PNAS RESEARCH ARTICLE | PHYSIOLOGY

Double transgenic neonatal porcine islets as an alternative source for beta cell replacement therapy

Nizar I. Mourad[a,b,1], Andrea Perota[c], Daela Xhema[a], Roberto Duchi[c], Irina Lagutina[c], Cesare Galli[b,c], and Pierre Gianello[a,b]

Edited by Melanie Cobb, The University of Texas Southwestern Medical Center, Dallas, TX; received May 13, 2024; accepted September 30, 2024

To be clinically efficient, beta cell replacement therapies such as pig islet xenotransplantation must ensure sufficient insulin secretion from grafted islets. While protection from host immune reaction is essential for islet engraftment and their subsequent functioning, intrinsic physiological properties of used cells are also a key factor. We have previously shown that islets with adenoviral-mediated expression of a dipeptidyl peptidase-resistant form of glucagon-like-peptide-1 (GLP-1) and a constitutively activated form of type 3 muscarinic receptor (M3R) in their beta cells have greatly improved insulin secretory response to glucose stimulation that is otherwise 4 to 10 times lower than human islets. Here, we describe in vitro characterization of the secretory function of pancreatic islets, derived from transgenic pigs expressing the GLP-1M3R cassette under the porcine insulin promoter (InsGLP-1M3R), and their usage to treat insulin-dependent diabetes in an immunodeficient mouse model. Our results show that InsGLP-1M3R islets isolated from neonatal and adult pigs secrete up to 15-fold more insulin in response to glucose stimulation compared to wild-type (WT) islets. They also proved to be more efficient in treating diabetes in a preclinical model as shown by a significantly higher percentage of normoglycemic recipients and higher porcine C-peptide levels up to 9 mo post implantation.

insulin secretion | porcine islets | xenotransplantation | type I diabetes | transgenic pigs

Significance

Our study describes the generation of genetically modified pigs from which "superislets" can be isolated. We characterized the secretory function of these islets in vitro and found them to be very good insulin secretors quantitatively and qualitatively similar to human islets. We also successfully used these islets to treat insulin-dependent diabetes in an immunodeficient pig-to-mouse xenotransplantation model. We believe that this line of transgenic pigs, the first of its kind, could become a preferred source of xenoislets for both preclinical and clinical research or even future use in the clinic as an alternative for human islets in the treatment of type I diabetes.

Porcine islets have the potential of becoming a reliable, theoretically unlimited source of pancreatic islets for beta cell replacement therapy. In order for this long-awaited promise to become clinical reality, several research groups have tackled different aspects of porcine islet xenotransplantation mainly mitigation of the host immune response by means of donor genetic modification (1), fine tuning and refinement of immunosuppression protocols (2) and/or cellular encapsulation to shield implanted islets from cellular and humoral components of the immune system (3). Porcine-born zoonosis is an important risk to be mitigated by means of careful donor screening and selection, strict housing and breeding conditions, and most importantly, efficient and reliable testing of the final product to be implanted, i.e., isolated pancreatic islets (4). An equally crucial aspect of porcine islet xenotransplantation is their capacity to produce insulin in amounts that are relevant to human physiology upon stimulation. Since porcine islets are known to secrete less insulin both in vivo and in vitro compared to their human counterparts, it has been proposed to 1) increase the number of implanted islets, 2) to optimize their implantation thus facilitating their access to nutrients and oxygen, and 3) to favor hypoxia-resistant and maturation-competent neonatal islets in an attempt to guarantee a satisfactory and long-lasting secretory function. Using one and more often a combination of aforementioned strategies, encouraging results have been published showing the efficacy of transplanted porcine islets in treating insulin-dependent diabetes in non-human primates (5–9) as well as in rodents (10–14). Successful outcomes of such studies can be summarized in three main objectives: i) restauration of normoglycemia and/or decreasing exogenous insulin dose if used, ii) detection of porcine c-peptide in the recipient during the follow-up period, and iii) return of hyperglycemia upon graft retrieval when technically feasible. All of these endpoints require very good islet secretory function in order to comply with the high insulin demands of non-human primates (15) or to compensate for the incompatibility between porcine and rodent insulin (16). Moreover, an improved islet secretory function would lessen the need to transplant extremely high numbers of porcine islets, a practice expected to worsen hypoxia, complicate surgery, and increase the cost of the procedure. We (17, 18) and others (19–21) have studied porcine islet in vitro insulin secretion and found it to be qualitatively mostly similar to that of human islets but quantitatively much less robust in terms of fold increase and amount of secreted insulin and this difference is even more striking when using less mature islets from neonatal piglets (22). Previously, we have shown that simultaneous pharmacological activation of protein

Author affiliations: [a]Pôle de chirurgie expérimentale et transplantation, Université catholique de Louvain, Brussels 1200, Belgium; [b]Pig For Life, Marche-en-Famenne 6900, Belgium; and [c]Laboratorio di Tecnologie della Riproduzione, Avantea, Cremona 26100, Italy

Author contributions: N.I.M., A.P., C.G., and P.G. designed research; N.I.M., A.P., D.X., R.D., and I.L. performed research; N.I.M. and A.P. analyzed data; and N.I.M. and A.P. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

Copyright © 2024 the Author(s). Published by PNAS. This article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

[1]To whom correspondence may be addressed. Email: Nizar.mourad@uclouvain.be.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2409138121/-/DCSupplemental.

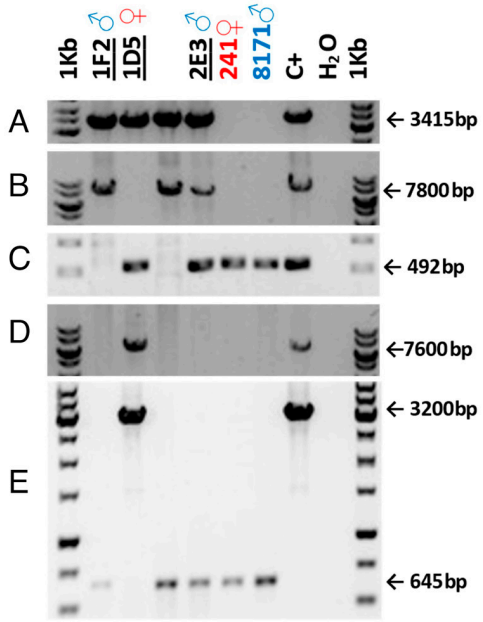
Published November 4, 2024.

Downloaded from https://www.pnas.org by Andrea Perora on November 16, 2024 from IP address 87.21.255.93.

kinase A (PKA; to mimic the effect of glucagon-like-peptide-1 [GLP-1]) and protein kinase C (PKC; to mimic parasympathic stimulation) produces an unexpected synergetic amplification of glucose-stimulated insulin secretion from neonatal and adults porcine islets and we reported similar observations after treating isolated islets with adenoviral vectors to express GLP-1 and constitutively activated muscarinic receptors at the beta cell level (17). Here, we describe the use of functionally enhanced porcine islets, isolated from the first transgenic InsGLP-1M3R herd, to study in vitro their secretory function and to treat in vivo insulin-dependent diabetes in a xenotransplantation pig-to-mouse model.

