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GLUCOSE METABOLISM IN PIGS EXPRESSING HUMAN GENES UNDER AN INSULIN PROMOTER

M. Wijkstrom¹, R. Bottino^{2,3}, H. Iwase¹, H. Hara¹, B. Ekser^{1,4}, D.J. van der Windt¹, C. Long¹, F. Toledo⁵, C. Phelps⁵, M. Trucco^{2,3}, D.K.C. Cooper¹, and D. Ayares⁶

¹Thomas E. Starzl Transplantation Institute, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

²Division of Immunogenetics, Children's Hospital of Pittsburgh, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

⁵Division of Endocrinology and Metabolism, University of Pittsburgh, Pittsburgh, PA, USA

⁶Revivacor, Inc., Blacksburg, VA, USA

Abstract

Background—Xenotransplantation of porcine islets can reverse diabetes in nonhuman primates. The remaining hurdles for clinical application include safe and effective T-cell directed immunosuppression, but protection against the innate immune system and coagulation dysfunction may be more difficult to achieve. Islet-targeted genetic manipulation of islet-source pigs represents a powerful tool to protect against graft loss. However, whether these genetic alterations would impair islet function is unknown.

Methods—On a background of $\alpha 1,3$ -galactosyltransferase gene-knockout (GTKO)/human (h) CD46, additional genes (hCD39, human tissue factor pathway inhibitor, porcine CTLA4-Ig) were inserted in different combinations under an insulin promoter to promote expression in islets (confirmed by immunofluorescence). Seven pigs were tested for baseline and glucose/arginine-challenged levels of glucose, insulin, C-peptide, and glucagon.

Results—This preliminary study did not show definite evidence of β -cell deficiencies, even when 3 transgenes were expressed under the insulin promoter. Of 7 animals, all were normoglycemic at fasting, and 5 of 7 had normal glucose disposal rates after challenge. All animals exhibited insulin, C-peptide, and glucagon responses to both glucose and arginine challenge; however, significant interindividual variation was observed.

Corresponding author: Martin Wijkstrom, MD, Thomas E. Starzl Transplantation Institute, UPMC Montefiore Hospital, Unit 7 South, 3459 Fifth Avenue Pittsburgh, PA 15213, Tel: 412-647-5800; Fax: 412-647-5736, wijkstrommn@upmc.edu.

³Current affiliation: Institute of Cellular Therapeutics, Allegheny Health Network, Pittsburgh, PA, USA;

⁴Current affiliation: Transplant Division, Department of Surgery, Indiana University School of Medicine, Indianapolis, IN, USA;

Conflict of interest

DA and CP are employees of Revivacor, Inc. No other author has a conflict of interest.

Author contributions

MW and RB participated in concept and study design, data collection, analysis and interpretation, and drafting of the article. HI, HH, BE, DJvdW, CL participated in critical revision of the article, collecting data and procedures. FT participated in analysis of data. CP, MT, DKCC, and DA participated in concept and study design, approval of the article.

Conclusions—Multiple islet-targeted transgenic expression was not associated with an overtly detrimental effect on islet function, suggesting that complex genetic constructs designed for islet protection warrants further testing in islet xenotransplantation models.

Keywords

arginine stimulation test; C-peptide; genetic engineering; glucagon; insulin; IVGTT; pig; xenotransplantation

Introduction

Porcine islet xenotransplantation in nonhuman primate models is becoming increasingly successful, with 6 groups reporting medium-to-long-term xenograft function using either adult/neonatal- ‘free’ or encapsulated islets (reviewed in (1–4)). Significant hurdles exist before clinical trials would be justified, but these may be overcome by genetically modifying the islet-source pig (5). While genetic modification may limit the instant blood-mediated inflammatory reaction (IBMIR), studies on α 1,3-galactosyltransferase gene-knockout (GTKO) mice demonstrated glucose intolerance (6;7). However, we have previously shown that the same phenotypic change does not cause glucose intolerance in pigs (8). Expressing genes under the insulin promoter may increase gene expression in the islet/beta cell compartment, but the effect on glucose metabolism is unknown. Nagasumi et al. used targeted over-expression of GPR40 (a G-protein-coupled receptor regulating free fatty acid-induced insulin secretion) under the insulin promoter in mice, and demonstrated improved insulin secretion and glucose metabolism, indicating that the insulin promoter may allow for normal or improved glucose metabolism (9).

