as enzyme competition and/or the control of sugar processing by glycosyltransferases, had provided some very interesting insights into the downregulation of xenogenetic epitopes.

Substrate competition in terminal glycosylation

This strategy for downregulating the α-Gal epitope capitalizes on the enzymatic competition for terminal glycosylation between α1,3GT and other glycosyltransferases for the common acceptor substrate in the trans-Golgi network by masking the α-Gal epitope by fucosylation or sialylation. Among them, α1,2FT-transgenic (Tg) pigs can still be found in several institutes [40,41].

Substrate competition in core glycosylation

β-D-mannoside, β1,4-*N*-acetylglucosaminyltransferase III (GnT-III) catalyzes the branching of *N*-linked oligosaccharides, producing a bisecting *N*-acetylglucosamine (GlcNAc) residue. Once a bisecting GlcNAc residue is added to the core mannose by GnT-III, the action of other competitive enzymes such as GnT-IV and GnT-V is prevented from introducing any additional tri-antennary structures into the Golgi stack. As a result, it is likely that all levels of *N*-linked sugar, including Gal and non-Gal antigens, are decreased. As a strong point of this strategy, overexpression of GnT-III clearly works on non-Gal antigens relate to *N*-linked sugar [42].

End-β-Gal-C

This enzyme was identified from a *Clostridium perfringens* culture media, has a strong activity for digesting α-Gal epitopes by cleaving the β-galactosidic linkage in the Galα1–3Galβ1–4GlcNAc (α-Gal epitope) structure. The gene for this enzyme was cloned, and they finally produced Tg-pigs [43].

NATURAL KILLER CELL AND OTHER INNATE CELLS

Increasing evidence has accumulated to suggest that NK cells play an important role in pig to human xenogenic cytotoxicity. In our previous study, the remodeling of glycoantigen, especially the α-Gal epitope, by a glycosyltransferase, such as GnT-III, α1,2FT and sialyltransferases, affects the susceptibility of pig endothelial cells to NK-mediated direct lysis in the absence of an antibody-mediated reaction [44]. Several other studies also demonstrated that uncharacterized receptors on human NK cells react with ligands containing the α-Gal epitope [45,46]. However, some studies do not support the evidence for direct recognition of α-Gal by NK cells [47,48]. Finally, Christiansen *et al.* [49] found the receptor, NKRP1A, which binds to the α-Gal epitope. The debate then seemed to be burnt down.

After that, the study gradually turned to other innate cellular response. Among them, the relation between α-Gal and Galectin3 on monocyte was identified, but still remains controversial [50]. In general, each lectin domain of the receptors has not so strong affinity and specificity to the carbohydrates (ligands), like antibody response.

PORCINE ENDOGENOUS RETROVIRUS INFECTIVITY AND N-LINKED SUGARS

In pigs, at least several proviral copies of PERV-A, PERV-B, and PERV-C are present in the genome. In addition, recombination between PERV-A and PERV-C advance the infectivity [51]. An analysis of *O*-linked sugars is less relevant to general retrovirus infectivity. On the contrary, most of the individual consensus *N*-linked glycosylation sites are indispensable for viral infectivity [52]. We addressed the effects of the remodeling of pig cell-surface glycoproteins, especially the high mannose type of *N*-linked sugars, on the susceptibility of PERV by the overexpression of α-1,2 mannosidase Ib (Man Ib), *N*-acetylglucosaminyltransferase I (GnT-I) and α-mannosidase II (Man II). The reduction in PERV infectivity by overexpression of each enzyme was approximately 50%. However, as an alternate strategy, we turn our attention to the carbohydrate-deficient glycoprotein (CDG) syndrome, especially type IV [53]. In spite of the features of the disease, the patient lived and the content of high-mannose type of *N*-linked sugars was diminished. Dolichyl-

phosphate mannosyltransferase (D-P-M) is associated with the early stages of *N*-linked sugar synthesis, and the gene cause CDG. The reduction in high mannose type *N*-linked sugars on pig cell surface glycoproteins by knockdown or KO of pig D-P-M represented a novel and potentially useful strategy for reducing PERV transmission to humans [54▪].

CONCLUSION

The current knowledge of carbohydrate antigens as related to xenotransplantation is summarized. A considerable amount of data related to carbohydrate antigens is now available, for example, as non-Gal antigen, NeuGc, Forrsman antigen, Galα1–3Lew^x, α-linked or β-linked GalNAc, β3-linked Gal, NeuAc, and SD^a-like antigens. However, at the same time, discrepancies between studies complicate this issue. As a result, further studies will be needed to completely understand this complicated area.

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Conflicts of interest

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There are no conflicts of interest.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 208).

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ORIGINAL ARTICLE

A lectin array analysis for wild-type and α-Gal-knockout pig islets versus healthy human islets

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Abstract
Purpose We performed lectin microarray analyses of islets from wild-type (WT) pigs and α1-3galactosyltransferase gene knockout (GKO) pigs and compared the results with the corresponding values for islets from healthy humans.
Methods Islets were isolated from the pancreas. After sonication and centrifugation, the proteins in the supernatant from each islet were labeled with Cy3 and applied to a lectin array.
Results Despite negligible expression of the Gal antigen on the adult pig islets (APIs), GKO-islets showed weaker signals, not only for GS-I-B4 but also for PNA, WFA, PTL-I, and GS-I-A4, than the WT islets, indicating reduced contents of α-linked GalNAc and Galβ1-3GalNAc. In comparing the islets of pigs vs. humans, human islets showed stronger signals for UEA-I, AAL, TJA-II, EEL, WFA, HPA, DBA, SBA and PTL-I, indicating that besides ABO blood type antigens, high levels of fucose and α-linked GalNAc are present. On the other hand, the high mannose form was very rich in the APIs.
Conclusion GKO reduced alpha-linked GalNAc, despite negligible expression of the Gal antigen on WT-API. On the other hand, the high-mannose form was richer in both APIs than in healthy human islets. These results provide useful information for future studies.

