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Review

PORCINE PANCREATIC ISLETS: FROM IN VITRO CHARACTERIZATION TO PRECLINICAL TRANSPLANTATION

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Summary

Porcine pancreatic islets could be used in the clinic as an alternative source of insulin-secreting cells in light of insufficient numbers of human pancreas donors. Prerequisites for clinical application include thorough understanding of porcine islet physiology as well as robust experimental *in vivo* trials using relevant models and permitting easy translation to humans. *In vitro* evaluation of pig islet secretory function and its comparison to that of human islets permit necessary adjustment of islet dose to be implanted, better choice of donor age and genetics, better understanding of treatment outcome and can serve as a basis for eventual modifications to boost function and survival. *In vivo* studies using the pig-to-non-human primate model are crucial to evaluate efficacy, safety and longevity of islet grafts. On the recipient side, refinement of immunosuppressive regimens and selection of an appropriate implantation site for free or encapsulated islets are equally important to promote diabetes reversal by the treatment and necessitate *in vivo* testing before application in humans.

Key words: porcine islets, insulin secretion, transplantation, diabetes

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INTRODUCTION

Alternative treatments of type I diabetes are based on the concept of restoring innate control of blood glucose with little or no need for time-consuming and quality of life-affecting monitoring, life-long insulin injections and severe restrictions. Insulin-secreting beta cells required to restore this lost endocrine function could be sourced from cadaveric donors, neo-formed islet-like organoids derived from undifferentiated or dedifferentiated stem cells [1] or from xenogeneic donors. Given that the number of human cadaveric donors will never satisfy the growing number of islet graft-requiring patients [2] and that large-scale production of stem-cell-derived beta cells still needs to overcome scientific, financial and ethical considerations [1], it appears that procurement of pancreatic islets from other animal species could provide a reliable, theoretically unlimited source for life-saving beta-cell replacement therapy. In this context, pigs are most frequently cited as ideal donors of endocrine pancreatic tissue given the historical use of porcine insulin to treat diabetes before and in some cases even after synthetic human insulin was made available in the early 80's. Other factors favoring pigs as islet donors include but are not limited to the possibility of genetically engineering these animals in an effort to render them immunologically [3] and physiologically [4] more compatible with human recipients. Scientific research has already identified key-factors favoring successful islet xenotransplantation in preclinical small and large animal models with main outcomes being graft survival and *in vivo* function leading to amelioration

or complete reversal of the diabetic state. Choosing donors of optimal age and strain [5], improvement of islet isolation and culture techniques, better understanding of pig islet physiology, determination of optimal implantation sites, refinement of immunosuppressive treatment and/or development of cellular encapsulation [5] together with genetic modifications aiming to optimize one or a combination of the aforementioned points are all key factors relevant to further development of the field. In the current article, we focus on reviewing in vitro studies dealing with regulation of porcine islet insulin secretion as well most recent preclinical studies of porcine islet xenotransplantation.

IN VITRO STUDIES OF ISOLATED PORCINE ISLETS

Compared to the extensive body of work in pancreatic islet physiology utilizing mostly murine and to a certain extent human islets, the number of studies dedicated to *in vitro* characterization of porcine islets is small. This probably stems from the higher requirements for pig housing and breeding in a research laboratory setting as well as more time- and money-consuming isolation techniques that are known to provide variable and somehow hard-to-predict islet yields. Following isolation, purification and culture, secretory function of obtained isolated islets could be studied by exposing them to various physiological and non-physiological insulin secretagogues in static incubation or preferably dynamic perifusion experiments to record hormone output as well as underlying metabolic changes.

Porcine islet insulin content

Before delving into characteristics of insulin secretion from porcine islets, a look into their insulin content is worthy. A few studies expressing adult pig islet insulin content normalized to islet size (insulin content per islet-equivalent) report very similar values varying from 616 to 807 µU/IEQ [6-9]. Such values are slightly higher than or similar to what is reported for human islets depending on the normalization method used as reviewed here [10]. Similar observations were made when insulin content was normalized to DNA content [11]. Data regarding neonatal porcine islet insulin content is less homogenous with different groups reporting ranges from 30 to 1300 µU/islet [6, 12-14] although this variability could be easily attributed to the lack of normalization. Interestingly, insulin content per cell aggregate was inversely correlated to the reported islet yield across these studies. In our hands, we calculate an insulin content of 175 µU/IEQ after normalization to islet size [6] consistent with a study reporting 6-fold less insulin in juvenile pig islets compared to adults after normalization to DNA content [6] (summary in Table I).