Pigs with multiple genetic modifications have been developed using combinations of somatic cell nuclear transfer and breeding. These modifications have been targeted to reduce graft injury from antibody, complement, inflammation, and T cells, and to inhibit platelet activation (10–25). Several of these manipulations may be beneficial for islet xenotransplantation.

In the present study, we tested seven pigs with three different combinations of genetic modifications aimed at providing protection against IBMIR (26) or providing local immunosuppression (Table 1). All pigs were GTKO, thereby rendering the animals without a target for the anti-galactose- α 1,3-galactose antibodies ubiquitously expressed in humans, apes, and Old World monkeys, and associated with hyperacute rejection of vascularized tissues (10). Islets from GTKO pigs transplanted into nonhuman primates have been demonstrated to have better immediate outcome than those from wild-type (WT) pigs (27). Islets from pigs expressing a human complement-regulatory protein, hCD46 (18), have been tested successfully previously by our group, and in one case maintained insulin-independent normoglycemia for >1 year (28). The combination of these two genetic modifications, GTKO/hCD46, is currently our standard background genetics on which new genes are added, including all animals in the current study. Islets prepared from some of the animals in this study were transplanted into nonhuman primates. Results from that *in vivo* study are reported separately (29).

Transgenes of relevance to the present study were as follows:- (i) hCD39, which mediates anticoagulant changes by metabolizing adenosine triphosphate (ATP) and thereby decreasing platelet activation and aggregation (20;30;31), (ii) human tissue factor pathway inhibitor (TFPI) which reduces tissue factor activity, and thereby should reduce the IBMIR by its anti-thrombotic and anti-inflammatory effects (22;32;33), and (iii) expression of the porcine fusion protein of the Fc region of immunoglobulin IgG1 and cytotoxic T lymphocyte-associated antigen 4 (pCTLA4-Ig) to provide local immunosuppression (24;34). To avoid any potential deleterious effects to the donor pigs that might result from constitutive expression of these bioactive molecules, and to optimize islet-specific expression of these transgenes, an insulin promoter was utilized (35).

Our laboratory has previously defined the islet secretory function of juvenile and young adult WT and GTKO pigs (8;36). The present study investigated the metabolic capacity of GTKO/hCD46 pigs expressing one or more of the above additional genes under the insulin promoter.

Materials and Methods

Animals

Seven adult Landrace Cross pigs (ages 14–36 months, weight 160–250 kg), all with GTKO/hCD46 (constitutive) background (Revivicor, Blacksburg, VA), were genetically-engineered to express one or more additional human or pig genes in the beta cells (Table 1). All animal studies were approved and conducted in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee and the *Principles of Laboratory Animal Care* formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). Control animals were described previously (8).

Vector construction

A modified version of the mammalian expression vector pCI-Neo (Promega, Madison, WI) served as a platform for the pancreatic islet-specific expression cassette (Figure 1). An 898bp region 5' to the coding region of the rat insulin II gene was amplified by PCR using purified high molecular weight rat DNA as template. This fragment serves as promoter in all vectors used to produce pigs with pancreas-specific expression of transgenes. This rat insulin II (rIns-II) promoter region, as a BglII/HindIII fragment, was inserted into the modified pCI-neo vector by digestion with restriction enzymes BglII and Hind III. The murine PDX-1 gene distal enhancer (483bp) was amplified by PCR using purified high molecular weight mouse DNA as template. The enhancer was inserted as a Bgl II/BamHI fragment 5' of the rIns-II promoter in the BglII restriction site to create the intermediate pInsII vector.

Multiple chicken β -globin insulator fragments were inserted into the vector at locations flanking the enhancer/promoter/transgene site/SV40late pA. The chicken β -globin insulator (227bp) was amplified by PCR using an in-house vector containing the insulator sequence.

ClaI/XbaI insulator fragments and SpeI/ClaI insulator fragments were generated from this amplification and introduced into the ClaI site at the 3' end of the pIns-II vector. A total of 4 insulator fragments were introduced at this site. Insulators for the 5' end of the cassette were prepared by excising a pair of insulator fragments from the vector containing the four 3' insulator fragments (above) with a ClaI digest, blunting this ClaI fragment with DNA polymerase I, large (Klenow) fragment, and inserting this blunt-ended fragment into a blunted BglII site in the rIns-II vector already containing the promoter, enhancer, and 3'-flanking insulators. This base was used for all subsequent introductions of transgenes for pancreatic islet-specific expression.

