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RESEARCH PAPER

An islet maturation media to improve the development of young porcine islets during in vitro culture

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ABSTRACT

Background: The use of pancreata from pre-weaned piglets has the potential to serve as an unlimited alternative source of islets for clinical xenotransplantation. As pre-weaned porcine islets (PPIs) are immature and require prolonged culture, we developed an islet maturation media (IMM) and evaluated its effect on improving the quantity and quality of PPIs over 14 days of culture.

Methods: PPIs were isolated from the pancreata of pre-weaned Yorkshire piglets (8–15 days old). Each independent islet isolation was divided for culture in either control Ham's F-10 media (n = 5) or IMM (n = 5) for 14 days. On day 3, 7 and 14 of culture, islets were assessed for islet yield, isolation index, viability, insulin content, endocrine cellular composition, differentiation of beta cells, and insulin secretion during glucose stimulation.

Results: In comparison to control islets, culturing PPIs in IMM significantly increased islet yield. PPIs cultured in IMM also maintained a stable isolation index and viability throughout 14 days of culture. The insulin content, endocrine cellular composition, and differentiation of beta cells were significantly improved in PPIs cultured in IMM, which subsequently augmented their insulin secretory capacity in response to glucose challenge compared to control islets.

Conclusions: Culturing PPIs in IMM increases islet yield, isolation index, viability, insulin content, endocrine cellular composition, differentiation of endocrine progenitor cells toward beta cells, and insulin secretion. Due to the improved islet quantity and quality after *in vitro* culture, the use of IMM in the culture of PPIs will assist to advance the outcomes of clinical islet xenotransplantation.

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Pre-weaned porcine islets; Type 1 diabetes; islet culture; culture media; in vitro culture

Introduction

Lifelong exogenous administration of insulin is currently being used as a treatment to manage

Type 1 diabetes.[1] Although it can improve the quality of life, daily treatment using intensive insulin therapy can lead to a threefold increase in the rate of severe hypoglycemia.[2] In addition, this practice is inconvenient to manage and can cause daily stress on a patient.[3] Allotransplantation of insulin-secreting islets by way of the Edmonton protocol can restore euglycemia and result in long-term insulin independence.[4,5] Even though islet transplantation has been shown to be a promising cure for Type 1 diabetes, this treatment is hampered by the scarcity of cadaveric donors.[6] Human donor selection is very particular because donor age, body mass index, and health affect the quality of islets isolated.[7] Touniversalize islet transplantation as a treatment, a need for more donors and maximal islet production must be identified.

In response to the high demand for islets, xenotransplantation of porcine islets has been extensively studied and shown as a viable alternative for islet allotransplantation.[8,9] Although adult porcine islets are mature and responsive to glucose challenge without the need for extended culture, islet isolation from adult pigs is costly and associated with substantial islet loss due to the increased islet fragility.[10] Young porcine islets isolated from neonatal (1–3 days old) or pre-weaned juvenile (8–15 days old) pigs have been reported to be a more cost-effective and scalable source of islets compared to adult porcine islets.[11,12] Our group has demonstrated a method to partially digest pre-weaned juvenile porcine pancreata without requiring prolonged husbandry and

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elaborated equipment.[13] Pre-weaned porcine pancreata yield a higher amount of islets per gram of pancreas compared to adult porcine pancreata, likely due to their immature exocrine component and inactive pancreatic enzymes.[13,14] Despite this advantage, the endocrine composition of pre-weaned porcine islets (PPIs) requires prolonged culture for development and adequate insulin secretion.[15,16] While pre-transplant culture is essential to improve the quality of PPIs, substantial islet loss occurs during this period.[17,18] Therefore, optimizing the culture media of PPIs to accelerate the *in vitro* culture period may reduce the culture time, mitigate islet loss, and improve islet development.