Glucose stimulation of insulin secretion

To be useful in transplantation, porcine islets are required to adjust their insulin secretion rate in a quantitatively and qualitatively similar fashion to that of the recipient organism. Islets isolated from neonatal pigs have been unanimously reported to be weak insulin secretors upon stimulation with glucose alone [6,11, 12,14-17]. This is reflected by low stimulation indices and low absolute amounts of secreted insulin owing to their lower insulin content and the immaturity of their beta-cell population as summarized in Table I. However, neonatal islets present the non-negligible advantage of undergoing maturation as evidenced by the increase of grafted islet insulin content [12,14] and beta-cell proportion [18,19] in xenotransplantation models. *In vitro*, long-term culture of neonatal islets requires a form of encapsulation to support islet integrity, survival and function, the latter being significantly improved during the culture period up to 20 weeks [20]. Only one study reported a remarkably high stimulation index of 23.9 with incubated neonatal pig islets as well as exceptionally high insulin content [13]. However, the authors reported absence of biphasic insulin secretion in response to glucose and lack of maturation after 4 weeks of transplantation to non-diabetic mice. In adult porcine islets, glucose elicits biphasic insulin secretion as shown in several studies utilizing the dynamic perifusion approach [6,7,11,16,21]. However, this response differs from that of human or non-human primate islets in many aspects. The magnitude of the response is the most striking and constantly noted difference in all reviewed studies as porcine islets show an average stimulation index of 3.4 ± 2.0 which is up to 10-fold lower than human islets [7]. One study noted the existence of a glucose memory phenomenon reflected by increased insulin secretion and a slightly higher stimulation index (4.1 *vs* 3.3) during a second exposure to the sugar in adult pig islets [22]. In a recent study by Smith et al. [16], the authors reported a higher stimulation index of 8.5 for perifused adult islets after normalizing insulin concentrations to DNA content. However, the ratio of stimulated to basal secretion is not specified when results were normalized to total insulin content. Another peculiar characteristic of the dynamics of insulin secretion from adult porcine islets that can be observed only during perifusion experiments is the relatively small 1st phase and the often ascending second phase [7,23]. This is of particular importance since a robust 1st phase is associated with efficient glucose homeostasis in humans and its loss is regarded as an early sign of endocrine pancreas function deterioration [24]. Upon washout of high glucose media and the return to resting glucose concentrations, the expected decrease in insulin secretion is often delayed and even preceded by a paradoxical off-response as reported in these studies [7,11,25]. Obviously, sudden changes of glucose concentrations induce unexpected secretory responses in pig islets. This is also the case when glucose concentration is increased stepwise since almost no changes were observed between 1 and 7 mM glucose and insulin secretion had only doubled by 10 mM glucose [7].

Table I. Insulin content and stimulation indices of isolated neonatal and adult porcine islets.

Age	Insulin content	S.I.	GSIS	1st author	Year	Ref
Neonatal	1.9 ng/islet	5.5	Incubation	Korbutt	1996	7
Neonatal	1 ng/islet	1.1	Incubation	Omer	2003	9
Neonatal	45.3 ng/islet	23.9	Incubation	Juang	2004	8
Neonatal	1.5 pg/ng DNA	-	Perifusion	Mueller	2013	6
Neonatal	175 µU/IEQ	4.4	Perifusion	Mourad	2017	1
Neonatal	-	4.4	Incubation	Lee	2018	10
Neonatal	0.05 ng/ng DNA	1.8	Perifusion	Smith	2018	11
Neonatal	100 pg/ng DNA	4.5	Incubation	Lau	2021	12
Adult	-	2.5	Perifusion	Takei	1994	16
Adult	-	2	Incubation	Rabuazzo	1995	21
Adult	-	2.8	Perifusion	Bertuzzi	1996	18
Adult	-	3.6	Perifusion	Gouin	1998	17
Adult	774 µU/IEQ	3	Perifusion	Brandhorst	1999	4
Adult	683 µU/IEQ	5	Incubation	O'Neil	2001	3
Adult	-	2.2	Perifusion	Bottino	2007	34
Adult	28 ng/IEQ	2	Perifusion	Dufrane	2007	2
Adult	9.2 pg/ng DNA	-	Perifusion	Mueller	2013	6
Adult	616 µU/IEQ	2.3	Perifusion	Mourad	2017	1
Adult	3.5 ng/ng DNA	8.5	Perifusion	Smith	2018	11