Human TFPI cDNA was fused to domains 3 and 4 and C-terminal sequence of human CD4 (37) to provide anchorage on the cell membrane, resulting in a fusion construct of 1.7kb. The extracellular region of porcine CTLA4 was ligated to the CH2/CH3 regions of human IgG (38) to produce a 1.6kb fusion construct. The human CD39 coding region inserted into the expression cassette was a 1.1kb fragment. These transgenes were ligated individually into the multiple cloning site downstream from the rIns-II promoter in the expression vector. Linear vector fragments, separated from backbone vector elements by restriction digest, were purified from agarose using β -agarase (New England Biolabs, Ipswich, MA), suspended in Tris-EDTA buffer (Fluka, St. Louis, MO), characterized by restriction digestion, and quantitated for use in transfection of porcine fibroblasts.

Transfection of porcine fibroblasts

Approximately 5 million cells were co-electroporated with 3 μ g of each of the transgene vectors and 0.5 μ g of the selectable marker vector. Forty-eight hours post-transfection, transfected cells were selected with the addition of 0.5 mg/mL of the antibiotic puromycin (InvivoGen, San Diego, CA) in 20 $\times$ 10cm dishes at a density of approximately 25,000 cells per dish. Media was changed 72 hours post-initiation of puromycin selection. Colonies were harvested 7 days post-initiation of selection. PCR analysis was performed to detect presence of the transgene.

Cell line derivation and nuclear transfer

The fibroblast cell lines used to produce the 3-, 4-, and 5- gene pigs were derived from GTKO/hCD46 fetal lines produced from breeding of GTKO pigs (39) with pigs transgenic for human CD46 with ubiquitous expression of hCD46 (18). The starting cell lines were confirmed by genotype and phenotype as homozygous GTKO and hCD46 transgenic. 3-GE cell lines (GTKO/hCD46/insCD39) were produced by transfection of the ins-hCD39 transgene into GTKO/hCD46 cells as described above. 4-GE cell lines (GTKO/hCD46/insTFPI/insCTLA4Ig) were produced by co-transfection of the ins-hTFPI and ins-pCTLA4Ig transgenes into GTKO/hCD46 cells. 3-GE pigs and 4-GE pigs were subsequently produced from these respective cell lines by nuclear transfer (cloning). A 4-GE fetal line (548A.3) was isolated from a cloned d60 fetus, and confirmed by genotype and phenotype as GTKO/hCD46/insTFPI/insCTLA4Ig. 5-GE cell lines (GTKO/hCD46/insTFPI/insCTLA4Ig/insCD39) were subsequently produced by transfection of the ins-hCD39 transgene into the 4-GE fetal fibroblast cells from the 548A.3 line.

Nuclear transfer procedures using 3-GE, 4-GE, or 5-GE cell lines were performed on *in vitro* matured oocytes (Desoto Biosciences, Christiansburg, VA) to produce multi-transgenic pigs (39). Electrical fusion and activation of reconstructed oocytes was performed using an ECM2001 Electro cell Manipulator (BTX, San Diego, CA). Fused and activated nuclear transfer embryos were held in culture in phosphate buffered NCSU-23 medium (40) for 1–4 h at 38.5°C, and then transferred to the oviduct of an estrus-synchronized recipient gilt (Large White/Duroc/Landrace).

Intravascular catheter placement

After 16 hours of fasting, two 3.2mm central venous catheters (S-50-HL, Tygon tubing, Murdock Industrial, Akron, OH) were inserted into the internal jugular vein under inhalational anesthesia. Pigs remained fasting until metabolic testing was completed 6–8hr after surgery.

Biochemical determinations

Porcine insulin was assayed by ELISA (ALPCO, Salem, NH). Porcine C-peptide and glucagon were assayed by RIA (Millipore, Billerica, MA). Blood glucose was measured using the TRUE track Smart System Glucose Monitor (Home Diagnostics, Ft. Lauderdale, FL).