1. Results

1.1. Transgene Integration of InsGLP-1M3R Cassette in Cloned Founders. The InsGLP-1M3R expression cassette was detected intact in all the selected founders (1F2, 1D5, and 2E3; 3,415 bp in Fig. 1*A*). The two different integration loci were successfully validated on both alleles in boars (Chromosome 9-V2G locus (23); transgenic allele = 7,800 bp in Fig. 1*B*; WT allele = 492 bp in Fig. 1*C*) and in sows (Chromosome 17; transgenic allele = 7,600 bp in Fig. 1*D*; Prokaryotic backbone allele = 3,200 bp in Fig. 1*E*).



Note 1Kb = Mass Ruler 1Kb ladder-Thermo FisherScientific; **1F2** and **2E3** = transgenic InsGLP-1M3R cloned boar; **1D5** = transgenic InsGLP-1M3R cloned sow; **241** = WT PERV-C negative-low PERV-A/B female line; **8177** = WT PERV-C negative-low PERV-A/B male line; **C+** = positive control: colony #2E3-8177 for cloned boars; colony #1D5-241 for cloned sow.

Fig. 1. InsGLP-1M3R founders genotyping. This image is an example of the results of the PCR screenings performed in founder cloned animals. An intact copy of InsGLP-1M3R expression cassette was detected in each transgenic line (1F2, 1D5, 2E3; 3,415 bp, *A*). As expected, only selected boars resulted positive for the transgenic insertion occurring in Chromosome 9 (V2G locus; 7,800 bp; *B*). Homozygous boar line 1F2 was negative for the detection of the V2G wild-type (WT) allele because the InsGLP-1M3R insertion occurred in both V2G alleles, while the hemizygous boar line 2E3 was positive (492 bp, *C*), because only one V2G allele was concerned by the Chromosome9-V2G transgene integration (492 bp, *C*). Moreover, the 1D5 sow line which was found to be positive for the transgenic insertion detected in chromosome 17 (7,600 bp, D) is also hemizygous for the integration of a prokaryotic backbone fragment occurring into the same locus (3,200 bp, *E*). In fact, an intact WT allele (645 bp) is not visible, as it happened for the WT lines (241 and 8,171) and for the male founders lines 1F2 and 2E3 (*E*).

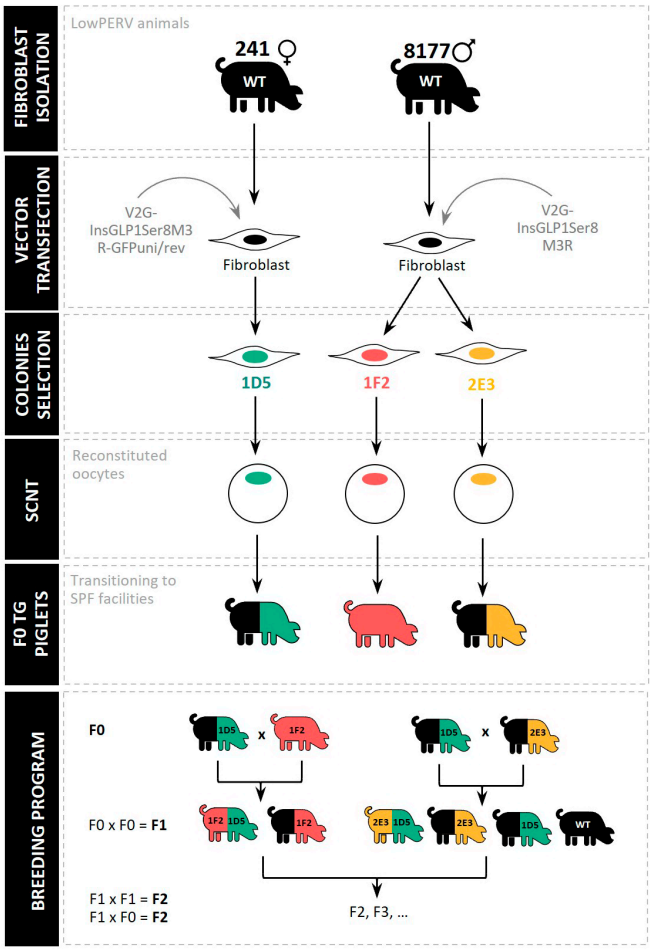


Fig. 2. Transgenic InsGLP-1M3R pig generation, breeding, and genotyping. One female (1D5) and 2 male (1F2 and 2E3) lines were generated. 1D5 is characterized by monoallelic integration of InsGLP-1M3R cassette in chromosome 17. 2E3 is characterized by monoallelic targeted integration of the cassette in V2G locus in chromosome 9 while 1F2 is characterized by biallelic integration in V2G. Breeding of founder transgenic pigs and consequent breeding of F1 individuals resulted in different allele combinations as schematized in the figure.

We finally confirmed that founders 1D5 and 2E3 were hemizygous and that 1F2 was homozygous for the InsGLP-1M3R integration (Fig. 1).

1.2. Piglet Genotyping. Among the obtained cloned piglets, healthy male and female individuals from each type were allowed to reach adult age and a breeding program was then started to obtain a herd of pigs carrying the InsGLP-1M3R cassette. Genotyping piglets from each subsequent farrowing allowed us to identify transgenic individuals and to determine their specific combination of alleles received from parents. As schematized in (Fig. 2), monoallelic and biallelic individuals were obtained. Each specific combination was identified and pancrea from each type w treated separately to evaluate in vitro islet function for each allele combination.

1.3. Pancreatic Hormone Secretion in Transgenic Pigs. Fasting blood glucose was lower in transgenic pigs compared to WT animals. Within 2 min of intravenous glucose injection, glycemia peaked at 507 mg/dL and 444 mg/dL in WT and transgenic pigs, respectively. It then gradually decreased and plateaued returning to similar levels in both groups (134 mg/dL and 142 mg/dL in WT and transgenic respectively) and following a similar dynamic course over the 90-min follow-up period (Fig. 3*A*). Insulin

Downloaded from https://www.pnas.org by Andrea Perora on November 16, 2024 from IP address 87.21.255.93.

