In vitro and *in vivo* expression of Gal α -(1,3)Gal on porcine islet cells is age dependent

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Abstract

The expression of Gal α -(1,3)Gal (α Gal) on porcine islet cells remains controversial. Several groups have reported that porcine islet endocrine cells do not express α Gal while we have shown in neonatal porcine islets (NPI) that β cells do express this antigen. We hypothesize that endocrine cells expressing α Gal on NPI are less mature cells that may have originated from ductal cells and that expression of this antigen disappears as they develop into fully mature β cells. Thus, we further examined α Gal expression on various porcine islet cell preparations and correlated this with the proportion of cytokeratin 7 (CK7)-positive ductal cells. *In vitro* and *in vivo* expression of α Gal and CK7 was significantly ($P < 0.05$) higher in less mature NPI cells

compared with matured NPI and adult porcine islet cells while the reverse was observed in the proportion of β cells. Moreover, a significantly higher proportion of CK7-positive cells was detected in the Gal-expressing population compared with non-expressing cells. In contrast, a higher proportion of β cells was observed in the Gal-negative population compared with the Gal-positive population. These data showed a reduced expression of α Gal and CK7 as porcine islet cells mature into β cells suggesting a possible role for α Gal in the maturation of pancreatic endocrine β cells.

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Introduction

Insulin independence in type 1 diabetes patients can now be achieved in a safe and reproducible manner when a sufficient islet cell mass (>9000 islets/kg body weight) is transplanted; however, this often requires more than one donor pancreas (Shapiro *et al.* 2000, Ryan *et al.* 2001). If islet transplantation is to become a widespread treatment for the majority of type 1 diabetes patients the supply of human pancreatic islet tissue will become a limitation. The shortage of available human allogeneic tissues for clinical transplantation has resulted in considering other sources of insulin-producing tissue. Porcine islets represent a practical alternative source of insulin-producing tissue since pigs are inexpensive, readily available, and they exhibit physiological and morphological characteristics comparable with humans. Adult porcine islets have been extensively studied; however, in most laboratories they are difficult to isolate or maintain in tissue culture. In contrast, we have previously developed a simple procedure for isolating abundant islets from neonatal pigs (Korbitt *et al.* 1996). Our group (Korbitt *et al.* 1996, Rayat *et al.* 2000) and

others (Yoon *et al.* 1999, Trivedi *et al.* 2001) have shown that neonatal porcine islets (NPI) are capable of correcting diabetes in immunoincompetent mice. In addition, we have also shown that these cell aggregates are composed of approximately 40–45% endocrine cells as well as 50% ductal cells (Korbitt *et al.* 1996), suggesting the enormous growth potential of this tissue.

One of the main obstacles in porcine-to-human xenotransplantation is the occurrence of hyperacute rejection. The carbohydrate moiety Gal α -(1,3)Gal (α Gal) is highly expressed on the surface of porcine endothelial cells and is immunoreactive to human preformed natural antibodies. This reaction leads to activation of the recipient's complement system and rapid destruction of the xenograft. The expression of α Gal on specific porcine islet endocrine cells remains unclear. Some studies have shown that α Gal is not expressed on adult or fetal porcine islet endocrine cells (Oriol *et al.* 1993, McKenzie *et al.* 1994, 1995a,b), while we have demonstrated that α Gal is expressed in both insulin- and glucagon-producing neonatal porcine islet cells (Rayat *et al.* 1998). A recent study has shown that fetal porcine islet cells consistently express high levels of

α Gal epitope, whereas most adult islet cells are void of this epitope (Bennet *et al.* 2000). The same group has also demonstrated that the strongest α Gal antigen expression was detected in capillary endothelial cells, duct epithelial cells, and occasional macrophages in fetal porcine islet cell cluster preparations (Strokan *et al.* 2000) while mature endocrine-like cells were completely negative for the α Gal antigen expression. However, some immature endocrine cells, scattered between the duct epithelial cells or inside the clusters, were weakly positive for α Gal antigen. Based on our previous study (Rayat *et al.* 1998), we hypothesize that immature endocrine cells and some ductal cells present in NPI are the cells that we previously detected to express α Gal antigen. To test this hypothesis, we further examined the presence of α Gal in NPI and correlated this expression with cytokeratin 7 (CK7)-positive ductal cells in various porcine islet cell preparations. The preparations include (1) islet cells isolated and cultured from 3-day-old neonatal pigs (non-mature NPI), (2) microencapsulated NPI (*in vitro* matured) and (3) NPI grafted into non-diabetic SCID mice (*in vivo* matured NPI) as well as (4) adult porcine islets.