Keywords Xenotransplantation · Glycoantigen · Lectin array · Islets

Abbreviations

WT	Wild-type
GKO	The α-Gal knockout
API	Adult pig islets
H–D	Hanganutziu–Deicher

Introduction

The worldwide shortage of donor organs has renewed interest in xenotransplantation [1, 2]. The pig represents an ideal source of tissue and organs for discordant xenografts for many reasons: anatomical, physiological, and ethical. The major xenoantigen responsible for rejection is a single carbohydrate structure, the α-Gal epitope (Galα1-3Galβ1-4GlcNAc-R) [3], which is expressed by most mammalian cells, including those of the pig.

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The pig pancreas is considered a suitable source of islets for clinical xenotransplantation [4]. There are two types of islet transplantation: that of neonatal porcine islet-like cell clusters (NPCCs) [5] and that of adult pig islets (APIs) [6]. The original wild-type (WT) API contains negligible levels of the α-Gal, but shows a clear antigenicity to human serum.

On the other hand, using somatic cell nuclear transfer technology (SCNT) [7], we produced genetically engineered pigs by heterozygous knockout (KO) of the α1-3galactosyltransferase (GalT) gene (GKO) [8–10]. Even after producing GKO pigs, most antigens, including the so-called non-Gal antigen, are still present on the pig cell surfaces. In our previous study, we described analyzing the glycans of endothelial cells and fibroblasts and compared the results of WT vs. GKO pigs, and of pigs vs. humans. However, changes in the glycoantigens of the islets of the GKO pig have not been extensively analyzed. In this study, lectin microarray analyses of APIs from WT and GKO pigs were carried out and the results compared with the corresponding values for healthy human islets.

Materials and methods

Pig islet isolation

Pig islet isolation and cultures were performed as previously described [11] with minimal modifications. Briefly, the duodenal-splenic lobe and connective lobe were distended intraductally by treatment with a cold enzyme solution of LiberaseHI (Roche Molecular Biochemicals, Indianapolis, IN, USA) diluted with Hanks' balanced salt solution. The distended glands were digested by the semi-automated continuous-filtration method at 37 °C and the cleaved islets were purified from non-islet tissue with a continuous density gradient in a COBE 2991 cell processor (COBE Blood Component Technology, Lakewood, CO, USA). Isolated islets were cultured overnight at 37 °C in a humidified atmosphere with 5 % CO_2 in Tohoku culture bags (Nipro, Osaka, Japan) [12] with medium 199 (Gibco) supplemented with 10 % heated inactivated porcine serum, 2 mM *N*-acetyl-L-alanyl-L-glutamine, 10 mM *N*-2-hydroxyethylpiperazine-*N*1-2-ethanesulfonic acid (HEPES), 100 IU/ml penicillin, 100 μg/ml streptomycin (Biochrom), and 20 μg/ml ciprofloxacin.

Human islet isolation and evaluation

Research grade human pancreata were obtained through two local organ procurement agencies (LifeGift, Fort Worth, TX and Southwest Transplant Alliance, Dallas, TX, USA). These agencies obtained a signed consent form from the family of the donor, to use the pancreas graft for research. Donor pancreata were procured by surgeons who belonged to the islet isolation team. Pancreatic ducts were preserved with ET-Kyoto solution [13] and the oxygen-charged static two-layer method [14] was used for transportation of all the procured pancreata.

Islets were isolated using the modified Ricordi method [15]. Serva collagenase NB1 with neutral protease (SERVA Electrophoresis, Heidelberg, Germany) or Liberase MTF with thermolysin (Roche Diagnostics GmbH, Penzberg, Germany) was used for digestion. Islets were purified using a continuous density gradient in a COBE 2991 cell processor (CaridianBCT, Inc., Lakewood, CO, USA). ET-Kyoto solution and iodixanol was used as density gradient solution [13].

The final preparation of islets was assessed by dithizone staining (Sigma Chemical Co., St. Louis, MO, USA) (2 mg/ml) to determine yield and purity. The islet yield was converted into a standard number of islet equivalents (IEQ, diameter standardized to 150 μm) in accordance with previous protocols [14, 16, 17]. Islet viability after purification was measured with fluorescein diacetate (10 μM) and propidium iodide (15 μM) staining [14, 16, 17]. The average viability of 50 islets was calculated. All of these procedures were carried out at the Baylor Research Institute, Texas, USA.

Ethical guidelines

This study was approved by the institutional review boards of Osaka University Graduate School of Medicine and the Baylor Research Institute, Texas, USA.

Preparation of samples

Each sample was washed with PBS, and sonicated in PBST (PBS containing 1.0 % Triton X-100). The whole cell lysate was frozen once at −80 °C and each sample was thawed on ice. After centrifuging at 14,000*g* for 5 min, the supernatant was collected.

Lectin microarray

The lectin microarray was prepared as previously described, with minor modifications [18, 19]. The glycoprotein from each cell was incubated with 10 μg of Cy3-succimidyl ester (Amersham Biosciences, Tokyo, Japan) for 60 min at room temperature in the dark. The resulting reaction product was loaded on a Sephadex-G25 spin column to remove the excess fluorescent reagent. After centrifugation, the volume of the collected solution was adjusted to 200 μl with probing buffer (Tris-buffered saline containing 1.0 % Triton X-100 and 500 mM glycine) and