Metabolic testing

An intravenous glucose tolerance test (IVGTT) and arginine stimulation test (AST) were performed sequentially, the AST >1hr after the IVGTT. Two baseline serum samples were obtained before each test and at intervals after challenge. The blood was sampled and stored at 4°C with 5% aprotinin 0.05 KIU/L added to C-peptide samples (Trasylol, Bayer Pharmaceuticals, Wayne, NJ). Serum was obtained within 2hrs and stored at –70°C.

Intravenous glucose tolerance test (IVGTT)

Glucose (dextrose 0.5g/kg body weight [D50]) was infused over <2min through one of the central venous lines. Time zero was at completion of glucose infusion. Blood was sampled twice at baseline, and at 1, 3, 5, 10, 15, 30, 45, 60, and 90 minutes after glucose infusion. Glucose disappearance rate (K_g) was calculated as the slope of the decline of the log-transformed value of blood glucose between 10 and 45 minutes, and represented the percent decline of blood glucose per minute. The acute C-peptide response (ACR_{Glu}) and the acute insulin response (AIR_{Glu}) were calculated as the basal level subtracted from the peak level 15 minutes after glucose infusion. The acute glucagon response (AGR_{Glu}) to glucose was calculated as the difference between the average baseline level and the nadir following stimulation by glucose infusion.

Arginine stimulation test (AST)

Seventy mg/kg of 30% arginine solution (prepared from L-arginine hydrochloride by a local pharmacy) was infused intravenously over 30 seconds. Blood samples were obtained at baseline, and at 2, 3, 4, and 5 minutes following the start of the infusion. The ACR_{Arg} , AIR_{Arg} , and AGR_{Arg} to arginine were calculated as the difference between the mean of

each protein at 2, 3, 4, and 5 minutes and the mean basal level. The desired tempo of infusing the arginine dose is 30 seconds; however, despite having a large-bore indwelling catheter, the initial attempt with arginine infusion required >90 sec when using the standard solution (R-Gene® 10; L-arginine hydrochloride; Pfizer, New York, NY). In order to deliver the arginine more rapidly (closer to 30 sec), a local pharmacy prepared a 30% arginine solution (30 g L-arginine per 100 mL diluant; Hieber's Pharmacy, Pittsburgh, PA) for all reported data.

Pancreatectomy

Donor pancreatectomy was performed as previously described (41). Briefly, fasting animals underwent pancreas procurement without warm time using 4°C University of Wisconsin solution until effluent was clear. The pancreas was excised, and biopsied for histological examination, as well as being processed for islet isolation and purification (28).

Histological examination (immunofluorescence)

Tissues were processed and frozen in OCT (Electron Microscopy Sciences, Hatfield, PA) and stored at -70°C. Frozen sections were cut for staining (10 µm), mounted onto gelatin coated slides, and fixed in acetone (Sigma Aldrich Chemicals, St. Louis, MO). Sections were stained with mouse anti-human CD46 (1:100, Thermo Fisher Scientific, Fremont, CA), mouse anti-CD152 (1:10, Serotec, Raleigh, NC), mouse anti-CD39 (1:10, BioLegend, San Diego, CA), rabbit anti-TFPI (1:10, American Diagnostic, Hauppauge, NY). Goat anti-insulin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) was used as double-stain on these sections as well as TO-Pro-3 iodide (Molecular Probes, Eugene, OR) for nuclear staining. Secondary antibodies were donkey anti-mouse Cy3, donkey anti-rabbit Cy3 (1:500, all from Jackson ImmunoResearch, West Grove, PA), donkey anti-rabbit Alexa 488 (1:500, both from Molecular Probes, Eugene, OR). Images were viewed at different magnifications and captured by using a Nikon confocal microscope (Nikon D-ECLIPSE C1, Japan).

Statistical analysis

Data are presented as descriptive statistics (mean ± SEM).