We report here a direct correlation between α Gal and CK7 expression on porcine islet cells both *in vitro* and *in vivo*. Our data show a reduction in the expression of α Gal and CK7 as porcine islet cells mature into insulin-producing cells. This suggests a possible role for α Gal in the maturation of pancreatic endocrine β cells and may explain in part why in some studies the expression of α Gal on endocrine cells was not detected. We also demonstrate for the first time an *in vitro* method of increasing the proportion of β cells in NPI using alginate capsules and further culture of islets in medium supplemented with autologous serum. This may have implication(s) for the choice of NPI as alternative islets for clinical transplantation.

Materials and Methods

Preparation of non-mature NPI and adult porcine islets

As previously described (Korbitt *et al.* 1996), Landrace–Yorkshire neonatal pigs aged 1–3 days old (1.5–2.0 kg body weight) of either sex were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreases were removed, cut into small pieces and digested with 2.5 mg/ml collagenase (Sigma, St Louis, MO, USA). After filtration through a nylon screen (500 μ m), non-mature spherical-shaped NPI were formed during 9 days of suspension culture in Ham's F10 medium (Gibco, Burlington, Ontario, Canada) containing 10 mmol/l glucose, 50 μ mol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, Ontario, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 10 mmol/l nicotinamide (BDH Biochemical, Poole, Dorset, UK), 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Adult porcine islets were isolated in Minneapolis from miniature swine Troll (age 18–19 months) as previously described (Hering *et al.* 1995). The pancreases were intraductally distended with University of Wisconsin solution (Du Pont, Bad Homburg, Germany) containing 0.4% (w/v) collagenase (Serva, Heidelberg, Germany) and digested in a continuous digestion-filtration device at 32 °C. Islets were purified using Ficoll–Na-diatrizoate density gradients on a Cobe 2991 cell separator (Cobe BCT, Inc., Lakewood, CO, USA) and then cultured at 37 °C for 24 h. Following culture, islets were shipped to Edmonton, and maintained in culture for an additional 24–48 h prior to being assessed, as described below.

In vitro maturation of NPI

After 9 days of culture, non-mature NPI were microencapsulated by suspension in 1.0 ml 0.72% (w/v) highly purified alginate dissolved in Hank's balanced salt solution (Rayat *et al.* 2000). Microcapsules (250–400 μ m) were formed by passing the alginate/islet suspension through an electrostatic generator followed by collection in a 120 mmol/l CaCl_2 solution containing 10 mmol/l HEPES and 0.01% Tween 20 for 10 min to allow formation of capsules. The capsules were washed by gravity sedimentation in supplemented Ham's F10 solution and cultured in the same medium with 10% autologous pig serum for an additional 7 days at 37 °C. After culture, alginate capsules were dissolved by incubating the encapsulated islets in calcium-free medium for 15 min at 37 °C. The *in vitro* matured islets were washed with calcium-free medium then dissociated into single cells as described below.

Preparation of single islet cell suspensions

Non-mature and *in vitro* matured NPI as well as adult porcine islets were dissociated into single cells by gentle agitation in calcium-free medium containing 15 μ g/ml trypsin (Boehringer Mannheim, Laval, Quebec, Canada) and 4 μ g/ml DNase (Boehringer Mannheim). The cell suspension was filtered through a 63 μ m nylon screen to remove cell clumps and then centrifuged through Percoll (Sigma) of 1.040 g/ml density to eliminate dead cells and debris. Cells were then washed with phosphate-buffered saline and analyzed either by flow cytometry or placed on microscopic glass slides (FGR Steinmetz Inc., Surrey, British Columbia, Canada) which were then fixed in Bouin's solution for 30 min, washed with 70% ethanol, and stored at 4 °C prior to morphological assessment.