The effects of various cytoprotective agents and growth factors on the differentiation of islet endocrine cells and islet function during *in vitro* culture have been extensively studied. Porcine islets cultured in Ham's F-12 and Medium 199 have been reported to have increased insulin secretion compared to those cultured in CMRL 1066 or RPMI 1640 medium.[19] Culturing murine and human in culture media supplemented with glutathione could decrease the production of reactive oxygen species, reduce the rate of apoptosis, and improve islet viability.[20] A mixture of insulin, transferrin, and selenium (ITS) has been used to promote the differentiation of human labia minora dermis-derived fibroblasts into insulin-producing cells.[21] Nicotinamide has been shown to increase the insulin content and insulin response during glucose challenge in neonatal porcine islets.[22] The antioxidant trolox, an H2O2-scavenging vitamin E analog, has been used in the digestion medium for the isolation of intact and viable porcine islets.[23] The use of heparin has been shown to prevent islet loss and protect islet graft against instant blood-mediated inflammatory reaction after islet transplantation without affecting insulin secretion.[24,25] Previous studies have demonstrated that the addition of a serine protease inhibitor during islet isolation reduces the activity of serine proteases and improves islet yield.[26,27] L-glutamine treatment in porcine islets has been reported to increase islet resistance against proinflammatory mediators and viability during culture.[28,29]

In an effort to improve the quantity and quality of PPIs during pre-transplant culture, we developed an islet maturation media (IMM) using previously studied cytoprotective agents and growth factors. The aim of this study was to determine whether culturing PPIs in IMM can minimize islet loss, improve islet insulin content, facilitate endocrine cell development, and increase insulin secretion over a 14-day culture period.

Results

Effects of IMM on the yield, isolation index, and beta-cell recovery of PPIs

The yield of PPIs cultured in either control media or IMM after 3, 7, and 14 days was expressed as both islet count and islet equivalent per gram of pancreatic tissue (IC/g and IEQ/g) and per pancreas (IC/pancreas and IEQ/pancreas). On day 3 of culture, islet cultured in IMM (10065 ± 1010 IC/g) had a significantly higher islet count compared to control islets (7029 ± 898 IC/g) (p <.05, Table 1). However, there was no significant difference in the amount of islet equivalent between control islets (9985 ± 1761 IEQ/g) and islets cultured in IMM (12600 ± 1573 IEQ/g) (p = NS, Table 1). After 7-day culture, the count of islets cultured in IMM (6065 ± 476 IC/g) was not significantly different than control islets (4593 ± 1183 IC/g) (p = NS, Table 1). In comparison to control islets (3058 ± 454 IEQ/g), islets cultured in IMM (7443 ± 1383 IEQ/g) had a twofold increase in the amount of islet equivalent (p < .05, Table 1). The islet count on day 14 of culture was similar between control islets (2121 ± 394 IC/g) and islets cultured in IMM (3050 ± 262 IC/g) (p = NS, Table 1). The amount of islet equivalent from islets cultured IMM (2534 ± 364 IEQ/g) was twofold higher than control islets (1082 ± 134 IEQ/g) after 14 days of culture (p < .01, Table 1).

After islet yield was normalized to recovery per pancreas, there was no significant difference in the islet counts per pancreas between control islets (day 3 = 37314 ± 5741 IC/pancreas, day 7 = 24746 ± 6598 IC/pancreas, day 14 = 10600 ± 1794 IC/pancreas) and islets cultured in IMM (day 3 = 51174 ± 6169 IC/pancreas, day 7 = 31263 ± 2589 IC/pancreas, day 14 = 15946 ± 1648 IC/pancreas) on day 3, 7, and 14 of culture (p = NS, Table 2). On day 3 of culture, control islets (52580 ± 9473 IEQ/pancreas) and islets cultured in IMM (64108 ± 8845 IEQ/pancreas) had a similar islet equivalent per pancreas (p = NS, Table 2). However, islets cultured in IMM (day

Table 1. Islet yield per gram of pancreatic tissue and isolation index after 3, 7, and 14 days of culture in either control media or IMM.