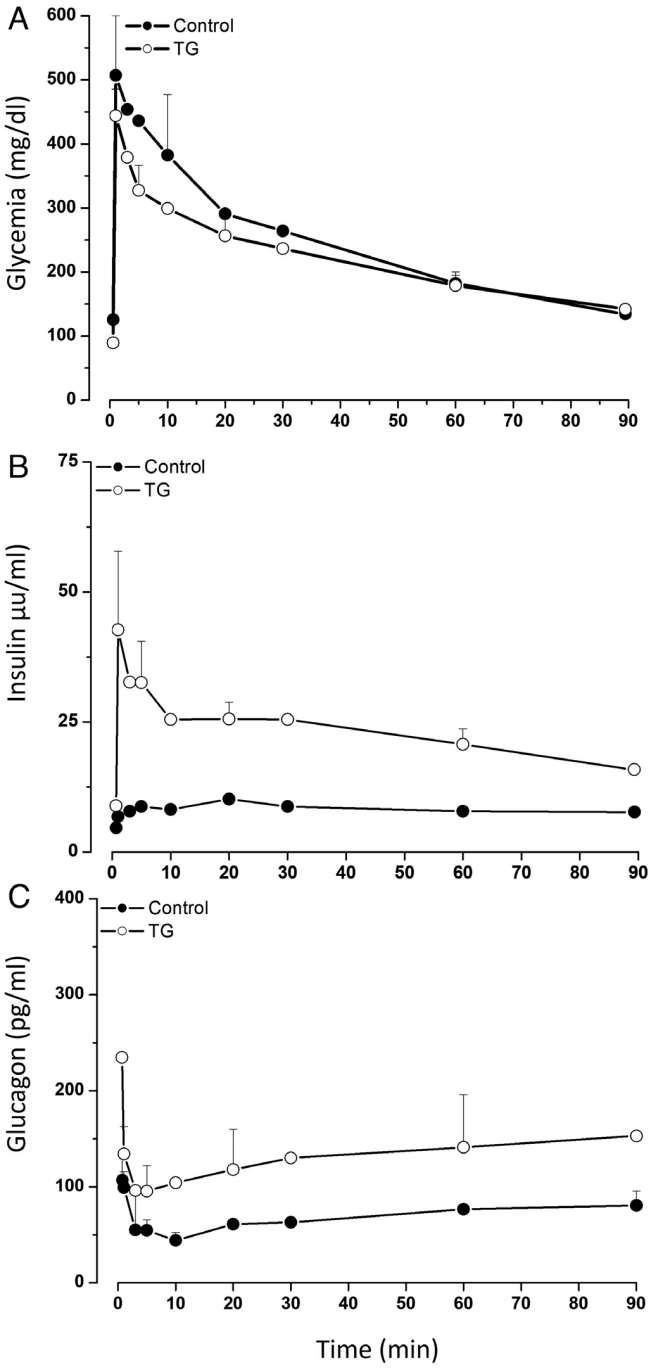


Fig. 3. In vivo islet secretory function in WT and InsGLP-1M3R pigs. Blood samples were taken at the indicated time intervals following intravenous glucose injection (0.5 g/kg) to determine glucose (*A*), insulin (*B*), and glucagon (*C*) concentrations in serum samples. Values are means ± SE from n = 2 to 9 experiments.

measurements performed at the same time points revealed a delayed twofold increase of insulin levels taking 20 min to occur in unmodified pigs compared to a much quicker 4.8-fold increase observed in transgenic pigs. Insulin levels remained significantly higher in InsGLP-1M3R pigs throughout the 90-min follow-up period as shown in Fig. 3*B*. Differences in glucagon secretion between the two groups were also observed as shown in Fig. 3*C*. Glucagon levels were found to be twofold higher in transgenic pigs at the fasting state. Following glucose injection, a 2.4-fold drop in glucagon concentration occurred in both groups, within 5 to 10 min in transgenic and WT animals, respectively.

1.4. Glucose-Stimulated Insulin Secretion. To evaluate the secretory function of control and modified islets, batches of 500 adult IEQ or 2,000 neonatal IEQ were stimulated with 15 mM glucose during dynamic perifusion experiments, and insulin secretion was measured over a period of 30 min for each batch of islets after a 30-min stabilization period at 1 mM glucose. The perifusion medium contained low glucose (1 mM, G1) during the first 10 min (–10 to 0) and then it was changed to high (15 mM, G15) glucose at t = 0 and remained so for the next 20 min (0 to 20). As shown in (Fig. 4*A*), neonatal porcine islets (NPIs) responded to high glucose stimulation with a 2.8-fold increase of insulin secretion in the case of WT islets compared to 4.7-fold with transgenic islets (G15/G1 ratio). This amelioration of islet secretory function is further showcased by the comparison of area under the curve (AUC) integration of the insulin response during 20 min of glucose stimulation as we observed a significant ($P = 0.0071$) 16-fold increase of the amount of secreted insulin compared to controls. Interestingly, we did not observe any significant differences between the secretory responses of islets derived from each type of transgenic pigs. In another set of experiments, we evaluated the good functioning of ATP (adenosine triphosphate)-dependent potassium channels (K-ATP) in transgenic islets. Following glucose stimulation, perifused islets were exposed to diazoxide (Dz) to open K-ATP channels and this resulted in complete inhibition of insulin secretion. Subsequent addition of tolbutamide which mimics the effect of glucose by closing K-ATP channels reversed the effect of Dz and significantly increased insulin secretion. Compared to control islets, transgenic islets responded as expected to these stimuli, the only difference being the significantly increased magnitude of the secretory response (Fig. 4*C*). Similar results were obtained when studying adult islets obtained from transgenic pigs. As shown in (Fig. 4*B*), adult InsGLP-1M3R islets had a higher stimulation index compared to controls (4.7 vs. 2.2 respectively). AUC comparison revealed a 15-fold amplification of the secretory response to high glucose thus confirming the significant improvement of islet insulin secretion following expression of the InsGLP-1M3R cassette in pigs at both neonatal and adult ages. This is further showcased when comparing the secretory response of WT and TG porcine islets to that of human islets as shown in panels (*A*) and (*B*).

1.5. Glucagon Secretion. We evaluated islet glucagon secretion during the same perfusion experiments performed to study insulin secretion. As shown in Fig. 4 *D* and *E*, inhibition of glucagon by high glucose was greater in the case of neonatal islets (six folds) compared to their adult counterparts (1.9 folds). Islets from transgenic pigs also showed a greater effect of glucose on glucagon secretion from neonatal islets (three folds) than from adult ones (1.3 folds). However glucagon levels remained higher in the case of transgenic islets before and after glucose stimulation.

1.6. Porcine Islet Xenotransplantation. Diabetes reversal defined as nonfasting glycemia <200 mg/dL occurred in all mice having received InsGLP-1M3R islets. In 12 out of 14 transgenic islet recipients, hyperglycemia amelioration was observed within 4 wk of islet implantation under the kidney capsule. In two mice, diabetes reversal took 9 and 14 wk to occur, respectively. From that point on, 100% of all transgenic islet recipients maintained glycemia <200 mg/dL throughout the 9-mo follow up period as shown in Fig. 5*A*. In comparison, the success rate was significantly lower in WT islet recipients: 6 diabetic mice had to be killed within 2 to 3 wk of the start of the experiment due to persistent severe hyperglycemia (>600 mg/dL) and significant weight loss (>20%) in the absence of exogenous insulin treatment. Among the remaining 9 WT islet recipients, only two mice

Downloaded from https://www.pnas.org by Andrea Perora on November 16, 2024 from IP address 87.21.255.93.