In vivo maturation of neonatal porcine islets

Non-mature NPI were transplanted under the left kidney capsule of non-diabetic C.B-17 SCID mice (C.B-*Igh-1^b* GbmsTac-*Prkdc^{scid}*-*Lys^{tg}* N7; Taconic Farms Inc., Germantown, NY, USA). Aliquots of 2000 NPI were aspirated into polyethylene tubing (PE-50), and spun to

Table 1 Cellular composition of non-mature, *in vitro*-matured neonatal porcine and adult pig islets. The numbers in parentheses represent the number of independent samples

	Percent of total cells				
	α Gal	Vimentin	CK7	Insulin	Glucagon
Islets					
Non-mature NPI	19.7 $\pm$ 3.3 ^a (14)	15.0 $\pm$ 3.9 ^b (12)	28.0 $\pm$ 4.2 ^{c,d} (14)	33.7 $\pm$ 3.8 ^e (13)	19.5 $\pm$ 3.0 (13)
<i>In vitro</i> -matured NPI	11.0 $\pm$ 2.8 (7)	7.6 $\pm$ 1.3 (7)	10.5 $\pm$ 1.8 (9)	42.1 $\pm$ 3.1 ^f (9)	23.3 $\pm$ 1.4 (9)
Adult pig	5.1 $\pm$ 1.3 (6)	1.4 $\pm$ 0.5 (4)	1.3 $\pm$ 0.5 (6)	82.8 $\pm$ 1.2 (6)	9.7 $\pm$ 3.2 (6)

Islets were dissociated into single cells and fixed in Bouin's solution. Cells were then stained with appropriate antibodies as described in Materials and Methods.

^a $P < 0.05$ vs *in vitro*-matured and adult pig; ^b $P < 0.05$ vs *in vitro*-matured and adult pig; ^c $P < 0.01$ vs *in vitro*-matured pig; ^d $P < 0.001$ vs adult pig; ^e $P < 0.001$ vs adult pig; ^f $P < 0.001$ vs adult pig (ANOVA).

Table 2 Percentage of CK7- and insulin-positive cells in α Gal-expressing and non-expressing NPI cells. Values are means $\pm$ S.E.M.

	<i>n</i>	Percentage of total cells		
		CK7 ^a	Insulin ^b	CK7/insulin
Cell population				
α Gal-positive	7	24.4 $\pm$ 10.4	11.7 $\pm$ 2.1	0.7 $\pm$ 0.2
α Gal-negative	9	1.8 $\pm$ 0.7	32.4 $\pm$ 3.8	1.4 $\pm$ 0.9

Cells expressing and not expressing α Gal were collected and fixed in Bouin's solution and then stained for CK7 and/or insulin.

^a $P < 0.003$; ^b $P < 0.005$ (Mann-Whitney *U* rank sum test).

form a pellet. The pellet of islets was gently placed under the kidney capsule with the aid of a micromanipulator syringe. Once the tubing was removed, the capsulotomy was sealed with a disposable high-temperature cautery pen (Aaron Medical Industries, St Petersburg, FL, USA). Graft-bearing kidneys were harvested on days 1, 7, 30, 50, and more than 200 days post-transplantation. Tissues were fixed in Bouin's solution, embedded in paraffin, and sectioned for morphological assessment. All recipient mice were cared for according to the standard set by the Canadian Council on Animal Care.

Histological analysis

Expression of α Gal on single islet cells and paraffin graft sections (4 μ m) was detected using biotinylated α Gal-specific BS-1 isolectin B4 from *Bandeiraea simplicifolia* (1:25 dilution; Sigma; see Rayat *et al.* 1998). CK7 expression was detected by treating the paraffin graft sections with 0.1 M citrate buffer. The sections were then heated for 1.5 min using a domestic microwave. Single islet cell preparations were exposed to citrate buffer and heated for 30 s. CK7 was detected using a monoclonal mouse anti-human CK7 antibody (1:200 dilution; Dako Diagnostics Canada Inc., Mississauga, Ontario, Canada) and biotinylated anti-mouse IgG secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA, USA). Monoclonal mouse anti-porcine vimentin (1:1000; Dako Diagnostics) was used as a marker for mesenchymal and/or rapidly dividing cells.