Total insulin content values are presented as reported in each study. Stimulation indices (S.I.) were calculated as the ratio of glucose-stimulated to basal insulin secretion with no distinction made between phases of insulin secretion. Reported glucose-stimulated insulin secretion (GSIS) tests utilized static incubation or dynamic perifusion methods.

Stimulus-secretion coupling in porcine islets

Given the disappointingly weak secretory response of pig islets and having established that islet insulin content is not a limiting factor, one can wonder whether a limiting step in glucose transport, its metabolism and subsequent triggering of cytosolic calcium concentration rise exists. Metabolic studies of adult primary porcine islet cells revealed no defects in glucose transport by GLUT-1 and/or GLUT-2 which was found to significantly exceed glucose phosphorylation as in rat and human islets [26,27]. Glucose phosphorylation and utilization, the following steps in stimulus-secretion coupling, were also found to happen at similar affinity and speed as in rodent islets [26]. This is also supported by the observation that glucokinase activation by GKA50 had no impact on insulin secretion from adult pig islets exposed to a gradual increase in glucose concentration alone [6]. A difference was however noted when measuring glucose-induced calcium changes in porcine islets with the rise of cytosolic calcium being small and delayed [23] compared to rodent [28] and human [29] islets. In our hands, changing glucose concentration from 1 to 15 mM induced no calcium oscillations in individual islets and a shy 1.5-fold increase in cytosolic calcium took 15 minutes to occur (Figure 1, previously unpublished).

Non-glucose modulators of porcine islet insulin secretion

Much less studied is the initiation of an insulin secretory response from porcine islets by amino acids. As with the response to glucose, stimulation of adult porcine islets by leucine, glutamine, arginine, alanine or a combination of these amino acids was found to be biphasic but of very low magnitude in 2 studies [7,11] compared to human islets [30]. Only one study investigated the effect of a combination of leucine and glutamine on insulin secretion from neonatal pig islets and reported a very low response that was nonetheless higher than the response to glucose [11].

Metabolic and neurohormonal amplification of porcine insulin secretion

Besides its clearly established role in triggering insulin secretion through direct effects on beta-cell electrical activity and cytosolic calcium, glucose amplifies the secretory response through a metabolic pathway that has been extensively investigated in rodent models and also demonstrated in human islets where it is thought to account for up to 50% of the total insulin response [31]. Such metabolic amplifying signals have been shown to be operational in porcine islets too: in the presence of either high concentrations of sulfonylurea tolbutamide, or a combination of K_{ATP} channel opener diazoxide and high KCl, glucose could further increase insulin secretion from adult porcine islets [7]. Under both conditions, the direct K_{ATP} channel-dependent pathway is bypassed through maximal activation of K_{ATP} channels by tolbutamide or their complete deactivation by diazoxide and additional secretion elicited by high glucose