	Day 3			Day 7			Day 14		
Media	IC/g	IEQ/g	Isolation index	IC/g	IEQ/g	Isolation index	IC/g	IEQ/g	Isolation index
Control	7029 ± 898$^{¥}$	9985 ± 1761$^{††¥¥}$	1.36 ± 0.14$^{†¥¥}$	4593 ± 1183	3058 ± 454	0.85 ± 0.09	2121 ± 394	1082 ± 134	0.55 ± 0.11
IMM	10065 ± 1010*$^{††¥¥}$	12600 ± 1573$^{†¥¥}$	1.60 ± 0.20	6065 ± 476$^{¥¥}$	7443 ± 1383*	1.36 ± 0.24	3050 ± 262	2534 ± 364**	1.34 ± 0.24*

IC/g: Islet count per gram of pancreatic tissue. IEQ/g: Islet equivalent per gram of pancreatic tissue. *p <.05 versus control media on the same day of culture. **p <.01 versus control media on the same day of culture. †p <.05 versus day 7 of culture in the same media. ††p <.01 versus day 7 of culture in the same media. $^{¥}$p <.05 versus day 14 of culture in the same media. $^{¥¥}$p < 0.01 versus day 14 of culture in the same media. Values represent mean ± SEM.

Table 2. Islet yield and total beta-cell recovery per pancreas after 3, 7, and 14 days of culture in either control media or IMM.

	Day 3			Day 7			Day 14		
Media	IC/pancreas	IEQ/pancreas	Beta cells/ pancreas (10^5)	IC/pancreas	IEQ/pancreas	Beta cells/ pancreas (10^5)	IC/pancreas	IEQ/pancreas	Beta cells/ pancreas (10^5)
Control	37314 ± 5741$^{¥}$	52580 ± 9473$^{††¥¥}$	1.21 ± 0.31	24746 ± 6598	15380 ± 2750	0.59 ± 0.067	10600 ± 1794	5463 ± 676	0.95 ± 0.12
IMM	51174 ± 6169$^{††¥¥}$	64108 ± 8845$^{¥¥}$	3.63 ± 0.94*	31263 ± 2589$^{¥}$	38714 ± 7679*	3.39 ± 0.86*	15946 ± 1648	12679 ± 1709**	1.55 ± 0.31

IC/pancreas: Islet count per pancreas. IEQ/pancreas: Islet equivalent per pancreas. *p <.05 versus control media on the same day of culture. **p <.01 versus control media on the same day of culture. †p <.05 versus day 7 of culture in the same media. ††p <.01 versus day 7 of culture in the same media. $^{¥}$p <.05 versus day 14 of culture in the same media. $^{¥¥}$p < 0.01 versus day 14 of culture in the same media. Values represent mean ± SEM.

7 = 38714 ± 7679 IEQ/pancreas, day 14 = 12679 ± 1709 IEQ/pancreas) had significantly higher islet equivalents per pancreas compared control islets (day 7 = 15380 ± 2750 IEQ/pancreas, day 14 = 5463 ± 676 IEQ/pancreas) on day 7 and 14 of culture ($p < .05$, $p < .01$, respectively, Table 2).

Control islets (1.36 ± 0.14 and 0.85 ± 0.09, respectively) and islets cultured in IMM (1.60 ± 0.20 and 1.36 ± 0.24, respectively) had a similar isolation index on both day 3 and day 7 of culture (p = NS, Table 1). On day 14 of culture, the isolation index was significantly higher in islets cultured in IMM (1.34 ± 0.24) when compared to control islets (0.55 ± 0.11) ($p < .05$, Table 1). Control islets on day 3 of culture had a significantly higher isolation index than control islets on day 7 and 14 of culture ($p < .05$, $p < .01$, respectively, Table 1). However, islets cultured in IMM had no significant differences in the isolation indices throughout 14 days of culture (p = NS, Table 1). The beta-cell recovery was significantly higher in islets cultured in IMM (day 3 = 3.63 ± 0.94 x 10^5 beta cells/pancreas, day 7 = 3.39 ± 0.86 x 10^5 beta cells/pancreas, day 14 = 1.55 ± 0.31 x 10^5 beta cells/pancreas) compared control islets (day 3 = 1.21 ± 0.31 x 10^5 beta cells/pancreas, day 7 = 0.59 ± 0.86 x 10^5 beta cells/pancreas, day 14 = 0.95 ± 0.12 x 10^5 beta cells/pancreas) on day 3 and 7, but not day 14 of culture ($p < .05$, $p < .05$, p = NS, respectively, Table 2).