The presence of pancreatic hormones was determined using guinea pig anti-porcine insulin antibody (1:1000 dilution; Dako Diagnostics) and a rabbit anti-porcine glucagon antibody (1:1000 dilution; Dako Diagnostics). Secondary antibodies were biotinylated goat anti-guinea pig IgG (1:200; Vector Laboratories) and goat anti-rabbit IgG (1:200 dilution; Vector Laboratories). The avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories) method with 3,3'-diaminobenzidine tetrahydrochloride (DAB; BioGenex, San Ramon, CA, USA) was used to visualize the positive reaction (brown color).

For double staining, paraffin sections were stained as described above with either biotinylated α Gal-specific lectin or mouse anti-human CK7 antibody for 30 min followed by 20 min with the appropriate biotinylated secondary antibody. The ABC/HP complex using DAB as chromagen was used to detect cells expressing α Gal or CK7. The same tissue sections were subsequently stained with guinea pig anti-porcine insulin antibody and biotin-conjugated goat anti-guinea pig secondary antibody. The avidin-biotin complex/alkaline phosphatase (Vector Laboratories) method and Fast Red as chromagen (Vector Laboratories) were used to obtain a red reaction.

Fluorescence-activated cell sorter (FACS) analysis

To further characterize the expression of α Gal on non-mature NPI, single islet cell suspensions were stained with fluorescein isothiocyanate (FITC)-conjugated BS-1

isolectin B4 (1:50 dilution; Sigma) for 1 h on ice, washed three times, and analyzed by flow cytometry (EPICS Elite ESP flow cytometer; Coulter, Hialeah, FL, USA). Cells expressing or not expressing α Gal were collected and subsequently stained for the co-expression of CK7 and/or insulin as described above. Positive controls for this experiment included porcine aortic endothelial cells stained with FITC-conjugated IB4 lectin using the same protocol for islet cells.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. of at least four independent observations. Significant differences between experimental groups were tested using ANOVA and unpaired *t*-test. A *P* value of 0.05 was considered to be statistically significant.

Results

Cellular composition of neonatal and adult porcine islets

The cellular composition of non-mature and *in vitro* matured NPI as well as adult porcine islets was assessed with respect to the proportion of cells expressing insulin, glucagon, α Gal, CK7 and vimentin (Table 1). Immunostaining for the expression of α Gal revealed a significantly ($P < 0.05$) higher proportion of α Gal-expressing cells in non-mature NPI ($19.7 \pm 3.3\%$) compared with those islets that were further matured *in vitro* ($11.0 \pm 2.8\%$). Moreover, this expression was further reduced in adult porcine islets ($5.1 \pm 1.3\%$). A similar pattern of expression was observed for CK7 and vimentin. The percentage of CK7- and vimentin-positive cells was significantly higher in non-mature NPI than in both *in vitro* matured NPI and adult pig islets. Non-mature NPI consisted of $28.0 \pm 4.2\%$ CK7-positive cells compared with 10.5 ± 1.8 and $1.3 \pm 0.5\%$ for *in vitro* matured NPI and adult pig islets respectively. Vimentin-positive cells in non-mature NPI was approximately twofold (15.0 ± 3.9 vs $7.6 \pm 1.3\%$) and tenfold (15.0 ± 3.9 vs $1.4 \pm 0.5\%$) higher than that observed in both *in vitro* matured NPI and adult pig islets respectively. In contrast to the reduction in α Gal, vimentin and CK7 expression, a significant increase ($P < 0.001$) in the proportion of insulin-positive cells in highly matured islet cell preparations was observed. In particular, non-mature NPI contained $33.7 \pm 3.8\%$ insulin-positive

cells compared with 42.1 ± 3.1 and $82.8 \pm 1.2\%$ in both *in vitro* matured NPI and adult porcine islets respectively.