Results

Fasting (baseline) metabolic parameters

Mean fasting blood glucose in the pigs was 99.4±4.6 mg/dL (range, 78 to 116 mg/dL); mean fasting serum C-peptide was 1.01±0.17 ng/mL (range, 0.41 to 1.29 ng/mL); mean fasting serum insulin was 20.6±3.25 pmol/L (range, 13.9 to 32.2 pmol/L); and the mean fasting serum glucagon was 116.2±16.6 pmol/L (range, 68 to 188 pmol/L) (Figure 2, Table 2). Reference mean fasting values in historical Revivicor WT controls (n=4, mean weight 124 ± 18 kg) were blood glucose 70.0±5.3 mg/dL, C-peptide 0.46±0.08 ng/mL, insulin 37.2±6.8 pmol/L, and glucagon 49.3±4.8 pmol/L; and, historical Revivicor GTKO controls (n=4, mean weight 124 ± 20 kg) were blood glucose 69.3.0 ± 2.5 mg/dL, C-peptide 0.50 ± 0.13 ng/mL, insulin 84.4 ± 23 pmol/L, and glucagon 64 ± 9.7 pmol/L (8).

Response to glucose

By 1 minute after completion of the dextrose infusion, the blood glucose exceeded 600 mg/dL (the upper limit of our glucometer) in all pigs (Figure 2 and Table 2). The mean glucose disposal rate (K_g ; percent decline/min) was 2.91 ± 0.37 (range, 1.55 to 4.12). The pig with the lowest K_g ($K_g=1.55$, 5-GE; P388-1) was found to have a positive blood culture (*Enterococcus faecalis*; no definite source identified). However, that same animal exhibited adequate C-peptide and insulin responses (see below, and Table 2). The mean acute C-peptide response to glucose (ACR_{Glu}) was 3.17 ± 0.34 ng/mL (range, 1.80 to 4.27 ng/mL). The mean acute insulin response to glucose (AIR_{Glu}) was 79.6 ± 18 pmol/L (range, 23.8 to 160 pmol/L). The mean acute decrease in serum glucagon to glucose challenge (AGR_{Glu}) was 78.9 ± 13.2 pmol/L (range, 43.3 to 143 pmol/L), corresponding to a mean decline of 67.9% from baseline. The inter-individual responses to glucose were significant, and did not correlate with the number of genetic manipulations in each pig. Historical WT controls exhibited better glucose disposal rates of 4.79 ± 0.99 %/min, diminished ACR_{Glu} of 1.28 ± 0.07 ng/mL, but improved insulin response (AIR_{Glu}) of 247 ± 53 pmol/L. The mean glucagon suppression (AGR_{Glu}) in WT pigs was significantly lower, 25.0 ± 3.6 pmol/L, which corresponds to a 51% decline from baseline levels. Historical Revivicor GTKO controls were exhibited similar responses as WT controls, except for slightly better glucose clearance (K_g , 5.45 ± 1.08 %/min), higher fasting serum insulin (84.4 ± 23 pmol/L), and increased insulin and C-peptide responses (AIR_{Glu} was 357 ± 48 pmol/L and ACR_{Glu} was 2.34 ± 0.13 ng/mL)(8).

Response to arginine

Blood glucose did not change following arginine infusion (Figure 3, Table 2). The mean acute C-peptide response to arginine (ACR_{Arg}) was 0.87 ± 0.12 ng/mL (range, 0.36 to 1.43 ng/mL). The mean acute insulin response to arginine (AIR_{Arg}) was 19.9 ± 6.9 pmol/L (range, 6.0 to 49.6 pmol/L). The mean acute glucagon response to arginine (AGR_{Arg}) was 133 ± 24.7 pmol/L (range, 51.9 to 220 pmol/L). As with fasting parameters, there was significant inter-individual variation, and no correlation with the number of genetic manipulations. In historical WT Revivicor controls, ACR_{Arg} was lower at 0.48 ± 0.13 ng/mL, AIR_{Arg} was similar at 58.5 ± 25.2 pmol/L, and the AGR_{Arg} was lower at 60.8 ± 19.5 pmol/L. Historical Revivicor GTKO pigs exhibited more robust insulin response (AIR_{Arg} 109 ± 14 pmol/L), similar C-peptide response (ACR_{Arg} 0.81 ± 0.20 ng/mL), and slightly diminished glucagon response (AGR_{Arg} 101 ± 29 pmol/L) (8).

Intra-islet expression of transfected gene products

Histology confirmed transgenic expression in all genetically-engineered pigs. To illustrate, immunofluorescence imaging of one 5-GE animal (GTKO/hCD46/hTFPI^{Ins}/pCTLA4-Ig^{Ins}/hCD39^{Ins}) was performed using target antibodies to insulin, CD152 (as a marker for CTLA4-Ig), hCD39, hCD46, and hTFPI to demonstrate expression in islets (Figure 4).