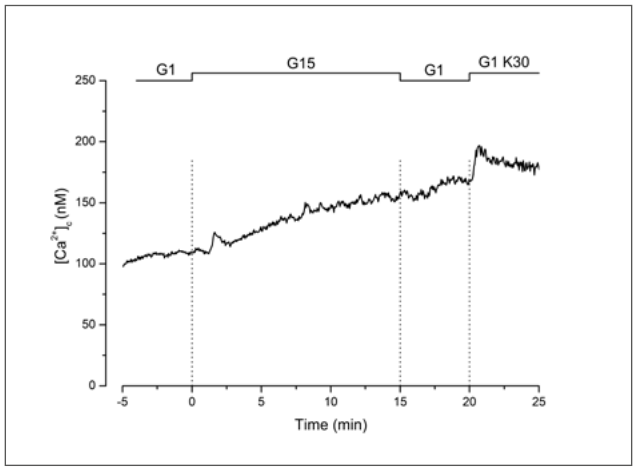

Figure 1. Glucose-induced changes of $[Ca^{2+}]_c$ in perifused adult pig islets. Cultured islets were loaded with the Ca^{2+} indicator furaPE3/AM (2 μM) for 2 hours at 37°C. Islets were then transferred into a chamber mounted on the stage of a microscope and maintained at 37°C. The fura-PE3 probe was excited at 340 and 380 nm, and emission was captured at 510 nm by a Quantem 512QC camera (Roper Scientific, Duluth, GA) and analyzed by the MetaFluor software (Universal Imaging, Downington, PA). Islets were first perifused with medium containing 1 mM glucose (G1) followed by consecutive stimulations with 15 mM glucose (G15) then high potassium (30 mM, K30) in the presence of G1. Stimulus-induced $[Ca^{2+}]_c$ changes were measured in individual islets and averaged for presentation as mean traces from 25 islets from 3 different preparations.

is attributed to metabolic amplification. Other amplifying pathways in the form of hormonal amplification exist. In this pathway, the rise of beta-cell cAMP and the ensuing activation of protein kinase A and Epac2 take center stage and significantly increase glucose-stimulated insulin secretion. Physiologically, this pathway is activated by glucagon-like peptide 1 (GLP-1) in what is known as the incretin effect. In in vitro studies, this paradigm is extensively used through addition of pharmacological agents such as forskolin or theophylline to significantly increase porcine islet insulin secretion in response to all stimuli cited before and has been instrumental in studying pig islet physiology especially when the effect of tested insulin secretagogues was too small to be appreciated [7]. Parasympathic neural stimulation of pancreatic islets mediated by acetylcholine binding to type 3 muscarinic receptors (M3R) expressed on beta cell membranes activates protein kinase C and potentiates the insulin secretory response to glucose [32]. Recordings from isolated perfused pig pancreas showed that vagus nerve stimulation or infusion of acetylcholine stimulated insulin secretion [33] and this was also the case when isolated pig islets were perifused with carbamylcholine [22] that directly activates protein kinase C via generation of its ligand diacylglycerol consistent with human islet sensitivity to cholinergic stimulation [34]. Interestingly, these paradigms can be used to ameliorate the insulin secretory response of pig islets and bring its amplitude closer to that of their human counterparts, which should favor successful islet preclinical and clinical xenotransplantation outcome and alleviate the need to transplant extremely large number of islets to compensate their low innate secretion. In this context, we have shown that concomitant pharmacological activation of PKA and PKC pathways produces a synergetic effect and greatly amplifies glucose-stimulated insulin secretion with stimulation indices as high as 12.5 and 6.1 in neonatal and adult pig islets respectively [6]. We then sought to induce these changes permanently by means of genetic modification and used adenoviral vectors to express a long-acting form of GLP-1 and a constitutively active form of M3R in porcine pancreatic islets. This resulted in a 4-fold increase of secreted insulin upon stimulation with 15 mM glucose compared to unmodified islets from both neonatal and adult age groups [6]. In light of these results, we established a herd of transgenic pigs carrying the aforementioned modifications specifically expressed at the beta-cell level and are currently evaluating the efficacy of neonatal islets isolated from these pigs in treating induced insulin-dependent diabetes in rodent and canine models.

Other factors affecting pig islet secretory response

As mentioned earlier, isolated islets can benefit from microenvironment enhancements to sustain their viability and secretory function *in vitro* post-isolation and *in vivo* post-implantation. Culture media supplementation with various growth factors including nicotinamide, exendin-4 or necrostatin-1 was efficient in improving islet insulin content and secretion [35,36]. In this context, islet encapsulation can serve not only to isolate implanted cells from the host immune system but also to provide a haven for an improved secretory response. As such, culture on porcine acellular dermis proved beneficial for neonatal islet maturation reflected by amelioration of their response to high glucose stimulation [22]. Additionally, encapsulation in an oxygen-generating scaffold promoted islet survival and higher insulin output during culture [15] while adjustment of the grade or concentration of encapsulation gels proved beneficial for *in vitro* function and *in vivo* performance of adult islets [37]. All experimental islet transplantation studies utilizing genetically modified pigs as islet donors indirectly evaluate islet secretory function as mirrored by the efficacy of the transplantation procedure in curing diabetes. However, this analysis does not permit discernment between pure islet function and the advantages procured by genetic modifications aimed to mitigate the immune response. Two studies have shown that multiple genetic modifications even when expressed under an insulin promoter did not cause impairment of glucose homeostasis in pigs [35,38] and did not affect *in vitro* insulin secretion by islets isolated from such pigs [39,40]. This is of great significance since pig islets that will be used in the clinic will be most probably sourced from herds carrying multiple genetic