Expression of CK7 and insulin in α Gal-expressing and non-expressing NPI cells

Following FACS analysis of dissociated non-mature NPI cells that had been stained with FITC-conjugated IB4-lectin, Gal-expressing and non-expressing cell populations were collected and the proportion of CK7- and insulin-positive cells in these two populations was determined by immunocytochemical staining (Table 2). The α Gal-expressing population was comprised of a significantly ($P < 0.003$) higher proportion of CK7-positive cells ($24.4 \pm 10.4\%$) than that observed in the α Gal-negative cell fraction ($1.8 \pm 0.7\%$). In contrast, the α Gal non-expressing cell population consisted of a significantly ($P < 0.005$) higher proportion of insulin-positive β cells than those present in the α Gal-positive cell fraction (32.4 ± 3.8 vs $11.7 \pm 2.1\%$ respectively). There was no significant difference in the number of cells staining double positive for both CK7 and insulin in either the α Gal-expressing or non-expressing populations (0.7 ± 0.2 vs $1.4 \pm 0.9\%$ respectively). These data suggested that a proportion of less mature endocrine β cells can express the α Gal xenoantigen while the majority of mature endocrine β cells lose the expression of this antigen.

In vivo expression of α Gal and CK7

To examine the fate of α Gal and CK7 expression *in vivo*, non-mature NPI were transplanted and allowed to mature under the kidney capsule of non-diabetic C.B-17 SCID mice. On day 1 post-transplantation, a high proportion of α Gal-positive cells were observed in the graft (Fig. 1A and B). A strong reaction of IB4 lectin with ductal epithelium and some centrally located islet β cells was noted. A similar intensity of positive immunostaining reaction for α Gal was also observed on day 7 (data not shown). However, by day 30 post-transplantation (Fig. 1C and D) a reduction in positive staining for α Gal was observed and this continued to decrease by day 50 post-transplantation (Fig. 1E and F). At these times, α Gal-expressing cells were mostly concentrated on luminal structures and some cells stained for both α Gal and insulin were still present. More than 200 days post-transplantation, the α Gal-positive cells were almost undetectable in the grafts while numerous clusters of β cells stained positive for insulin comprised the majority of the graft (Fig. 1G and H).

Figure 1 α Gal and insulin staining on NPI xenografts following transplantation into C.B-17 SCID mice. Grafts were harvested on days 1 (A and B), 30 (C and D), 50 (E and F) and more than 200 days (G and H) post-transplantation. α Gal-positive cells are shown as brown structures whereas insulin-positive cells are stained red. α Gal expression was highly expressed on day 1 then decreased by day 30 and day 50 and was subsequently non-detectable more than 200 days post-transplantation. Scale bars represent 100 μ m for A, C, E and G and 50 μ m for B, D, F and H.

To determine whether an association exists between α Gal with less mature islet cells *in vivo*, islet xenografts were stained with anti-CK7 and anti-insulin antibodies. Similar to that observed in α Gal expression, a strong reaction of anti-CK7 antibody to ductal epithelium was detected in less mature islet preparations (Fig. 2). On day 1 post-transplantation, the ductal epithelia were present mostly around the β cells that were arranged as small cell clusters closely attached to the ductal epithelium (Fig. 2A and B). Some clusters contained one or two β cells and some were devoid of β cells (clear round structures). On day 7 post-transplantation, the epithelial duct cells began to form more luminal structures that contained cells positive for insulin and CK7 as observed in Fig. 1. On day 30 post-transplantation, the CK7-positive ductal cells were largely arranged as luminal structures consisting of a higher proportion of insulin-positive cells budding off from the CK7-positive ducts (Fig. 2C and D). At this time, the majority of islet clusters containing β cells were present closely adjacent to the kidney. Few CK7-positive ducts could be identified on day 50 post-transplantation and these ducts were mostly located in the center of the graft (Fig. 2E and F). More than 200 days post-transplantation, no CK7-positive ductal cells were detected and the grafts consisted mainly of insulin-positive cells (Fig. 2D and H). These data showed that pig β cells were derived from ducts present in the islet preparations that also express α Gal xenoantigen.