Discussion

In this study, we assessed 7 pigs on the GTKO/hCD46 background with 1 to 3 additional genes under the insulin promoter. Five of 7 animals had normal glucose disposal after

IVGTT with $K_g > 2$. One pig had a K_g of 1.55, which is abnormal, but could possibly be explained by a concurrent infection, as the animal had a positive blood culture (enterococcus). Another pig had a K_g of 1.83, with no apparent explanation. AK_g of < 1 signifies diabetes, whereas a K_g between 1 and 2 is considered to be glucose-impaired.

All 7 animals secreted insulin, C-peptide, and glucagon in response to arginine stimulation; none of them were diabetic (assuming the human definition can be translated to pigs; fasting blood glucose 126 mg/dL). The absolute measurements of metabolic parameters showed some differences between the current GTKO/hCD46 animals with 1 to 3 transgenic modifications under the insulin promoter and previous WT and GTKO pigs (8;36). In those studies, the animals underwent glucose and arginine challenge; animals were younger and had lower body weight (5–13 months, 70–160 kg body weight); summarized and compared to the current results in Table 3. As compared with the previously studied Revivicor WT and GTKO pigs, the current animals exhibited trends towards being older, higher body weight, higher fasting blood glucose, higher fasting C-peptide, higher fasting glucagon, higher C-peptide response to glucose, and increased glucagon response to intravenous arginine. In contrast, the current series of animals exhibited trends towards lower glucose disposal rate (K_g), lower fasting insulin levels, and lower insulin response to intravenous arginine and glucose. The C-peptide response to arginine was comparable between all current and historical controls. It is possible that some of these differences between groups are due to longer fasting times at the time of testing (about 24 hours) than in control animals (after 16 hours).

The pigs in the current study (median body weight 200 kg) had decreased glucose clearance after IV glucose challenge, but exhibited a substantial response by C-peptide, insulin, and glucagon. It is possible that this increased response is due to the greater age and/or weight of the animals tested, and not due to the genetic modifications. Since the acute effects of arginine were less affected than glucose responses, it is possible that the glucose-stimulated metabolic amplifying pathway capacity of the β -cell is affected, but not production and processing of insulin. Arginine stimulates acute release of these peptides by direct β -cell depolarization, whereas insulin release via glucose is mediated by ATP (closure of ATP-sensitive potassium channels thereby causing Ca^{2+} influx leading to depolarization) in addition to the (not yet clearly defined) metabolic pathway (42;43).

Graham et al performed a detailed comparison of the metabolic demands of humans, nonhuman primates, and WT pigs (44). The average fasting blood glucose in the current study was identical to that reported by Graham in WT pigs. In contrast, however, we documented a more robust ACR_{Arg} of 0.87 ± 0.12 ng/mL than reported by Graham (mean 0.3 ng/mL); possibly even more accentuated by the way ACR_{Arg} was calculated; in the current study we calculated the difference between baseline and average C-peptide level between 2 and 5 minutes after arginine stimulation, whereas Graham used the peak level following stimulation. It is unclear what factors account for these differences. Pig size should not be a factor in this comparison, since animal weights were comparable. It is possible that the species-specific metabolic capacity accounts for the metabolic demand mismatch described by Graham et al (44). This is further supported by the study of insulin secretion capacity of islets from different species by the same group (45).

A detailed comparison between these studies may not be valuable. The current study was not undertaken to determine whether pigs with up to 3 transgenic modifications under the insulin promoter were “normal”; the goal was to provide preliminary evidence that the glucose metabolism is acceptable in these pigs, with appropriate baseline measurements and with responses to metabolic challenge suggesting satisfactory β -cell function as measured by glucose normalization and appropriate release of C-peptide, insulin, and glucagon.

Indeed, in our recent studies in the pig-to-diabetic monkey islet transplantation model, when primary graft function was obtained, islets from one of these pigs maintained normoglycemia in a monkey for a period of >1 year (28).