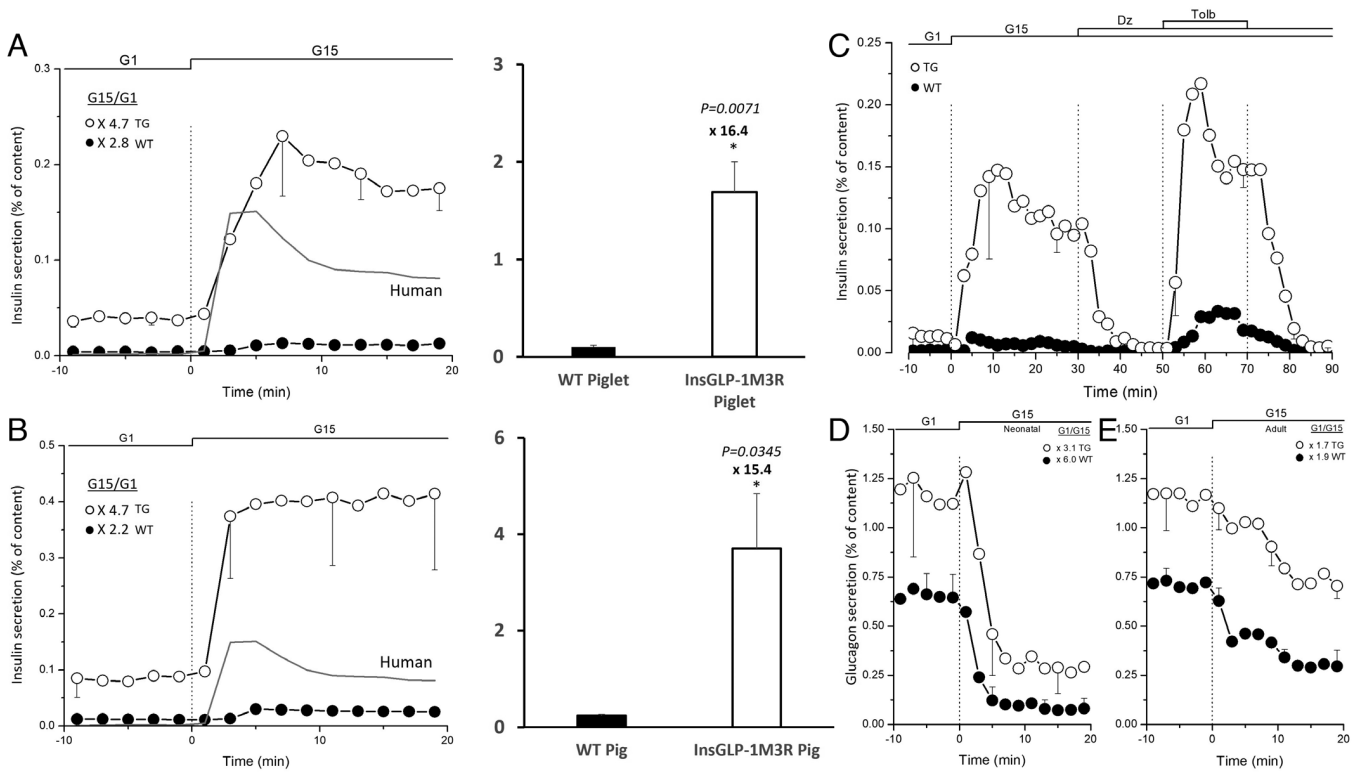


Fig. 4. In vitro secretory function of WT and InsGLP-1M3R isolated islets. Insulin and glucagon secretion from neonatal (*A*, *C* and *D*) and adult (*B* and *E*) porcine islets were evaluated using dynamic islet perifusion. *A*: batches of 1000 IEQ islets from neonatal WT (●, WT) and InsGLP-1M3R (○, TG) piglets were perifused with 1 mM glucose (G1) then stimulated with 15 mM glucose (G15) as shown on top of the figures. Stimulation indices (G15/G1 ratio) and fold-increase (TG vs. WT) are shown. Area under the curve integration of insulin secretion during 20 min of G15 stimulation is represented by columns on the right panel. *B*: Batches of 200 IEQ islets from adult pigs were perifused similarly to experiments in *A*. *C*: Batches of 1,000 IEQ from neonatal WT (●) and InsGLP-1M3R (○) piglets were exposed to 1 mM glucose (G1) followed by 30-min stimulation with 15 mM glucose (G15). K-ATP channels were then closed by 100 μM Dz during 20 min then opened by adding 500 μM tolbutamide to the perifusion medium for an additional 20 min. At the end of the experiment, G15 was replaced with G1 as shown. *D* and *E*: similar perifusion experiments to those shown in *A* and *B* using neonatal (*D*) and adult (*E*) islets to evaluate glucagon secretion. In all experiments, islets in perifusion chambers were retrieved at the end of the experiments and lysed to determine total insulin or glucagon content. Insulin secretion from adult human islets is showed in (*A*) and (*B*) for comparison. Results are expressed as percentages of total insulin or glucagon content. Values are means ± SE from n = 10 to 50 experiments. Statistical significance was evaluated using unpaired two-tailed *t* tests.

achieved normoglycemic nonfasting blood glucose levels 3 wk post implantation in one case and as late as 33 wk in the other although we speculate this was mainly due to partial endogenous beta cell regeneration for the latter (Fig. 5*A*). Serum samples taken from graft recipients on a weekly basis were used to quantify porcine c-peptide indicative of porcine beta cell function. As shown by the scatter plots in Fig. 5*B*, porcine c-peptide could be detected in 100% of both WT and transgenic islet recipients as early as 3 wk postimplantation and up until graft removal 9 mo later. Consistent with glycemia measurements, porcine c-peptide levels were significantly higher in the transgenic islet group compared to the WT group (79 vs. 34 pM, respectively, median values). In seven transgenic and two WT islet recipients, the graft-bearing kidney could be successfully excised without immediate postsurgical complications. Within 3 to 4 d, all nine nephrectomized mice returned to hyperglycemic blood glucose values (>200 mg/dL) and remained so for the rest of the follow-up period. As expected, porcine c-peptide became undetectable upon graft removal. Individual data for each graft recipients are illustrated in *SI Appendix*, Figs. S1 and S2. Immunohistological analysis of graft-bearing kidneys revealed the presence of numerous insulin-positive and less abundant glucagon-positive cells organized in large islet-like clusters under the kidney capsule as shown by representative sections from transgenic (Fig. 5 *C* and D) and control (Fig. 5 *E* and *F*) group individuals in Fig. 5.

2. Discussion

Today, it is clear that porcine islets that might be used in clinical transplantation will be sourced from genetically modified pigs, most probably from multitransgenic pigs. A number of such potential xenogeneic pancreatic islet donors already exist and have entered the process of preclinical (5, 6, 24) and even clinical (25) assessment. All of the existing genetic modifications aim to reduce immunogenicity of porcine tissues by suppressing expression of carbohydrate antigens (Gal, Neu5Gc, and SID) or by expressing human coagulation-regulatory factors (thrombomodulin and CD39) anti-inflammatory factors (hemeoxygenase-1 and A20) or cellular rejection inactivators (CD47, HLA-E, LEA29Y, and CTLA-Ig) (26). Knock-out of growth hormone receptor deemed necessary in the case of porcine organ transplantation is not relevant for the islet field, whereas genome-wide knock-out of porcine endogenous retrovirus (PERV) pol gene can eliminate the risk of PERV transmission (27). Indeed, this porcine endogenous retrovirus is expressed variably in all porcine tissues but have been found to be particularly enriched in pancreatic islets (28) and to infect human cells in coculture experiments (29). Surprisingly, little attention has been given to physiological considerations embodied in the quality and quantity of insulin secreted by porcine islets. From a qualitative point of view, porcine insulin differs from its human counterpart by one amino-acid in its β-chain (30), but this difference might be enough to initiate an antibody reaction in humans and decrease its efficacy

Downloaded from https://www.pnas.org by Andrea Perora on November 16, 2024 from IP address 87.21.255.93.