Table 1 List of lectins used and their reported specificities

Abbreviation	Lectin	Reported specificity
LTL	*Lotus tetragonolobus*	Fucα1-3(Galβ1-4)GlcNAc, Fucα1-2Galβ1-4GlcNAc
PSA	*Pisum sativum*	Fucα1-6GlcNAc, α-D-Glc, α-D-Man
LCA	*Lens culinaris*	Fucα1-6GlcNAc, α-D-Glc, α-D-Man
UEA-I	*Ulex europaeus* I	Fucα1-2Galβ1-4GlcNAc
AOL	*Aspergillus oryzae*	Fucα1-6GlcNAc (core fucose)
AAL	*Aleuria aurantia*	Fucα1-6GlcNAc, Fucα1-3(Galβ1-4)GlcNAc
MAL-I	*Maackia amurensis*	Siaα2-3Galβ1-4GlcNAc
SNA	*Sambucus nigra*	Siaα2-6Gal/GalNAc
SSA	*Sambucus sieboldiana*	Siaα2-6Gal/GalNAc
TJA-I	*Trichosanthes japonica* I	Siaα2-6Gal/GalNAc
PHA(L)	*Phaseolus vulgaris* (L.)	Tri/tetra-antennary complex-type *N*-glycan
ECA	*Erythrina cristagalli*	Galβ1-4GlcNAc
RCA120	*Ricinus communis*	Galβ1-4GlcNAc
PHA(E)	*Phaseolus vulgaris* (E)	Bi-antennary complex-type *N*-glycan with outer Gal and bisecting GlcNAc
DSA	*Datura stramonium*	(GlcNAcβ1-4)n, Galβ1-4GlcNAc
GSL-II	*Griffonia simplicifolia* II	Agalactosylated tri/tetra antennary glycans, GlcNAc
NPA	*Narcissus pseudonarcissus*	High-mannose, Manα1-6Man
ConA	*Canavalia ensiformis*	High-mannose, Manα1-6(Manα1-3)Man
GNA	*Galanthus nivalis*	High-mannose, Manα1-3Man
HHL	*Hippeastrum hybrid*	High-mannose, Manα1-3Man, Manα1-6Man
ACG	*Agrocybe cylindracea*	Siaα2-3Galβ1-4GlcNAc
TxLC-I	*Tulipa gesneriana* I	Manα1-3(Manα1-6)Man, bi- and tri-antennary complex-type *N*-glycan, GalNAc
BPL	*Bauhinia purpurea alba*	Galβ1-3GalNAc, GalNAc
TJA-II	*Trichosanthes japonica* II	Fucα1-2Galβ1→ or GalNAcβ1→ groups at their nonreducing terminals
EEL	*Euonymus europaeus*	Galα1-3Gal, blood group B antigen
ABA	*Agaricus bisporus*	Galβ1-3GalNAc
LEL	*Lycopersicon esculentum*	GlcNAc trimers/tetramers
STL	*Solanum tuberosum*	GlcNAc oligomers, oligosaccharide containing GlcNAc and LacNAc
UDA	*Urtica dioica*	GlcNAcβ1-4GlcNAc, Mixture of Man5–Man9
PWM	*Phytolacca americana*	(GlcNAcβ1-4)n
Jacalin	*Artocarpus integrifolia*	Galβ1-3GalNAc, GalNAc
PNA	*Arachis hypogaea*	Galβ1-3GalNAc
WFA	*Wisteria floribunda*	GalNAcβ1-4GlcNAc, Galβ1-3(-6)GalNAc
ACA	*Amaranthus caudatus*	Galβ1-3GalNAc
MPA	*Maclura pomifera*	Galβ1-3GalNAc, GalNAc
HPA	*Helix pomatia*	α-Linked terminal GalNAc
VVA	*Vicia villosa*	α-Linked terminal GalNAc, GalNAcα1-3Gal
DBA	*Dolichos biflorus*	GalNAcα1-3GalNAc, blood group A antigen
SBA	*Glycine max*	α- or β-linked terminal GalNAc, GalNAcα1-3Gal
Calsepa	*Calystegia sepium*	Mannose, maltose
PTL-I	*Psophocarpus tetragonolobus* I	α-Linked terminal GalNAc
MAH	*Maackia amurensis*	Siaα2-3Galβ1-3(Siaα2-6)GalNAc
WGA	*Triticum unlgaris*	Chitin oligomers, Sia
GS-I-A4	*Griffonia simplicifolia* I-A4	α-Linked GalNAc
GS-I-B4	*Griffonia simplicifolia* I-B4	α-Linked Gal

These data was collected by Moritex Corporation

then incubated for 2 h at room temperature, to inactivate the residual fluorescent reagent completely. To each well on the glass slide, 60 μl of the Cy3-labeled glycoprotein solution was applied in triplicate and the solution was incubated overnight at 20 °C in a humidified chamber. After the binding reaction was complete, a fluorescence image of the array was acquired using an evanescent-field fluorescence scanner, Glycostation Reader (Moritex, Yokohama, Japan). All data were analyzed with Array Pro analyzer version 4.5 (Media Cybernetics, Bethesda, MD, USA). The acquired data were differentially analyzed using normalized signals obtained for all lectins, with the lectin showing the strongest signal intensity (max intensity) being assigned a value of 1.0. Table 1 lists the lectins we used.

Results and discussion

Analysis of each sample

Glycoantigens are major obstacles to xenotransplantation. Even after producing GKO pigs, non-Gal antigens remain on the surface of pig cells. Lectin microarray analyses were first performed to assess the changes in glycan structures between WT and GKO pigs, and their differences in pigs vs. humans were then examined. The experiments were performed three times using a different source of WT pig and human samples in each run. Concerning the GKO-pig, only one line has been established in our institute, and islets from the same pig were divided into three samples, which were then used in each experiment (Fig. 1a–c). To compare each result, we summarized the data in Fig. 2a, b and Table 2. Comprehensive statistical analyses were difficult because of the limited sample numbers. Thus, we applied the distinct difference in values that shows over twice the gap.

Changes of the glycans in GKO pigs

GKO islets showed weaker signals, downregulated by over 50 % for PNA, WFA, PTL-I, GS-I-A4, and GS-I-B4, and by almost 50 % for EEL. On the other hand, the signal for VVA was increased by over twice the values by the knocking down of the α-Gal (Fig. 2). The changes in EEL and GS-I-B4 represent the direct effect of the knocking out of α-Gal, because both lectins bind to α-Gal.

Similar to the changes in endothelial cells and fibroblasts of the GKO pigs in our previous study [20], the values of the lectins related to GalNAc, such as GS-I-A4 and PTL-I, were clearly down-regulated. Moreover, Galβ1-3GalNAc [21], for which PNA and WFA have specificity, was also reduced in the GKO-API. Whether these changes, in addition to α-Gal, are related to the antigenicity of pigs to humans is unknown. On the other hand, the value for VVA, which binds to α-linked terminal GalNAc and GalNAcα1-3Gal units, was elevated. However, considering the changes in GS-I-A4 and PTL-I, the elevation of VVA suggests that the changes in GalNAcα1-3Gal are unrelated to the terminal GalNAc, the Thomsen–Friedenreich (T–Tn) antigen. Fortunately, this antigen was reduced in the GKO pigs. The changes in GalNAcα1-3Gal might be related to the antigenicity of pig tissue to humans [20, 22].