Our study had several limitations. These pigs were produced by cloning, which is labor-intensive and expensive. To compensate for the limited number of multi-gene animals of each genotype, and as all had at least one transgene modification under the insulin promoter, we summarized the results by combining all animals into one group for comparison with WT and GTKO historical controls. This comparison was not made to make any assertions on functional difference with statistical significance, since we only had 2–3 animals with each genotype. Other limitations were related to the larger size of the pigs. First, despite two large-bore indwelling intravascular catheters, the infusion of arginine and, in particular, dextrose was slower than ideal. For a 250 kg animal, we needed to infuse 250 mL of 50% dextrose (higher concentrations were not released by our pharmacy due to risks of crystallization). Even at maximal infusion rates, the infusion took 2 minutes. This slower infusion rate could potentially have altered the absolute values of blood glucose, C-peptide, insulin, and glucagon shortly after infusion, but the total dextrose given was identical to comparative studies (8;36;44). To compensate for this, when calculating the Kg, we used time-points for blood glucose sampling between 10 and 45 minutes, rather than between the standard 5 and 30 minutes, thus minimizing the impact of any early potential distortion. Indeed, the intraindividual correlation of the log-transformed BG was 98–99% during this time-period, indicating stable rate of BG decline. Second, controlled oral glucose tolerance tests were not feasible in awake animals of this size for safety reasons and oral intake variability. Of note, we have observed markedly altered responses to glucose and arginine in anesthetized animals (data not shown); thus, all experiments were conducted in fully awake animals. Third, it is unknown whether production of animals by cloning vs. breeding impacts function; this question could be addressed in future studies.

A conservative conclusion we can draw is that there is no definite evidence that animals with 1 to 3 transgenic modifications under the insulin promoter on the GTKO/hCD46 background have β -cell deficiencies to the point that further evaluation should be discouraged. Further testing of genetically engineered pig islet donors is warranted in relevant *in vivo* xenotransplant systems. These *in vivo* experiments will address whether the metabolic capacity of islets from pigs with multiple transgenic modifications suffices to consistently restore and maintain normoglycemia in recipient animals over time (29). It is possible that the metabolic differences identified in this study may require some consideration in pig-to-human islet xenotransplantation, or may signal the need to examine a larger data set. Our demonstration that pigs with multiple islet-specific genetic modifications have near-physiologic islet responses is encouraging for the continued development of islet-

directed expression (or suppression) of genes that may improve the survival and function of pig islets after transplantation into nonhuman primates or humans.

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Abbreviations

ACR	acute C-peptide response
AGR	acute glucagon response
AIR	acute insulin response
AST	arginine stimulation test
ATP	adenosine triphosphate
CTLA4-Ig	cytotoxic T lymphocyte-associated antigen 4 immunoglobulin
GTKO	α 1,3-galactosyl transferase gene-knockout
IBMIR	instant blood-mediated inflammatory reaction
IVGTT	intravenous glucose tolerance test
K_g	glucose disappearance rate
TFPI	tissue factor pathway inhibitor
WT	wild-type

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Figure 1.

Diagram of the expression vector used to produce islet-specific transgene-expressing pigs. Transgenes (TFPI, CTLA4-Ig, CD39) were cloned into an expression cassette driven by the rat ins-II promoter and the murine PDX-1 distal enhancer. Multiple copies of the chicken β -globin insulator flank the cassette to limit interaction with adjacent genes.

Figure 2.

Glucose (A), C-peptide (B), Insulin (C), and glucagon (D) in genetically-engineered pigs after intravenous glucose challenge. Glucose was infused over 2 minutes, and completed at time zero. Data are presented as mean $\pm$ SEM.

Figure 3.

Glucose (**A**), C-peptide (**B**), Insulin (**C**), and glucagon (**D**) in genetically-engineered pigs after intravenous arginine challenge. Arginine was given between time zero and 30 seconds. Data are presented as mean $\pm$ SEM.

hTFPI   CD152 (CTLA4-Ig)   hCD39   hCD46

Figure 4.

Immunofluorescent staining for hTFPI, hIgG1 (CTLA4-Ig), hCD39, and hCD46. All were double stained for both insulin and nuclei (TO-Pro-3).