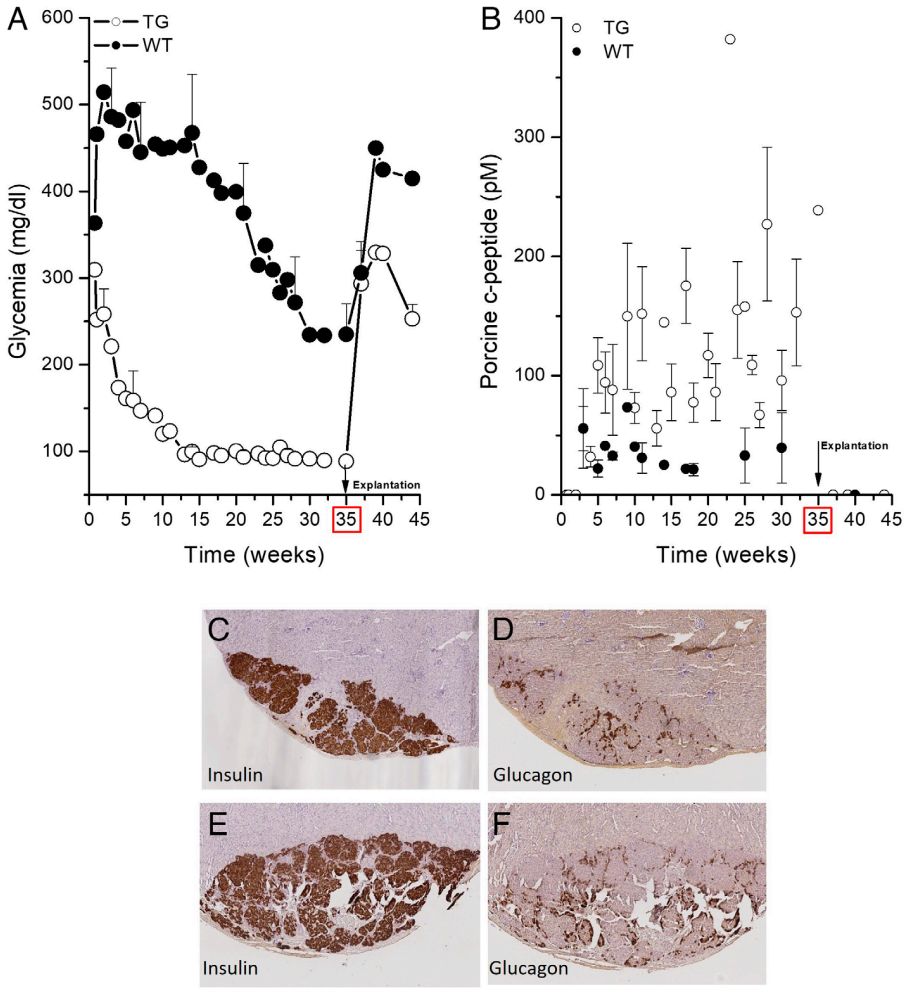


Fig. 5. Transplantation of WT and InsGLP-1M3R NPIs to diabetic immunodeficient mice. 2,500 IEQ InsGLP-1M3R (○) and WT (●) islets were transplanted under the left kidney capsule of streptozotocin-treated diabetic NSG® mice. Mean nonfasting blood glucose (*A*) and porcine C-peptide (*B*) levels during the 40-wk follow-up period are represented for n = 14 TG islet recipients and n = 9 WT islet recipients. Graft bearing kidneys were removed at t = 35 wk and processed for immunohistochemistry. Values are means ± SEM from n = 14 and n = 9 TG and WT islet recipient, respectively. Representative kidney sections stained for insulin and glucagon are shown in panels *C* and *D* (TG islet recipients) *E* and *F* (WT islet recipients).

(31). However expression of human proinsulin gene in pigs might eliminate this risk as previously described (32). With regard to quantitative islet secretory function, none of the transgenic pig models tackled the issue of low insulin secretion from pig islets. Only two studies showed that expression of immunomodulatory genes under an insulin promoter (33) or knock-out of porcine xenoantigens (34) do not affect glucose homeostasis nor in vitro insulin secretion from islets isolated from these genetically modified animals. Using adenoviral transfection, we have previously shown that concomitant expression of GLP-1 and mutated M3R in porcine beta cells results in amplification of glucose-induced insulin secretion (17). We have now built upon these findings and produced a line of transgenic pigs with permanent and stable expression of these modified proteins at the beta cell level resulting in a 15-fold increase of the secretory response to glucose stimulation as benefit. Indeed, GLP-1 binding to its receptor or direct activation of adenylyl cyclase and the ensuing rise of cAMP (cyclic adenosine monophosphate) and consequent activation of PKA and EPAC2 (exchange protein directly activated by cAMP 2) are known to amplify glucose-induced insulin secretion in mouse (35), human (36), and porcine (17) islets. Similarly, binding of acetylcholine to M3R receptors or pharmacological activation of PKC also amplifies the secretory response to glucose (17, 37). In mice, these amplifying pathways have been hypothesized to affect converging mechanisms without additional amplification upon simultaneous activation (38). Conversely, we have shown a synergetic effect between the two pathways in pigs and observed an increase of the size of readily releasable insulin granule pool as a possible mechanism of this amplification in pig islets expressing GLP-1 and constitutively activated M3R (17). The effect of this genetic modification was as significant on neonatal islets as on adult ones. However, after 8 d of culture, the insulin content of neonatal islets is fourfold lower compared to adult islets but a longer culture period on a collagen matrix has been shown to permit further maturation of neonatal islets, whereas adult islets loose this capacity as they age (39). In a recent study, we investigated the regulation of glucagon secretion from porcine islets and found glucose to exert more drastic effects on glucagon than on insulin secretion especially in neonatal age in what appears to be a characteristic of porcine islets both in vivo and in vitro after isolation (18). Here, we confirm these findings and report higher glucagon secretion from InsGLP-1M3R islets compared to WT islets. This glucagon counter regulation might explain almost normal glucose homeostasis in transgenic pigs despite higher insulin secretion as showcased during intravenous glucose tolerance tests. To validate the superiority of InsGLP-1M3R islets in vivo, we transplanted them to diabetic immunodeficient mice where their survival is minimally affected by the host immune response and not threatened by low oxygen and nutrient availability during early implantation in an encapsulated form. Compared to

Downloaded from https://www.pnas.org by Andrea Perora on November 16, 2024 from IP address 87.21.255.93.