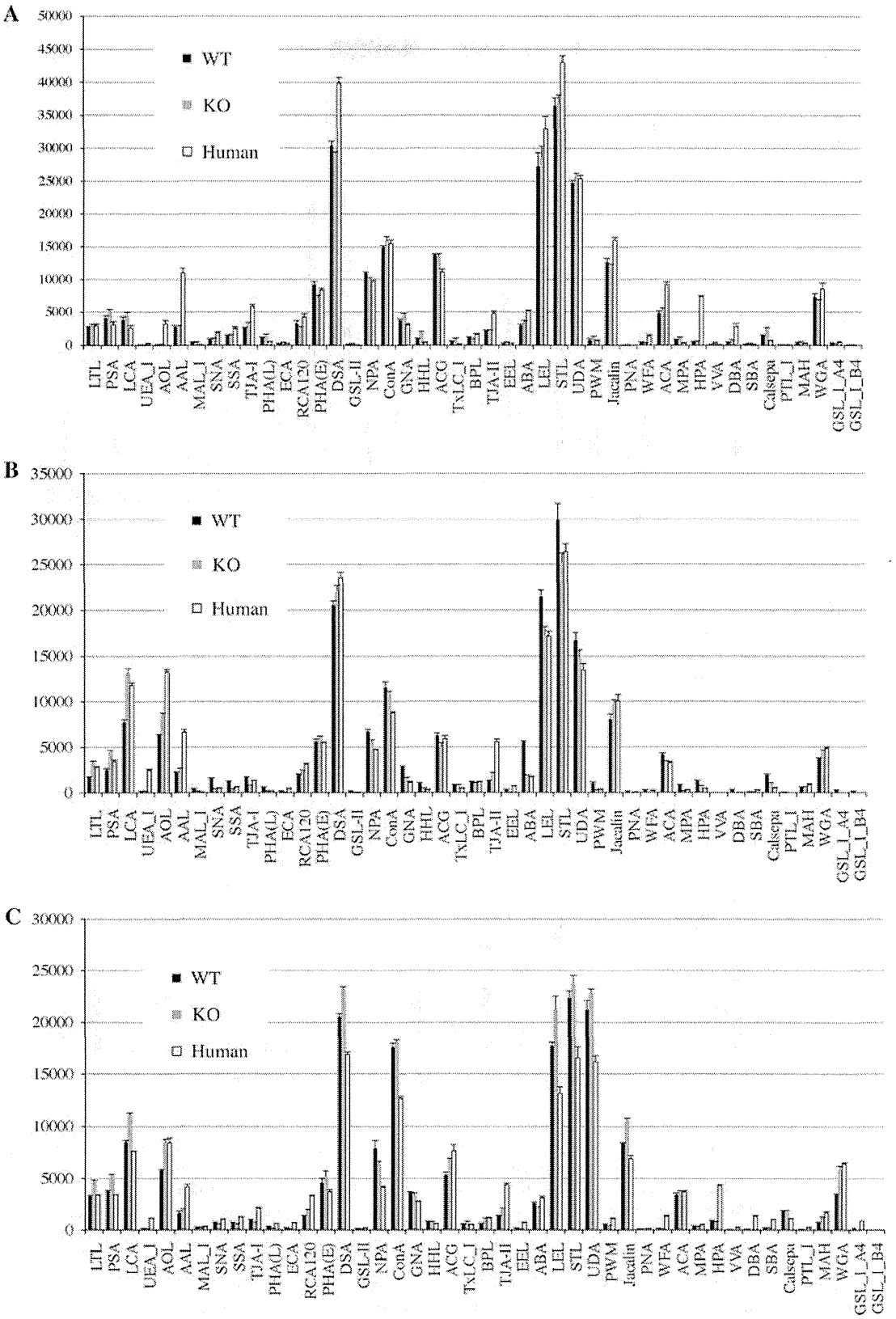
Fig. 1 Comparison of glycan profiles among the wild-type (WT) and ▶ α1-3galactosyltransferase gene knockout (GKO) pig and human islets. **a** Net intensity. The differences in the islets among the WT and GKO pigs and humans were analyzed. *Columns* indicate the signal intensity for experiment 1 and are the average ± SD of triplicate determinations. **b** Net intensity. The differences in the islets among the WT and GKO pigs and humans were analyzed. *Columns* indicate the signal intensity for experiment 2 and are the average ± SD of triplicate determinations. **c** Net intensity. The differences in the islets among the WT and GKO pigs and humans were analyzed. *Columns* indicate the signal intensity for experiment 3 and are the average ± SD of triplicate determinations

Differences between WT-API and GKO-API and human islets in the reactivity of a lectin

UEA-I, AAL, TJA-II, EEL, WFA, HPA, DBA, SBA, and PTL-I are strongly bound to human islets and the differences in their values were more than double those for both APIs. On the other hand, the values for MAL-I, HHL, Calsepa, and GS-I-B4 were higher in both APIs than in humans (Fig. 2; Table 2).

The data for UEA-I, AAL, and TJA-II indicate that human islets are rich in fucose. While EEL has specificity for Galα1-3Gal, it also binds strongly to the blood group B antigen. The high value of DBA in humans indicates the existence of the blood group A antigen in the human samples obtained from subjects with A (Fig. 1a), B (Fig. 1b) and AB (Fig. 1c) blood types. In our previous study, the values of the lectins HPA, SBA, PTL-I, as well as GS-I-A4, human endothelial cells, and fibroblasts, were lower in humans than in pigs [20]. It is noteworthy that the expression of lectins related to α-linked GalNAc is rich in human islets. On the other hand, because of the elevated values of NPA, ConA, GNA, HHL, and Calsepa in both APIs, the data indicate that both APIs are richer than human islets in high mannose form *N*-glycans. Moreover, the data for MAL-I suggest that of the sialyl glycans, α2-3 sialyl acid is rich in WT-API.