Table 1

Description of genetic modifications of the 7 pigs included in this study

Animal ID	Abbreviated Name	Genetic
F474-3 F474-7 P462-4	3-GE	GTKO/hCD46/hCD39 ^{Ins}
P388-3 P384-2	4-GE	GTKO/hCD46/hTFPI ^{Ins} /pCTLA4-Ig ^{Ins}
P388-2 P388-1	5-GE	GTKO/hCD46/hTFPI ^{Ins} /pCTLA4-Ig ^{Ins} /hCD39 ^{Ins}

Table 2
Summary of metabolic data and responses to glucose (IVGTT) and arginine (AST) challenge in individual pigs.

Animal name	F474-3	F474-7	P462-4	P388-3	P384-2	P388-2	P388-1	Mean ± SEM
Weight [kg]	200	250	200	200	200	160	200	201 ± 9.9
Fasting BG [mg/dL]	100	78	104.5	116.5	96	93.5	107.2	99.4 ± 4.6
Fasting C-peptide [ng/mL]	1.08	1.29	0.41	1.125	0.81	0.62	1.74	1.01 ± 0.17
Fasting Insulin [pmol/L]	15.6	18.3	14.6	19.7	39.1	15.1	21.8	20.6 ± 3.25
Fasting Glucagon [pmol/L]	82.5	106.6	77.4	115.7	67.9	151.4	187.9	116.2 ± 16.6
Kg [mg/dL/min]	2.82	1.83	2.84	3.26	3.96	4.12	1.55	2.91 ± 0.37
ACR-Glu [ng/mL]	3.21	4.27	1.80	3.69	2.49	4.12	2.65	3.17 ± 0.34
ATR-Glu [pmol/L]	160.1	106.5	23.8	32.3	95.5	52.3	86.9	79.6 ± 18.0
AGR-Glu [pmol/L]	-55.8	-77.3	-43.3	-92.6	-47.2	-93.0	-143	-78.9 ± 13.2
ACR-Arg [ng/mL]	0.91	1.43	0.78	0.36	0.69	0.98	0.95	0.87 ± 0.12
ATR-Arg [pmol/L]	6.16	14.1	10.3	10.8	6.0	42.3	49.6	19.9 ± 6.9
AGR-Arg [pmol/L]	51.9	172.9	93.9	219.7	107.1	187.7	99.6	133.3 ± 24.7

Abbreviation: BG, blood glucose

Table 3

Summary comparison of baseline metabolic data and responses to glucose (IVGTT) and arginine (AST) challenge in pigs in the current study compared with GTKO and WT pigs on an identical background. Data are expressed as mean $\pm$ SEM.

	Current Study	GTKO	WT
n	7	4	4
Weight [kg]	201 $\pm$ 9.9	124 $\pm$ 20	124 $\pm$ 18
Fasting BG [mg/dL]	99.4 $\pm$ 4.6	69.3 $\pm$ 2.5	70.0 $\pm$ 5.3
Fasting C-peptide [ng/mL]	1.01 $\pm$ 0.17	0.50 $\pm$ 0.13	0.46 $\pm$ 0.08
Fasting Insulin [pmol/L]	20.6 $\pm$ 3.25	84.4 $\pm$ 23	37.2 $\pm$ 6.8
Fasting Glucagon [pmol/L]	116.2 $\pm$ 16.6	64.0 $\pm$ 9.7	49.3 $\pm$ 4.8
Kg [mg/dL/min]	2.91 $\pm$ 0.37	5.45 $\pm$ 1.08	4.79 $\pm$ 0.99
ACR-Glu [ng/mL]	3.17 $\pm$ 0.34	2.34 $\pm$ 0.13	1.28 $\pm$ 0.07
AIR-Glu [pmol/L]	79.6 $\pm$ 18.0	356.7 $\pm$ 48	246.8 $\pm$ 53.0
AGR-Glu [pmol/L]	-78.9 $\pm$ 13.2	-20.7 $\pm$ 4.6	-25.0 $\pm$ 3.6
ACR-Arg [ng/mL]	0.87 $\pm$ 0.12	0.81 $\pm$ 0.20	0.48 $\pm$ 0.13
AIR-Arg [pmol/L]	19.9 $\pm$ 6.9	108.5 $\pm$ 13.8	58.5 $\pm$ 25.2
AGR-Arg [pmol/L]	133.3 $\pm$ 24.7	101.1 $\pm$ 28.7	60.8 $\pm$ 19.5