Discussion

NPI constitute an attractive source of xenogeneic insulin-producing tissue for clinical transplantation (Korbutt *et al.* 1996, Yoon *et al.* 1999, Rayat *et al.* 2000, Trivedi *et al.* 2001); however, several aspects of the model need further investigation before this tissue can be considered for treating patients with type 1 diabetes. In particular, the susceptibility of porcine pancreatic endocrine cells to immunologic destruction remains an active field of research in xenotransplantation. We have previously shown that the xenoreactive α Gal xenoantigen is expressed on NPI cells and its expression is not restricted to non-endocrine cells (Rayat *et al.* 1998). Furthermore, our previous study also demonstrated that the proportion of β cells staining positive for α Gal residues was greater in freshly isolated (less mature) preparations than those allowed to mature further during a 9-day culture period (Rayat *et al.* 1998). We therefore hypothesized that less

mature porcine islet β cells express α Gal antigen and, once fully matured into islet endocrine cells, α Gal expression disappears.

In the present study, we have demonstrated using both *in vitro* and *in vivo* models of islet maturation that α Gal expression on NPI cells significantly decreases with maturation of the cells. We have found that α Gal is highly expressed in less mature NPI cells and that its expression decreases once the cells differentiate into fully mature islet β cells. This reduction in α Gal expression occurred when non-mature 9-day-cultured NPI were allowed to mature *in vitro* by microencapsulation followed by an additional 7 days culture with serum or when they were transplanted under the kidney capsule of non-diabetic C.B-17 SCID mice. In non-mature NPI, α Gal was expressed on 20% of the islet cells then, following further *in vitro* maturation, the proportion of α Gal-expressing cells decreased to 11% and further reduction (5%) was observed in fully developed adult porcine islets. Interestingly, the proportion of endocrine cells but not β cells was comparable between non-mature NPI and *in vitro* matured NPI but not with adult pig islets. We found that the proportion of glucagon-secreting cells as well as polypeptide (8.7 and 10.4% respectively, data not shown) or somatostatin-secreting cells (8.2 and 8.0% respectively, data not shown) were significantly higher in these two preparations compared with adult porcine islets (4.5% for polypeptide and 4.0% for somatostatin). It is possible that these cells might have undergone apoptosis during maturation to maintain the homeostasis in the pancreas as more β cells are formed. The observation that the proportion of vimentin- and CK7-positive ductal cells decreased while the proportion of β cells increased during the *in vitro* maturation process supports the concept that our *in vitro* system of NPI cell maturation is a valid model of porcine islet maturation. For example, during this culture period it is plausible that CK7-positive cells differentiate into hormone-expressing cells and thereby suggests a mechanism for the change in the relative proportions of these cells.

To further characterize the phenotype of α Gal-expressing cells present in non-mature NPI, we used FACS analysis to isolate α Gal-expressing cells from those that do not. Our data showed that ~25% of the α Gal-positive population co-expressed CK7 and only ~12% were insulin positive. On the other hand, the α Gal-negative population consisted of <2% of the cells co-expressing CK7 and ~33% were insulin positive. It is possible that α Gal is expressed in a subpopulation of less mature NPI cells that are potentially precursors of

Figure 2 CK7 and insulin staining on NPI xenografts from C.B-17 SCID mice. On day 1 (A and B) both CK-7 (brown stain) and insulin-positive cells (red stain) are scattered within the graft. Some cells stained positive for both CK7 and insulin can be detected and some islets are devoid of staining. On day 30 (C and D) and day 50 (E and F) a high proportion of insulin-positive cells appeared to be budding off from aggregates of CK7-positive ductal cells. More than 200 days (G and H) post-transplantation these CK7-positive cells were no longer detected as β cells positive for insulin comprised the majority of the xenograft. Scale bars represent 100 μ m for A, C, E and G and 50 μ m for B, D, F and H.

hormone-secreting cells or transitional cytodifferentiated forms of insulin/glucagon cells that eventually lose α Gal expression upon becoming fully differentiated into a mature form. It is generally believed that during fetal and neonatal development, islet neogenesis occurs from precursor cells that bud off from pancreatic ductules (Bouwens *et al.* 1994). Since the present study has demonstrated that 24% of the α Gal-expressing cells present in the non-mature NPI expressed the ductal cell marker CK7, it is possible that these cells are islet endocrine cell precursors.