In relation to the non-Gal antigens, besides the Hanganutziu–Deicher (H–D) antigens such as α2-3- and α2-6-linked NeuGc [23–25], it was reported that patients who had received transplanted pig islets as therapy had Abs against terminal α-linked GalNAc units, especially the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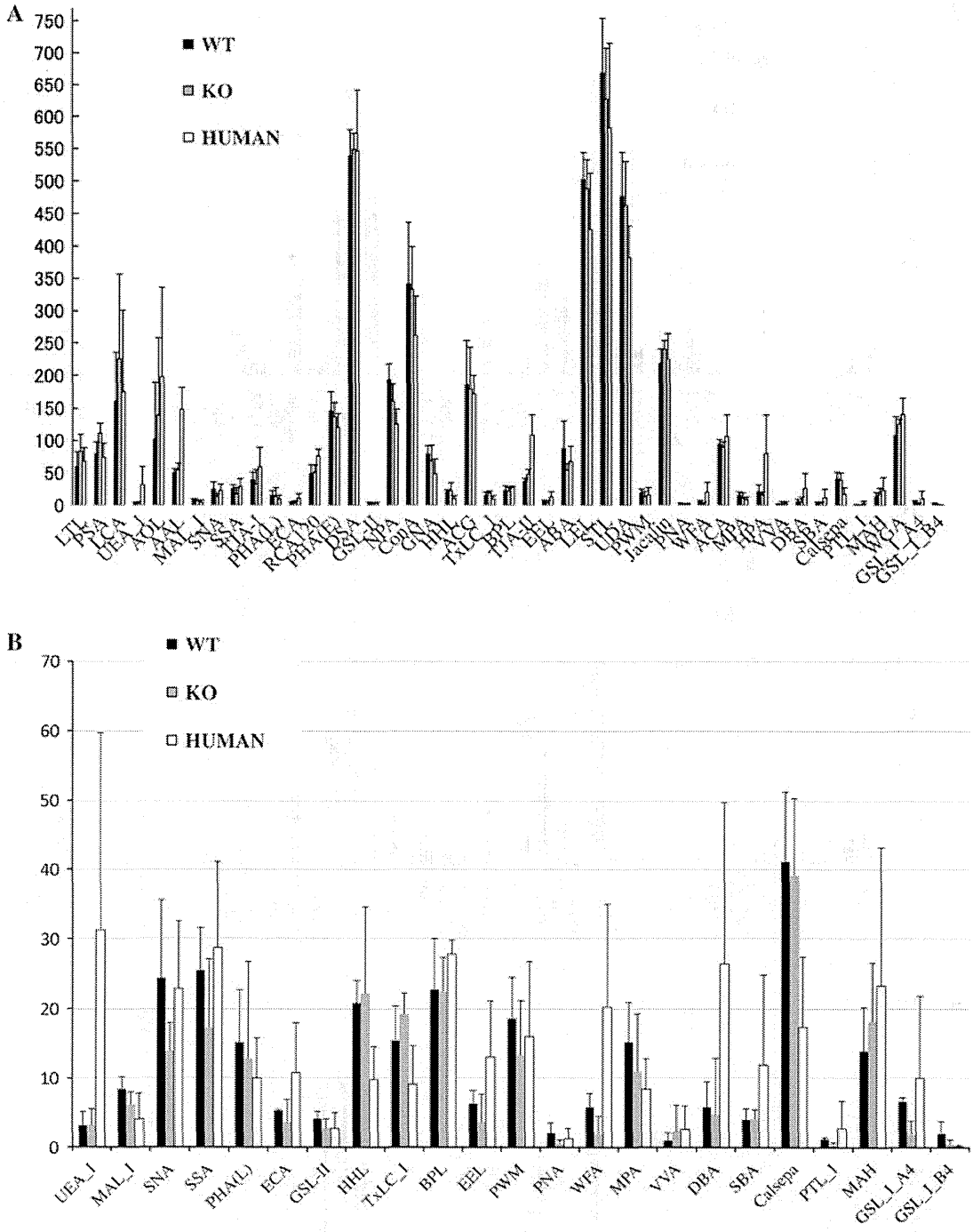

Fig. 2 Comparison of glycan profiles among the wild-type (WT) and α1-3galactosyltransferase gene knockout (GKO) pig and the human islets. **a** Summary of the normalized glycan intensity. The differences in the islets among the WT and GKO pigs and humans were analyzed. The obtained signals of net intensities were normalized by the total intensity of each experiment and the average value of three experiments was calculated. *Columns* indicate the signal intensity and are the average ± SD of three experiments. **b** Summary of the normalized glycan intensity for small peaks. *Columns* indicate the signal intensity and are the average ± SD of three experiments

Forssman epitope, Galα1-3Lew^x, Neu5Acα2-3Galβ1-3GlcNAc, and linear and branched oligomannose structures [21, 26, 27]. However, it was not possible to detect the H–D antigen in this study, because there is no available lectin that binds to NeuGc. Although the degree of antigenicity to humans remains unknown, it is certain that

Table 2 Summary of the actual values of the average of the normalized intensities