modifications. Viral pathogens represent a risk inherent to the use of living xenogeneic material in the clinic for obvious biosafety considerations but also because some viruses from the Picornaviridae family have been shown to impair human and pig islet functionality even before their negative effect on viability became apparent thus emphasizing once again the importance of careful selection and testing of donor animals [41].

Glucagon secretion by porcine islets

Despite losing some during the isolation procedure, pig islets contain alpha-cells and therefore secrete glucagon *in vitro* and after transplantation. We recently investigated glucose regulation of glucagon secretion by perifused neonatal and adult porcine islets and reported inhibition of glucagon secretion by high glucose as expected [42]. Interestingly, our observations show greater impact of glucose fluctuations on glucagon than on insulin secretion, a characteristic that seems distinctive of porcine islets.

RECENT PROGRESS IN PRECLINICAL PIG ISLET XENOTRANSPLANTATION

Pig-to-non-human primate islet transplantation is often regarded as the final step in preclinical research before transition to the clinic. Indeed, successful control of diabetes in this model could easily translate into pig-to-human islet transplantation due to similarities in the immune system as well as better compatibility of porcine insulin compared to rodent models [43]. However, one should keep in mind that higher insulin demands in non-human primates such as macaques suggest that porcine islets could perform better in humans and that potentially lower doses of islets would be required to obtain similar clinical results. Recent preclinical studies of porcine islet xenotransplantation follow three main axis: i) refinement and establishment of new immunosuppression regimens; ii) genetic modification of donor pigs to improve engraftment and reduce immunogenicity; iii) using encapsulation to permit engraftment without immunosuppression.

Immunosuppressive regimens

Different combinations of immunosuppressive drugs have been tested as summarized in Table II. Costimulation pathway blockade using CTLA4Ig (belatacept or abatacept) [44-49] or anti-CD40 [44,45,50], T-cell depletion with antithymocyte globulin (ATG) [45,46,50] or anti-CD2 [44] together with other clinically available conventional treatments sustained graft survival and function up to 22 months following intraportal [45-51] or peritoneal [44] implantation. Although such regimens were efficient in suppressing B- and T-cell mediated rejection, they failed to regulate innate immunity manifested by chronic systemic inflammation accompanying long-term graft survival [48]. In one study, addition of tocilizumab to the basal immunosuppressive regimen to block pro-inflammatory IL-6 pathway was efficient in reducing inflammation measured by C-reactive protein assay but resulted in delayed vascularization of the graft as a secondary effect although this was not detrimental to graft survival and function [50]. Although use of anti-CD154mAb is not clinically approved, it still is part of several successful regimens and it did not cause thromboembolic complications in non-human primates in a study by Bottino et al. [52].

Genetic modifications

Three of the reviewed recent studies used genetically modified pig donors. All of these modifications aimed to mitigate the host immune response via expression of human complement regulators CD46, CD55, CD59 and/or porcine CTLA4-Ig on α-galKO background [49,51,52]. However, it is not clear to what extent did the genetic modifications contribute to islet engraftment and survival since heavy immunosuppression was employed in all studied groups. Nonetheless, Hawthorne et al. suggested in their study that use of genetically modified pigs permitted diabetes reversal with significantly lower doses of islets compared to unmodified donors [49] while Rao et al. reported that expression of human leukocyte antigen G1 (HLA-G1), a potent immunomodulatory molecule, in porcine cells suppressed the human and monkey T, B, and NK cell response *in vitro* [53].