Effects of IMM on the viability of PPIs

The viability of islets cultured in either control media or IMM throughout 14-day culture was assessed by Calcein AM and propidium iodide staining. Control islets on day 3 (82.57 ± 4.23%) of culture had significantly lower viability compared to control islets on day 7 (97.40 ± 0.68%) and day 14 (95.03 ± 0.51%) of culture ($p < .01$, $p < .05$, respectively, Figure 1). Islets cultured in IMM on day 3 (92.88 ± 2.16%), 7 (94.00 ± 1.18%), and 14 (96.20 ± 1.66%) of culture were significantly more viable than control islets on day 3 of culture ($p < .05$, $p < .05$, $p < .01$, respectively, Figure 1). The viability of islets cultured in IMM was similar on day 3, 7, and 14 of culture (p = NS, Figure 1).

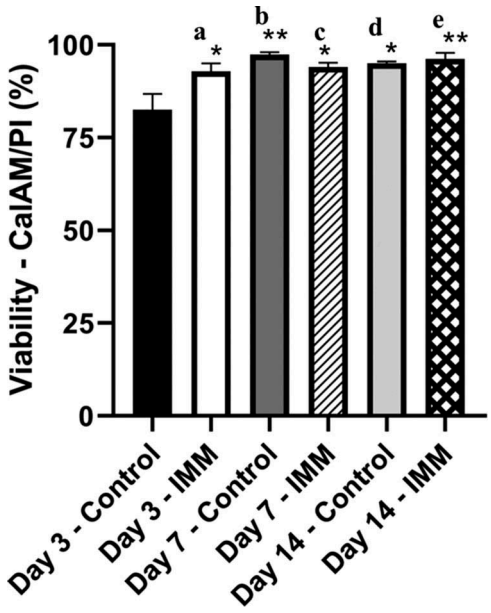

Figure 1. Islet viability after 3, 7, and 14 days of culture in either control media or IMM. 100 islet equivalents were stained with Calcein AM (CalAM) and propidium iodide (PI) for 30 minutes. Stained islets were analyzed using a microplate reader. The islet viability was calculated by the equation: CalAM-positive cells/(CalAM-positive cells + PI-positive cells) x 100. n = 5 for each group. *p <.05. **p <.01. Data expressed as mean ± SEM. a. Day 3 – Control vs. Day 3 – IMM; b. Day 3 – Control vs. Day 7 – Control; c. Day 3 – Control vs. Day 7 – IMM; d. Day 3 – Control vs. Day 14 – Control; e. Day 3 – Control vs. Day 14 – IMM.

Effects of IMM on the insulin content of PPIs

There was no significant difference in the insulin content of control islets on day 3 (24.89 ± 5.11 pg insulin/ng DNA), 7 (33.08 ± 6.70 pg insulin/ng DNA), and 14 (25.29 ± 4.59 pg insulin/ng DNA) of culture (p = NS, Figure 2). The insulin content of islets cultured in IMM on day 3 (35.10 ± 4.31 pg insulin/ng DNA) of culture was not significantly different on day 7 (51.13 ± 4.31 pg insulin/ng DNA) of culture, but was significantly lower compared to islets on day 14 (60.83 ± 6.15 pg insulin/ng DNA) of culture (p = NS, $p < .05$, respectively, Figure 2). Islets cultured in IMM on day 7 and 14 of culture had a twofold increase in insulin content compared to control islets on day 3 of culture ($p < .05$, $p < .01$, respectively, Figure 2). Control islets on day 7 of culture had significantly lower insulin content than islets cultured in IMM on day 14 of culture ($p < .05$, Figure 2). Culturing islets in IMM resulted in a twofold increase in insulin