	WT	KO	Human
LTL	59.78 ± 22.87	83.36 ± 26.19	67.50 ± 21.05
PSA	79.90 ± 17.88	111.08 ± 15.26	73.50 ± 22.11
LCA	160.20 ± 75.83	225.42 ± 131.24	175.17 ± 125.68
UEA-I	3.16 ± 1.97	3.19 ± 2.36	31.30 ± 28.39
AOL	102.0 ± 86.62	139.01 ± 118.69	197.85 ± 137.77
AAL	49.82 ± 6.48	54.37 ± 10.36	147.36 ± 33.77
MAL-I	8.44 ± 1.75	6.20 ± 1.82	4.08 ± 3.74
SNA	24.40 ± 11.18	13.89 ± 4.07	22.89 ± 9.69
SSA	25.52 ± 6.08	17.23 ± 9.89	28.77 ± 12.35
TJA-I	38.73 ± 12.46	32.33 ± 22.49	59.21 ± 29.88
PHA(L)	15.16 ± 7.53	12.81 ± 13.93	10.02 ± 5.77
ECA	5.33 ± 0.14	3.62 ± 3.30	10.78 ± 7.17
RCA120	48.55 ± 14.07	51.62 ± 10.69	76.26 ± 10.18
PHA(E)	144.64 ± 30.54	136.87 ± 21.41	120.39 ± 20.92
DSA	538.46 ± 41.34	548.82 ± 25.11	546.27 ± 94.94
GSL-II	4.17 ± 0.97	2.74 ± 1.35	2.70 ± 2.30
NPA	193.10 ± 24.648	160.29 ± 26.20	125.03 ± 23.30
ConA	340.80 ± 95.58	332.86 ± 65.91	262.01 ± 60.31
GNA	78.67 ± 13.14	68.64 ± 23.71	48.53 ± 22.91
HHL	20.85 ± 3.18	22.20 ± 12.41	9.79 ± 4.76
ACG	184.78 ± 69.05	178.59 ± 64.47	171.96 ± 27.68
TxLC-I	15.42 ± 4.95	19.21 ± 3.05	9.09 ± 5.57
BPL	22.77 ± 7.26	22.45 ± 4.87	27.83 ± 2.00
TJA-II	36.26 ± 5.36	47.98 ± 7.29	108.29 ± 31.05
EEL	6.33 ± 1.87	3.55 ± 4.10	13.04 ± 8.05
ABA	87.00 ± 43.20	54.33 ± 10.09	67.94 ± 22.98
LEL	502.26 ± 40.94	488.57 ± 44.14	425.76 ± 86.56
STL	668.64 ± 84.80	627.05 ± 79.72	582.64 ± 132.00
UDA	476.55 ± 67.26	462.96 ± 67.08	382.33 ± 48.65
PWM	18.61 ± 5.89	13.29 ± 7.84	16.00 ± 10.761
Jacalin	218.27 ± 22.52	239.98 ± 13.65	225.14 ± 39.141
PNA	2.10 ± 1.39	0.50 ± 0.51	1.26 ± 1.44
WFA	5.786 ± 2.00	1.87 ± 2.61	20.26 ± 14.77
ACA	94.30 ± 7.22	89.752 ± 8.29	106.22 ± 33.16
MPA	15.25 ± 5.70	10.93 ± 8.35	8.38 ± 4.38
HPA	21.01 ± 10.45	16.26 ± 4.71	79.92 ± 59.28
VVA	0.94 ± 1.14	2.22 ± 3.85	2.57 ± 3.39
DBA	5.81 ± 3.63	4.71 ± 8.16	26.45 ± 23.27
SBA	4.01 ± 1.54	4.06 ± 1.38	11.90 ± 12.95
Calsepa	41.14 ± 10.07	39.08 ± 11.19	17.33 ± 10.06
PTL-I	1.11 ± 0.31	0.24 ± 0.43	2.69 ± 3.97
MAH	13.93 ± 6.26	18.11 ± 8.47	23.30 ± 19.86
WGA	107.17 ± 29.33	125.27 ± 7.25	139.96 ± 24.83
GS-I-A4	6.62 ± 0.55	1.81 ± 2.00	9.99 ± 11.77
GS-I-B4	1.99 ± 1.695	0.45 ± 0.62	0.14 ± 0.23

NeuGc is antigenic to humans [28]. On the other hand, the relationship between the high mannose form in both APIs and the reported oligomannose structure is unclear. A detailed analysis of this structure will be needed to confirm this. Our finding of APIs being rich in mannose is nevertheless considered to be of importance.

All three types of islets were cultured overnight in medium containing some kind of heterogenous sugar; therefore, there is some chance of human cells taking in a special monosaccharide, such as NeuGc. However, the changes in the glycosylation of the cells within one night were minimal and should not have affected the results of the lectin blot assay. Unfortunately, a specific structural analysis for each glycan was not possible in this study, but future studies will include HPLC analyses to find the key glycan of the API that produces the antigenicity to humans. Several reports have been published on HPLC analysis [22]; however, most have proposed the existence of each glyco-structure as a non-Gal antigen, so more detailed structural analyses are needed. From these, we will try to identify the pig genes that produce the non-Gal antigenicity. As a next step, we will try to produce new KO pigs using a nuclear transfer technique.

In conclusion, in spite of the negligible expression of the Gal antigen on WT-API, α-linked GalNAc and Galβ1-3GalNAc were reduced in the GKO-API. In comparing pigs and humans, the high-mannose form is originally richer in both APIs than in humans, whereas human tissue appears to contain higher levels of α-linked GalNAc, possibly in the form of an *N*-glycan as a feature of the islet tissue.

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Conflict of interest None of the authors has a conflict of interest.

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RESEARCH ARTICLE

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Characterization of the ICSI-Mediated Gene Transfer Method in the Production of Transgenic Pigs

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SUMMARY

Understanding the behavior of transgenes introduced into oocytes or embryos is essential for evaluating the methodologies for transgenic animal production. We investigated the expression pattern of a transgene transferred to porcine eggs by intracytoplasmic sperm injection-mediated gene transfer (ICSI-MGT) or pronuclear microinjection (PN injection). The introduction of the EGFP gene by ICSI-MGT yielded significantly more embryos with non-mosaic transgene expression ($P < 0.01$). In the ICSI-MGT group, 61.5% (24/39) of the embryos were EGFP-positive in all their component blastomeres at the morula stage, while fewer than 10% of such embryos were EGFP-positive in the PN-injection group. Using three types of transgenes, ranging from 3.0 to 7.5 kb in size, we confirmed that approximately one in four fetuses obtained by ICSI-MGT was transgenic, suggesting that ICSI-MGT is a practical method for transgenic pig production. Southern blot analysis of 12 transgenic fetuses produced by ICSI-MGT revealed that the number of integrated transgene copies varied from 1 to 300, with no correlation between transgene size and the number of integrated copies. Fluorescence in situ hybridization analysis revealed that the transgenes were randomly integrated into a single site on the host chromosomes. Together, these data indicate that multiple-copy, single-site integration of a transgene is the primary outcome of ICSI-MGT in the pig and that ICSI-MGT is less likely than PN injection to cause transgene integration in a mosaic manner.