Table 1. Comparison of actual and potential islet sources for beta cell replacement therapy

	Human islets	Porcine islets				hPSC-derived
		Neonatal		Adult		
Islet isolation	Variable/difficult	Reproducible/easy		Variable/difficult		Easy (iPSC)
Yield (IEQ/pancreas)	170 to 200 × 10^3	25 to 40 × 10^3		80 to 300 × 10^3		–
Insulin content (μU/IEQ)	201 to 922	35 to 285		500 to 800		720
% of content/min during high glucose stimulation	0.07	WT	TG	WT	TG	0.04
		0.01	0.09	0.01	0.13	
Stimulation index	2.1 to 8.8	2.8	4.7	2.2	4.7	2.2
In vitro culture (days)	0 to 1	8		0 to 1		34
Risk factors	Availability	Xenopathogens				Tumorigenicity
Immune reaction	Immunosuppression	Immunosuppression/Encapsulation/GM				Avoidable

WT islets, InsGLP-1M3R neonatal islets were more efficient in treating diabetes as evidenced by significantly higher percentage of normoglycemic recipients as well as higher blood porcine C-peptide levels upon islet implantation under the kidney capsule. For both islet types recipients, removal of the graft-bearing kidney 35 wk posttransplantation resulted in return to hyperglycemia therefore confirming that the observed amelioration is due to the graft and not to endogenous beta cell regeneration as confirmed by absence of insulin staining in pancreata from killed recipients. Previous studies have shown successful reversal of diabetes in rodents using WT NPIs. However, in most cases, relatively large numbers of islets ranging from 4,000 (40) to 12,000 (12) IEQ per recipient were necessary to achieve this. In our hands, 2,500 IEQ of InsGLP-1M3R islets were enough to reverse hyperglycemia over a long follow-up period of 9 mo without using exogenous insulin treatment. Both of our in vitro and in vivo data presented in this study show that InsGLP-1M3R islets are better insulin secretors compared to unmodified porcine islets. As summarized in Table 1, their secretory function is equivalent to that of human islets and superior to that of human pluripotent stem cell–derived islets suggesting that they could be used in the clinic at doses similar to those currently in use with human islets (41) (5,000 to 10,000 IEQ/kg). Unmodified porcine islets are characterized by a flat insulin response to a sharp increase of glucose concentration, whereas InsGLP-1M3R islets respond in a biphasic pattern comparable to what is seen with human islets (Fig. 4 *A* and *B*). This is of significant impact since a lower dose of such functionally enhanced islets would lessen oxygen needs of the graft and lower the cost of this therapy by requiring less pig donors/patient while providing enough insulin to meet the receiver's physiological needs to maintain normoglycemia. Although we found no difference between islet secretory function of islets derived from InsGLP-1M3R pigs with different genotypes, we chose to continue our future herd breeding program using a single genotype characterized by a single monoallelic targeted integration of the InsGLP-1M3R cassette to guarantee stable integration and expression of the transgenic cassette across generations and to fulfill regulatory requirements related to usage of transgenic animal-derived tissues in the clinic.

3. Methods

All experiments requiring use of animals were submitted for review and approval by the local ethics committee.

3.1. InsGLP-1M3R Cassette. Our expression cassette inserted in a customized targeting vector was organized as follows: porcine insulin promoter (InsGLP-1M3R), Igk secretion signal, 31-amino-acid GLP-1 (7–37) modified by a mutation substituting alanine at position 8 by a serine (A8S), and type 3 muscarinic receptor (M3R) constitutively activated by a single point mutation substituting glutamine at position 490 by a leucine (Q490L) as previsously described (17, 42).

3.2. Genotyping of Cloned InsGLP-1M3R Founders. Genotype of cloned InsGLP-1M3R founders (boar 1F2; boar 2E3; sow 1D5/PERV-C negative low PERV--A/B) was determined by PCR analysis (LaTaq, TakaraBio, Saint-Germain-en-Laye, France). Detection of InsGLP-1M3R expression cassette and determination of each genomic locus where the transgenes were integrated (Chromosome 9-V2G locus (23) for males; Chromosome 17 for females) was achieved using the primer pairs (Table 2) and the amplification conditions (Table 3), previously validated in our laboratory. The InsGLP-1M3R transgenic herd was obtained by natural breeding of selected founders.

3.3. Piglet Genotyping. Tail biopsy samples were taken from piglets 2 to 3 d post farrowing and stored at −20 °C or processed immediately. DNA extraction was performed using DNeasy Blood & Tissue kits from Qiagen (Antwerp, Belgium) according to the manufacturer's instructions. Each sample was analyzed for InsGLP-1M3R cassette detection as well as the integration site and the number

Table 2. Primer pairs used for InsGLP-1M3R founders and piglets genotyping

PCR assay	Forward oligo (FW)	Reverse oligo (RV)	Target
A	GAGTTCAGCTGAGCTGGCT	TGCAGGTCGAGGGATCTT	InsGLP-1M3R detection
B	TCTTCCTGGATTCACGGC	TTCCCCACAACTTCTCCG	InsGLP-1M3R integration in founder boars (1F2 hemizygous and 2E3 homozygous; Chromosome 9-V2G locus[23])
C	TGCTCCTAATGCAGTGTTGC	CATGCTGTAGGTTCTTTGTAGC	WT allele (Chromosome 9-V2Glocus[23])
D	TGCAGGTCGAGGGATCTT	GGAGCACTTAACACTCACATAG	InsGLP-1M3R integration in founder sows (1D5 hemizygous; Chromosome 17)
E	AACAGCAATAGCCCCAAAC	GGAGCACTTAACACTCACATAG	Prokaryotic backbone integration in founder sows (1D5 hemizygous; Chromosome 17)

Downloaded from https://www.pnas.org by Andrea Perora on November 16, 2024 from IP address 87.21.255.93.

Table 3. PCR conditions for InsGLP-1M3R founders and piglets genotyping

Assay ID	PCR conditions	Amplicon (bp)
A	94 °C 2 min; 9 × (94 °C 30 s; 72 °C 30 s −1 °C/cycle; 72 °C 2 min); 30 × (94 °C 30 s; 64 °C 30 s; 72 °C 2 min); 72 °C 7 min	3,415
B	94 °C 2 min; 10 × (94 °C 30 s; 68 °C 30 s −1 °C/cycle; 72 °C 7 min 30 s); 30 × (94 °C 30 s; 59 °C 30 s; 72 °C 7 min 30 s + 7 s/cycle); 72 °C 7 min	7,800
D	94 °C 2 min; 40 × (94 °C 30 s; 55 °C 30 s; 72 °C 30 s); 72 °C 7 min	492
C	94 °C 2 min; 10 × (94 °C 30 s; 60 °C 30 s; 72 °C 12 min); 25 × (94 °C 30 s; 60 °C 30 s; 72 °C 12 min + 12 s/cycle); 72 °C 7 min	7,600
E	94 °C 2 min; 35 × (94 °C 30 s; 60 °C 30 s; 72 °C 3 min); 72 °C 7 min	3,200 (Prokaryotic); 645 (WT)

of alleles by PCR (TakaraBio, Saint-Germain-en-Laye, France) as described for founders animals.