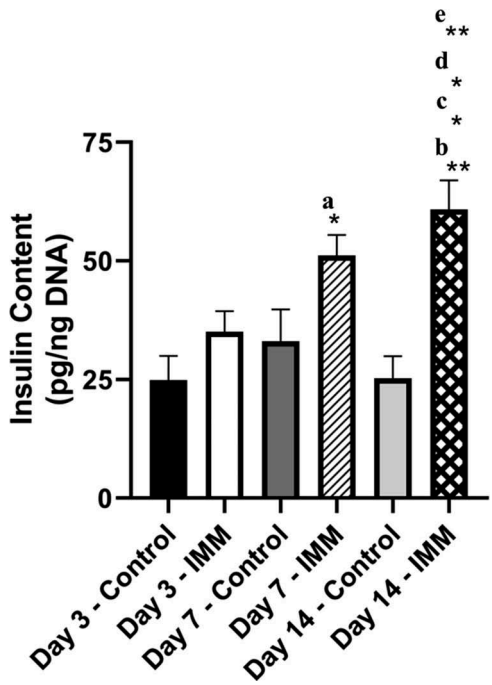

Figure 2. Islet insulin content after 3, 7, and 14 days of culture in either control media or IMM. 150 islet equivalents were lysed and sonicated. The insulin content was measured using standard porcine insulin ELISA and normalized to the DNA content of each sample. n = 5 for each group. *p <.05. **p <.01. Data expressed as mean ± SEM. a. Day 3 – Control vs. Day 7 – IMM; b. Day 3 – Control vs. Day 14 – IMM; c. Day 3 – IMM vs. Day 14 – IMM; d. Day 7 – Control vs. Day 14 – IMM; e. Day 14 – Control vs. Day 14 – IMM.

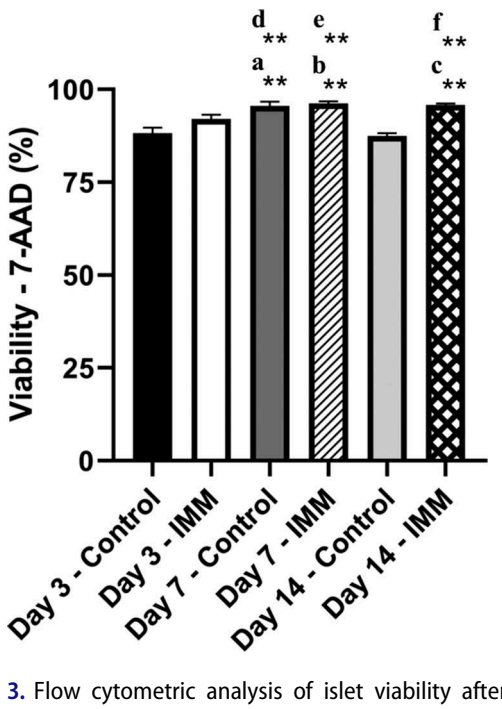

Figure 3. Flow cytometric analysis of islet viability after 3, 7, and 14 days of culture in either control media or IMM. 3000 islet equivalents were dissociated using Accutase, stained with 7-AAD viability dye, and analyzed by flow cytometry. n = 5 for each group. *p <.05. **p <.01. Data expressed as mean ± SEM. a. Day 3 – Control vs. Day 7 – Control; b. Day 3 – Control vs. Day 7 – IMM; c. Day 3 – Control vs. Day 14 – IMM; d. Day 7 – Control vs. Day 14 – Control; e. Day 7 – IMM vs. Day 14 – Control; f. Day 14 – Control vs. Day 14 – IMM.

content compared to control islets on day 14 of culture ($p < .01$, Figure 2).

Effects of IMM on cellular viability and composition of PPIs

7-AAD staining and flow cytometry were used to determine the viability of dispersed islet cells after 14-day culture. The viability of control islet cells on day 3 (88.24 ± 1.43%) of culture was significantly lower than control islet cells on day 7 (95.57 ± 1.12%) of culture, but not on day 14 (87.48 ± 0.74%) of culture ($p < .01$, p = NS, respectively, Figure 3). Control islet cells on day 7 of culture had significantly higher viability than control islet cells on day 14 of culture ($p < .01$, Figure 3). There was no significant difference in the cellular viability of islets cultured in IMM on day 3 (92.04 ± 1.18%), 7 (96.22 ± 0.57%), and 14 (95.78 ± 0.42%) of culture (p = NS, Figure 3). In comparison to control islets on day 3 of culture, the cellular viability of islets cultured in IMM on day 7 and 14 of culture was significantly higher ($p < .01$, $p < .01$, respectively, Figure 3). The viability of islet cells from islets cultured in IMM on day 7 and 14 of culture was significantly higher than control islet cells on day 14 of culture ($p < .01$, $p < .01$, respectively, Figure 3).