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INTRODUCTION

With the advances made in biomedical research in recent years, pigs have attracted increasing attention

Abbreviations: EGFP, enhanced green fluorescent protein; FISH, fluorescence in situ hybridization; ICSI [-MGT], intracytoplasmic sperm injection [-mediated gene transfer]; IVF, in vitro fertilization; PN injection, pronuclear microinjection; SCNT, somatic cell nuclear transfer.

as a potentially valuable experimental animal model for many preclinical applications. Their value for research is enhanced by the physiological and anatomical similarities between pigs and humans (Lunney, 2007). Additionally, transgenic porcine models of human disease are expected to provide a substantial contribution to the development of new treatments and drugs (Petters et al., 1997; Rogers et al., 2008; Umeyama et al., 2009). Genetically modified pigs that act as a source of organs/tissues for xenotransplantation or tissue engineering have also been investigated (Matsunari and Nagashima, 2009; Woo et al., 2009). Furthermore, transgenic pigs that can be used as bioreactors for producing therapeutic proteins may have a significant impact on the pharmaceutical industry (Van Cott et al., 1999). Yet, genetically modified pigs that fulfill all the required features for a model system (e.g., those that faithfully mimic the serious symptoms of human diseases) may require repeated trials for their successful establishment. In addition, a significant effort and cost is required to establish and maintain genetically modified animals of a large domestic species compared to rodent species (Wall, 1997). Therefore, it is important to maximize the efficiency of the methods used to produce genetically modified pigs.

Currently, various techniques can be used to create transgenic animals. DNA pronuclear microinjection (PN injection), for example, is a widely used technique in mice, rabbits, sheep, and pigs (Gordon et al., 1980; Hammer et al., 1985). Another technique with a high application potential is somatic cell nuclear transfer (SCNT), using donor nuclei with genetic modifications (Wilmut et al., 1997; Hyun et al., 2003). In this technique, an exogenous gene is introduced into nuclear donor cells, and the integration and expression of the gene are checked at the cellular level; suitable cells are then used to create clones. This technique can be used not only to introduce an exogenous gene but also to "knockout" an endogenous gene (Lai et al., 2002).

Lavitrano et al. (1989) proposed sperm-mediated gene transfer as a relatively easy gene introduction technique. In this method, in vitro fertilization (IVF) or artificial insemination is performed using sperm to which exogenous DNA is attached. The technique is based on the concept of using sperm as a vector for foreign DNA, and Lavitrano et al. demonstrated that transgenic mice and pigs could be created by this method (Lavitrano et al., 1989; Sperandio et al., 1996). In an alternative approach, Perry et al. (1999) generated transgenic mice by intracytoplasmic sperm injection (ICSI) using sperm that had been co-incubated with an exogenous transgene. This technique is termed ICSI-mediated gene transfer (ICSI-MGT) and has proved to be a viable method for creating transgenic animals, not only in mice, but also in rats and pigs (Kato et al., 2004; Yong et al., 2006).

More recently, we showed that disease-model pigs could be produced very efficiently using a method that combines ICSI-MGT with SCNT (Umeyama et al., 2009). We generated transgenic fetal pigs carrying the human mutant hepatocyte nuclear factor 1α (HNF1α) gene, a gene associated with the development of diabetes, using ICSI-MGT. We then cloned transgenic pigs by SCNT of fibroblast cells established from the transgenic fetuses. The cloned pigs that shared the same genetic background could then be used to examine the characteristics of the diabetes induced by the genetic modification. In other words, by combining the easy and reliable ICSI-MGT method with SCNT, we developed an approach that is highly practical for the creation of disease-model pigs. It is essential to characterize all aspects of gene transfer by ICSI-MGT before applying this system more generally for producing genetically modified pigs.

To date, we have obtained interesting findings from transgenic cloned pigs produced by ICSI-MGT combined with SCNT. We created a total of 43 transgenic cloned pigs from two different lines of transgenic fetal cells obtained by ICSI-MGT, and all the clones contained the expected transgene (Kurome et al., 2006; Umeyama et al., 2009). This finding suggested that the transgene was integrated into fetal tissues after ICSI-MGT in a non-mosaic manner. In the present study, we tested that this conclusion was correct by comparing the in vitro expression patterns of a transgene introduced into porcine eggs using either ICSI-MGT or PN injection. The integration pattern of the transgene introduced into porcine IVM oocytes by ICSI-MGT was determined by fluorescence in situ hybridization (FISH) and flow cytometric analysis of the resultant fetuses.

RESULTS

EGFP Expression in Transgenic Porcine Embryos Produced Using ICSI-MGT or PN Injection

The in vitro development of porcine embryos after introduction of the EGFP gene by ICSI-MGT or PN injection is shown in Table 1. In total, 314 oocytes received the EGFP gene by ICSI-MGT, 128 (40.8%) of these developed to the morula stage, and 39 (12.4%) exhibited EGFP expression in the whole or on some parts of the embryo (EGFP-positive embryos). By comparison, of the 128 embryos that received a PN injection 12 hr after IVF, 44 (34.4%) reached the morula stage, and 17 (13.3%) of these were EGFP-positive. Thus, the proportions of EGFP-positive embryos were similar in these two groups. Although developmental rate of the embryos to the morula stage was similar when PN injection was performed 20 hr after IVF (71/177; 40.1%), the proportion of EGFP-positive embryos was significantly lower (12/177, 6.8%, $P < 0.05$).

All the blastomeres from EGFP-positive morulae were isolated to determine the proportion of blastomeres that were positive for EGFP expression (Fig. 1). In the ICSI-MGT group, 61.5% (24/39) of the embryos were EGFP-positive in all of their blastomeres (Fig. 1A–D; Table 1). Conversely, only 5.9% of embryos (12 hr injection group) and 8.3% (20 hr injection group) of the morulae in the PN injected groups were EGFP-positive in all of their blastomeres; these proportions were significantly lower ($P < 0.01$) than that obtained by the ICSI-MGT method (Table 1).