3.4. Intravenous Glucose Tolerance Test. To study in vivo secretion of insulin and glucagon in WT and InsGLP-1M3R adult pigs, we performed glucose tolerance tests by administering an intravenous glucose bolus (0.5 g/kg bodyweight) in an auricular vein. Blood samples were taken before and at 1, 3, 5, 10, 20, 30, 60, and 90 min after glucose injection using a catheter placed on the other ear. Blood glucose was immediately measured using Aviva nano glucometer (Accu-Chek) and serum samples were prepared and stored at −80 °C for subsequent insulin and glucagon assays.

3.5. Islet Isolation. Pancreata from WT and transgenic adult pigs (>200 kg) were harvested aseptically at a local slaughterhouse (A. de Marbaix center, Louvain-la-Neuve), infused with 1 mL/g cold modified University of Wisconsin solution and transported at 4 °C in the same solution to the main laboratory in Brussels. Pancreata were digested with collagenase NB8 (4.4 U/g pancreas weight) from Serva (Heidelberg, Germany). The digested tissue was filtered through a prepurification column before islet purification on a discontinuous Ficoll gradient. Pancreatic islets were then collected, washed, counted, and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS (foetal bovine serum), 1% penicillin, 1% streptomycin, 5 mM glutamine, and 5 mM glucose. For isolation of NPIs, we used a modification of a method originally described by Korbutt et al.(22). Briefly, pancreata were harvested from exsanguinated piglets, cut into 1 to 3 mm^3 pieces in cold HBSS (Hanks balanced salt solution) supplemented with 0.25% bovine serum albumin (BSA), 10 mM HEPES, 10 mM glucose, 1% penicillin, 1% streptomycin, and 0.1% amphotericin B. Enzymatic digestion was done using 10 mg/ pancreas collagenase V from Sigma (Darmstadt, Germany). Digested tissue was then filtered on a 500 μm mesh, washed three times in cold HBSS and cultured in HAM-F10 supplemented with 0.5% BSA, 50 μM IBMX, 10 mM glucose, 2 mM glutamine, 10 mM nicotinamide, 1% penicillin, and 1% streptomycin during a period of 8 d to allow maturation of islet cells and removal of exocrine tissue.

3.6. In Vitro Insulin and Glucagon Secretion Assays. Secretory function of isolated neonatal and adult islets was studied by dynamic islet perifusion experiments. The working medium was a bicarbonate-buffered solution containing 120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 24 mM $NaHCO_3$, 1 mg/mL BSA, and varying concentrations of glucose and test substances as indicated in the figures. Batches of 200 to 1,000 IEQ (islet equivalent) were placed in perifusion chambers, covered with 8 μm cellulose filters, and sealed. Test solutions kept at 37 °C and gassed continuously to stabilize pH around 7.2 were pumped at a flow rate of 1 mL/min. Effluent fractions were collected at 2-min intervals and saved for insulin and glucagon assays using radioimmunoassay kits (Millipore, Billerica, MA). At the end of the experiments, islets were recovered and their insulin and glucagon content (no significant difference between WT and TG) were determined after extraction in acid-ethanol (75% ethanol, 180 mM HCl from Merck).

3.7. Diabetes Induction, Transplantation, and Recipient Follow-Up. Diabetes was induced in fasted female NSG® (NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJ) mice from Charles-River (France) by intraperitoneal injection of freshly prepared streptozotocin solution (100 mg/kg). Diabetes was confirmed after two consecutive nonfasting daily glycemia measurements >200 mg/mL. In 65% of cases, a second injection with the same dose was necessary to achieve a diabetic state. All surgery and following sample collection were performed under a ventilated hood to avoid microbiological contamination risks of immunodeficient mice. Islet transplantation was performed between 7 and 10 d following the first streptozotocin injection. Under general anesthesia, a small incision was made on the left side, and the left kidney was exposed and maintained in position using sterile cotton swabs. The renal capsule was lifted and cut open using a 27G 3/4 needle allowing the introduction of PE50 tubing (BD Intramedic, USA) containing 2,500 IEQ NPIs. A 250 μL Hamilton syringe connected to the PE50 tubing was then used to carefully push the islets under the renal capsule before it was cauterized and the incision sewed with sutures. Graft recipient follow-up consisted in weekly weight and nonfasting glycaemia measurements as well as monthly porcine C-peptide measurements. For this purpose, 200 μL of blood were drawn from the tail and sera were kept at −80 °C until porcine C-peptide was assayed by ELISA (enzyme-linked immunosorbent assay; Mercodia, Sweden). At the end of the experiment (35 wk postimplantation), left nephrectomy was performed and follow-up continued for 2 to 5 wk with the same measurements as before explantation. At euthanasia, a final blood sample was taken and the pancreas was removed for histological analysis.

3.8. Immunohistochemistry. Graft-bearing kidneys and pancreata from mice WT and InsGLP-1M3R islet recipients were processed for immunohistochemistry. Five-μm sections were stained for insulin (ab9569, abcam, UK) and glucagon (BSB5580, Bio SB, USA). Images were acquired using Leica SCN400 slide scanner and the SlideViewer software.

3.9. Presentation of Results and Statistics. Pancreatic hormone secretion values are presented as means ± SE. Glycemia and porcine C-peptide measurements are presented as individual values. Statistical significance was evaluated using two-tailed unpaired t test (Graphpad InStat). Results were considered statistically different for $P < 0.05$.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. This work benefited from financial support by EU FP7 grant 601827 "XENOISLET" and "Service public de Wallonie, SPW Economie, Emploi, Recherche" Grant "8318 SPW."

1. N. I. Mourad, P. Gianello, Gene editing, gene therapy, and cell xenotransplantation: Cell transplantation across species. *Curr. Transplant. Rep.* **4**, 193–200 (2017).
2. M. L. Graham *et al.*, Clinically available immunosuppression averts rejection but not systemic inflammation after porcine islet xenotransplant in cynomolgus macaques. *Am. J. Transplant* **22**, 745–760 (2022).
3. O. Korsgren, Islet encapsulation: Physiological possibilities and limitations. *Diabetes* **66**, 1748–1754 (2017).
4. J. Denner, Xenotransplantation of pig islet cells: Potential adverse impact of virus infections on their functionality and insulin production. *Xenotransplantation* **30**, e12789 (2023).
5. W. J. Hawthorne *et al.*, Xenotransplantation of genetically modified neonatal pig islets cures diabetes in baboons. *Front. Immunol.* **13**, 898948 (2022).
6. R. Bottino *et al.*, Safe use of anti-CD154 monoclonal antibody in pig islet xenotransplantation in monkeys. *Xenotransplantation* **24**, (2017).
7. R. W. Holdcraft *et al.*, Long-term efficacy and safety of porcine islet macrobeads in nonimmunosuppressed diabetic cynomolgus macaques. *Xenotransplantation* **29**, e12747 (2022).
8. B. Ludwig *et al.*, Favorable outcome of experimental islet xenotransplantation without immunosuppression in a nonhuman primate model of diabetes. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 11745–11750 (2017).