Immunoisolation

Alginate encapsulation of adult wild-type pig islets permitted graft survival and function in NHP recipients treated with targeted immunosuppression. Under these conditions, graft failure was found to be due to hypoxia rather than immune rejection [44] underlining once again the importance of oxygen supply for prolonged islet graft survival. Indeed, islet encapsulation in an oxygen reservoir-containing device (BetaO_2) permitted maintenance of normoglycemia up to 190 days despite 50% reduction of exogenous insulin injection and no immunosuppression [54]. Similarly, islets embedded in agarose macrobeads and transplanted intraperitoneally to diabetic monkeys allowed significant (> 30%) reduction of daily insulin requirements and survived up to 7 years without any immunosuppression [55].

CONCLUSIONS

Porcine islets share more similarities than differences with human islets. They respond to an array of nutrient and pharmacological stimuli. Their secretory response is qualitatively often comparable to that of human islets but quantitatively lower in terms of secreted insulin despite similar hormone content. A few *in vitro* studies have highlighted this deficiency and showed that it is not limited by glucose metabolism in porcine beta-cells but suggested that poor calcium signaling in isolated pig islets might explain their low insulin secretion. Beyond the obvious solution of using

Table II. Preclinical trials of pig-to-NHP islet xenotransplantation.

Recipient		Donor		Dose	Immunosuppression	Site	Encapsulation	Follow-up	1st author	Year	Ref
n		Age	GM								
14	Cynomolgus	Adult	GTKO hCD46 hCD39 hTFPI CTLA4Ig	-	Anti-CD154	IP	-	365 days	Bottino	2017	47
3	Cynomolgus	Adult	WT	20000	-	SC	$BetaO_2$	6 months	Ludwig	2017	49
8	Cynomolgus Rhesus	Adult	WT	48000-91000	CTLA-Ig Anti-CD154 Anti-CD40	P	Alginate microcapsules	20-70 days	Safley	2018	39
9	Rhesus	Adult	WT	77000-100000	Betalacept Anti-CD40 Tacrolimus	IP	-	266 days	Shin	2018	40
5	Rhesus	Adult	WT	87000-100000	ATG Anti-CD40 Tacrolimus Tocilizumab	IP	-	22-176 days	Min	2018	45
12	Rhesus	NN	GKO hCD46	39000-115000	Basilixmab ATG Belatacept Tacrolimus	IP	-	14-40 days	Gao	2021	46
7	Rhesus	Adult	WT	55000-100000	ATG Adalimumab Tocilizumab Tacrolimus Abatacept	IP	-	34-222 days	Kim	2021	42
3	Rhesus	Adult	WT	523000*	ATG Adalimumab Tocilizumab Tacrolimus Abatacept	IP	-	237 days	Kim	2021	41
25	Cynomolgus	Adult	WT	20130	CTLA-Ig Basilix Tacrolimus	IP	-	288 days	Graham	2022	43
5	Baboons	NN	GTKO CD55 CD59	9000-56000	Anti-CD2 Anti-CD154 Belatacept Tacrolimus	IP	-	22 months	Hawthorne	2022	44
6	Cynomolgus	Adult	WT	9800-22900	-	P	Agarose beads	7 years	Holdcraft	2022	50

Non-human primates (NHP) received pancreatic islets from adult or neonatal (NN) pigs. Donors were genetically modified (GM) or wild-type (WT). Transplanted islet dose is indicated as IEQ/kg recipient body weight except for * where mean total IEQ is indicated. Islets were transplanted in the portal vein (IP), subcutaneously (SC) or in the peritoneal space (P).

more islets to compensate for weak function, genetic modification of existing amplifying pathways, improved culture conditions and usage of function-boosting encapsulation materials represent novel strategies to improve *in vitro* and *in vivo* insulin secretion. At the pre-clinical level, recent studies have focused on establishing clinically acceptable immunosuppressive treatments that can be further lightened by using less immunogenic genetically modified islets while cellular encapsulation carries the promise of immunosuppression-free xenotransplantation.

Conflict of interest statement

The authors have no conflict of interest.

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Author contributions

NM: wrote the manuscript, performed experiments and prepared tables and figures; PG: provided critical review and guidance and assisted in writing.

Ethical consideration

Not applicable.

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