The frequencies of embryos in which the majority (81% or more) of the component blastomeres were EGFP-positive were compared between the ICSI-MGT

TABLE 1. In Vitro Development and Occurrence of Mosaic EGFP Expression in Porcine Embryos Following Gene Introduction by the ICSI-MGT or PN-Injection Method

Transgene injection methods	Oocytes or embryos injected	Embryos developed to morulae (%)[a]	EGFP-positive embryos (%)[b]	Embryos consist of only EGFP-positive blastomeres (%)[c]
ICSI-MGT	314	128 (40.8)	39 (12.4)[e]	24/39 (61.5)[g]
PN-injection; 12 hr[d]	128	44 (34.4)	17 (13.3)	1/17 (5.9)[h]
PN-injection; 20 hr[d]	177	71 (40.1)	12 (6.8)[f]	1/12 (8.3)[h]

e,f: different letters within columns denote significant differences ($P < 0.05$).
g,h: different letters within columns denote significant differences ($P < 0.01$).
[a]Percentage of morulae/injected embryos.
[b]Percentage of EGFP-positive embryos/injected embryos.
[c]Percentage of embryos consist of only EGFP-positive blastomeres/analyzed EGFP-positive embryos.
[d]Time after fertilization.

and PN injection groups. In the ICSI-MGT group, 82% of embryos had 81–100% EGFP-positive blastomeres, whereas in the PN injection groups the frequencies were 5.9% (12 hr injection group) and 8.3% (20 hr injection group); these PN frequencies were significantly lower than those obtained by the ICSI-MGT method ($P < 0.01$). In addition, 70% of the morulae of the PN injection groups had 40% or fewer EGFP-positive blastomeres, indicating frequent occurrences of mosaic expression.

Characteristics of Transgenic Fetus Production by ICSI-MGT

The efficiency of ICSI-MGT in producing transgenic porcine fetuses was examined using three transgenes that ranged in size from 3.0–7.5 kbp: CAG-EGFP, PI-HNF1-αP291fsinsC, and α-myosin heavy chain (α-MHC)-calreticulin (Table 2). Transfer of 1,264 embryos that had been injected with one of the three transgenes into 12 recipients gave rise to 57 fetuses (Table 2). The proportion of embryos developing into fetuses after transfer was similar among the transgenes, ranging from 4.1% (17/417, CAG-EGFP) to 5.1% (23/454, α-MHC-calreticulin). Furthermore, the production efficiency of transgenic fetuses was similar among the transgenes: 23.5% (4/17) for CAG-EGFP; PI-HNF1αP291fsinsC; and 30.4% (7/23) for α-MHC-calreticulin.

Primary fibroblast cultures were successfully established from 13 of the 15 transgenic fetuses (Table 3). Southern-blot analysis of the cells showed that the number

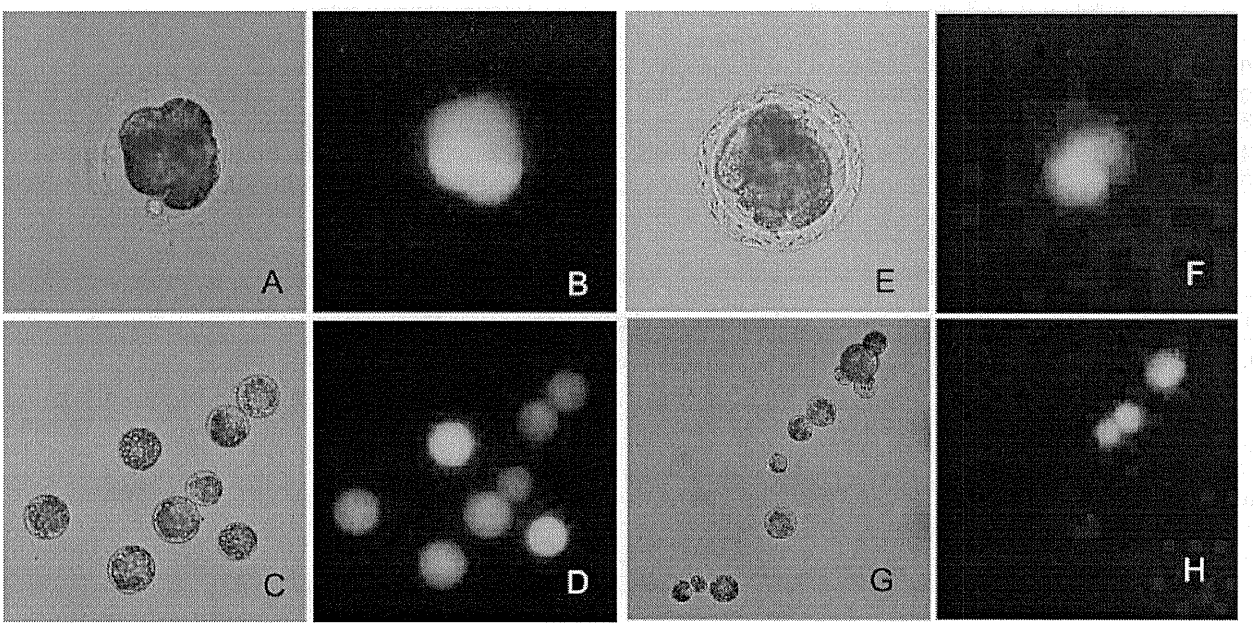

Figure 1. EGFP expression in porcine morulae following gene transfer using ICSI-MGT. **A–D**: Bright field (A) and fluorescence microscopy (B) images of a morula showing EGFP expression throughout the embryo. EGFP expression was present in all blastomeres of this embryo (C,D). **E–H**: Bright field (E) and fluorescence microscopy (F) images of a morula showing EGFP expression in part of the embryo. EGFP expression was present only in some blastomeres of this embryo (G,H), indicating mosaic transgene expression.