Downloaded from https://www.pnas.org by Andrea Perora on November 16, 2024 from IP address 87.21.255.93.

9. J. M. Kim *et al.*, Long-term porcine islet graft survival in diabetic non-human primates treated with clinically available immunosuppressants. *Xenotransplantation* **28**, e12659 (2021).
10. L. Wolf-van Buerck *et al.*, Engraftment and reversal of diabetes after intramuscular transplantation of neonatal porcine islet-like clusters. *Xenotransplantation* **22**, 443–450 (2015).
11. R. W. Holdcraft, P. R. Dumpala, B. H. Smith, L. S. Gazda, A model for determining an effective in vivo dose of transplanted islets based on in vitro insulin secretion. *Xenotransplantation* **25**, e12443 (2018).
12. K. Ajima *et al.*, A porcine islet-encapsulation device that enables long-term discordant xenotransplantation in immunocompetent diabetic mice. *Cell Rep. Methods* **3**, 100370 (2023).
13. H. Arefanian *et al.*, Yield, cell composition, and function of islets isolated from different ages of neonatal pigs. *Front. Endocrinol. (Lausanne)* **13**, 1032906 (2022).
14. L. W. Buerck *et al.*, LEA29Y expression in transgenic neonatal porcine islet-like cluster promotes long-lasting xenograft survival in humanized mice without immunosuppressive therapy. *Sci. Rep.* **7**, 3572 (2017).
15. A. Casu *et al.*, Metabolic aspects of pig-to-monkey (Macaca fascicularis) islet transplantation: Implications for translation into clinical practice. *Diabetologia* **51**, 120–129 (2008).
16. A. R. Pepper, C. Gall, D. M. Mazzuca, C. W. Melling, D. J. White, Diabetic rats and mice are resistant to porcine and human insulin: Flawed experimental models for testing islet xenografts. *Xenotransplantation* **16**, 502–510 (2009).
17. N. I. Mourad, A. Perota, D. Xhema, C. Galli, P. Gianello, Transgenic expression of glucagon-like peptide-1 (GLP-1) and activated muscarinic receptor (M3R) significantly improves pig islet secretory function. *Cell Transplant* **26**, 901–911 (2017).
18. N. I. Mourad, D. Xhema, P. Gianello, In vitro assessment of pancreatic hormone secretion from isolated porcine islets. *Front. Endocrinol. (Lausanne)* **13**, 935060 (2022).
19. D. Dufrane, M. Nenquin, J. C. Henquin, Nutrient control of insulin secretion in perifused adult pig islets. *Diabetes Metab.* **33**, 430–438 (2007).
20. K. R. Mueller *et al.*, Differences in glucose-stimulated insulin secretion in vitro of islets from human, nonhuman primate, and porcine origin. *Xenotransplantation* **20**, 75–81 (2013).
21. R. Bottino *et al.*, Isolation outcome and functional characteristics of young and adult pig pancreatic islets for transplantation studies. *Xenotransplantation* **14**, 74–82 (2007).
22. G. S. Korbutt *et al.*, Large scale isolation, growth, and function of porcine neonatal islet cells. *J. Clin. Invest.* **97**, 2119–2129 (1996).
23. A. Fiorenzo *et al.*, EP2921048B1 (2015).
24. R. Bottino *et al.*, Pig-to-monkey islet xenotransplantation using multi-transgenic pigs. *Am. J. Transplant* **14**, 2275–2287 (2014).
25. S. Singireddy *et al.*, Genetic engineering of donor pig for the first human cardiac xenotransplantation: Combatting rejection, coagulopathy, inflammation, and excessive growth. *Curr. Cardiol. Rep.* **25**, 1649–1656 (2023).
26. D. K. C. Cooper, S. S. Raza, R. Chaban, R. N. Pierson III, Shooting for the moon: Genome editing for pig heart xenotransplantation. *J. Thorac. Cardiovasc Surg.* **166**, 973–980 (2023).
27. L. Yang *et al.*, Genome-wide inactivation of porcine endogenous retroviruses (PERVs). *Science* **350**, 1101–1104 (2015).
28. N. I. Mourad, C. Crossan, V. Cruikshank, L. Scobie, P. Gianello, Characterization of porcine endogenous retrovirus expression in neonatal and adult pig pancreatic islets. *Xenotransplantation* **24**, 12311 (2017).
29. D. Niu *et al.*, Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. *Science* **357**, 1303–1307 (2017).
30. G. E. Sonnenberg, M. Berger, Human insulin–therapeutic progress? *Dtsch. Med. Wochenschr* **108**, 927–929 (1983).
31. A. J. Clark *et al.*, Biosynthetic human insulin in the treatment of diabetes: A double-blind crossover trial in established diabetic patients. *Lancet* **2**, 354–357 (1982).
32. B. Cho *et al.*, Production of genetically modified pigs expressing human insulin and C-peptide as a source of islets for xenotransplantation. *Transgenic Res.* **28**, 549–559 (2019).
33. M. Wijkstrom *et al.*, Glucose metabolism in pigs expressing human genes under an insulin promoter. *Xenotransplantation* **22**, 70–79 (2015).
34. A. Salama *et al.*, Neu5Gc and alpha1–3 GAL xenoantigen knockout does not affect glycemia homeostasis and insulin secretion in pigs. *Diabetes* **66**, 987–993 (2017).
35. N. I. Mourad, M. Nenquin, J. C. Henquin, cAMP-mediated and metabolic amplification of insulin secretion are distinct pathways sharing independence of beta-cell microfilaments. *Endocrinology* **153**, 4644–4654 (2012).
36. J. C. Henquin, D. Dufrane, M. Nenquin, Nutrient control of insulin secretion in isolated normal human islets. *Diabetes* **55**, 3470–3477 (2006).
37. N. I. Mourad, M. Nenquin, J. C. Henquin, Amplification of insulin secretion by acetylcholine or phorbol ester is independent of beta-cell microfilaments and distinct from metabolic amplification. *Mol. Cell Endocrinol.* **367**, 11–20 (2012).
38. Y. Yang, K. D. Gillis, A highly Ca2+-sensitive pool of granules is regulated by glucose and protein kinases in insulin-secreting INS-1 cells. *J. Gen. Physiol.* **124**, 641–651 (2004).
39. N. I. Mourad, P. Gianello, Long-term culture and in vitro maturation of macroencapsulated adult and neonatal porcine islets. *Xenotransplantation* **26**, e12461 (2019).
40. A. Citro *et al.*, Directed self-assembly of a xenogeneic vascularized endocrine pancreas for type 1 diabetes. *Nat. Commun.* **14**, 878 (2023).
41. S. Forbes *et al.*, The impact of islet mass, number of transplants, and time between transplants on graft function in a national islet transplant program. *Am. J. Transplant* **22**, 154–164 (2022).
42. D. Gautam *et al.*, Beneficial metabolic effects caused by persistent activation of beta-cell M3 muscarinic acetylcholine receptors in transgenic mice. *Endocrinology* **151**, 5185–5194 (2010).

Downloaded from https://www.pnas.org by Andrea Perora on November 16, 2024 from IP address 87.21.255.93.