To examine further the association of α Gal expression with maturation of porcine islet cells into β cells *in vivo*, non-mature NPI were transplanted under the kidney capsule of non-diabetic C.B17-SCID mice and then subsequently examined for the expression of α Gal and insulin. Grafts in these mice matured with time after transplantation as assessed by the significant increase in relative proportion of β cells. In contrast, α Gal-positive cells were most prominent on days 1 and 7 post-transplant and were no longer detected after 50 days. This pattern of α Gal expression further suggests that α Gal is highly expressed in immature developing porcine islet tissue and that its expression is dependent upon the maturity of the tissue. It has been shown that, during development, specific antigens are expressed at early developmental stages only to disappear as the cells mature (Kukowska-Latallo *et al.* 1990). Since our data have demonstrated that expression of α Gal is age dependent, this possibly suggests that its expression may play a role in the development of porcine islets. Exactly what this role is remains to be determined. Furthermore, the function(s) of many oligosaccharides present on glycolipids and glycoproteins is not known because of the complicated pathways that have evolved to synthesize them. It has, however, been suggested (Kukowska-Latallo *et al.* 1990) that oligosaccharides expressed on cell-surface proteins provide a protective cellular coating that allows changes in cellular shape and motility. These cellular properties are expected to play a more significant role in less mature/developing islet precursor cells in the native porcine pancreas. For example, during islet β -cell neogenesis, newly formed β cells are believed to bud from ductules and initially appear as extra-islet endocrine cells until they completely develop into distinct intact islet structures (Wang *et al.* 1995, Bouwens & Pipeleers 1998, Strokan *et al.* 2000). Similarly, we have shown, in the neonatal porcine pancreas, that the islet endocrine cells are not arranged as islets but rather randomly scattered as single cells or small clusters of cells frequently arranged alongside or within the duct lining (Korbitt *et al.* 1996). Taken together, α Gal and possibly other oligosaccharides may play a role in the migration of endocrine precursor cells from the ductules that ultimately leads to islet formation at a later developmental stage.

In our initial publication describing the isolation of NPI cell aggregates (Korbitt *et al.* 1996), we demonstrated that,

following transplantation of non-mature NPI under the kidney capsule of diabetic nude mice, the graft β -cell mass increased 20- to 30-fold. More recently, Trivedi *et al.* (2001) demonstrated that this increase in β -cell mass is attributable to proliferation of existing β cells and differentiation of ductal cells into β cells. In the present study, we have shown that non-mature NPI contain a high proportion of precursor CK7-positive ductal cells and that, following implantation into C.B-17 SCID mice, the proportion of CK7-positive cells decreased while the majority of the islet cells stained positive for insulin. Thus, the present data are in agreement with other studies (Strokan *et al.* 2000, Trivedi *et al.* 2001), thereby providing additional support for the concept that pancreatic ductal cells exhibit the inherent ability to differentiate into insulin-producing β cells. Moreover, the presence of more CK7-positive cells in non-mature NPI compared with *in vitro* matured NPI and adult pig islets indicates the existence of islet endocrine precursors in non-mature NPI, thereby denoting that this tissue constitutes an attractive model to study islet cell neogenesis.

It is conceivable that less mature NPI cells that express α Gal may be more susceptible to humoral-induced xenograft rejection than fully matured islet endocrine cells that have lower α Gal expression. It has, however, been previously demonstrated (McKenzie *et al.* 1995) that expression of α Gal in adult porcine islets can be increased under a variety of tissue culture conditions including varying proportions of CO₂ with oxygen. Therefore, if α Gal expression in adult porcine islets can also be increased post-transplantation, possibly by immunological activation, then NPI and adult pig islets may be equally susceptible to antibody-mediated hyperacute rejection.

We conclude from this study that expression of α Gal on NPI cells is highly associated with the age of the tissue and that by day 200 post-transplantation its expression is no longer detectable. Furthermore, by using *in vitro* and *in vivo* models of NPI maturation, the present study demonstrates that, as the islet tissue develops into mature β cells, the proportion of CK7-positive cells decreases while the relative number of insulin-positive cells increases. Taken together, these results suggest that α Gal-expressing cells are less mature NPI cells.

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