There was no significant difference in the beta-cell composition of control islets on day 3 (2.65 ± 0.32%) and 7 (2.83 ± 0.47%) of culture (p = NS, Figure 4A). A fourfold increase in the level of beta cells was observed in control islets on day 14 (11.12 ± 1.20%) of culture compared to day 3 and 7 of culture ($p < .01$, $p < .01$, respectively, Figure 4A). On day 3 (5.07 ± 0.79%) of culture, islets culture in IMM had a similar beta-cell composition to those on day 7 (8.31 ± 1.29%) of culture. The beta-cell composition of islets cultured in IMM on day 14 (12.20 ± 1.6%) of culture was twofold higher than islets on day 3 of culture; however, this was not significantly different than islets on day 7 of culture ($p < .01$, p = NS, Figure

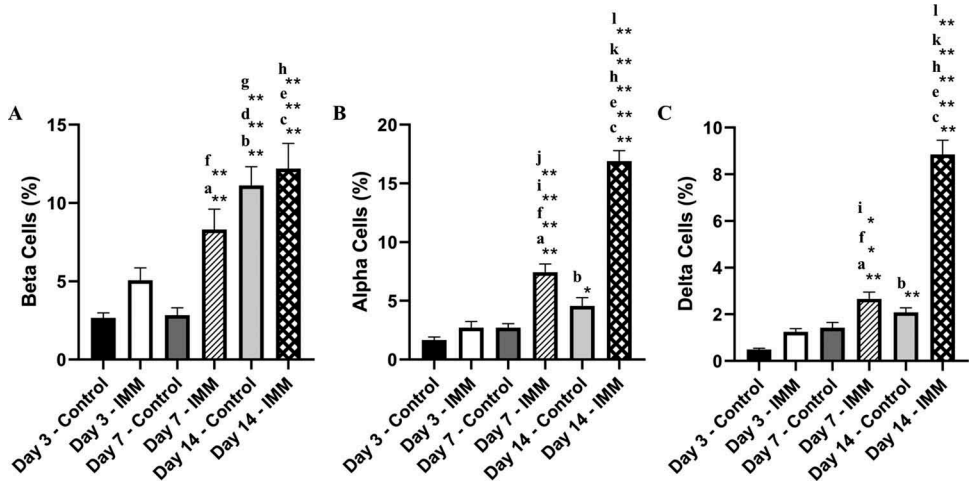

Figure 4. Flow cytometric analysis of islet cellular composition after 3, 7, and 14 days of culture in either control media or IMM. 3000 islet equivalents were dissociated using Accutase, stained with insulin, glucagon, and somatostatin antibodies, and analyzed by flow cytometry. (A) The percentage of beta cells. (B) The percentage of alpha cells. (C) The percentage of delta cells. n = 5 for each group. *p <.05. **p <.01. Data expressed as mean ± SEM. a. Day 3 – Control vs. Day 7 – IMM; b. Day 3 – Control vs. Day 14 – Control; c. Day 3 – Control vs. Day 14 – IMM; d. Day 3 – IMM vs. Day 14 – Control; e. Day 3 – IMM vs. Day 14 – IMM; f. Day 7 – Control vs. Day 7 – IMM; g. Day 7 – Control vs. Day 14 – Control; h. Day 7 – Control vs. Day 14 – IMM; i. Day 3 – IMM vs. Day 7 – IMM; j. Day 7 – IMM vs. Day 14 – Control; k. Day 7 – IMM vs. Day 14 – IMM; l. Day 14 – Control vs. Day 14 – IMM.

4A). In comparison to control islets on day 3 of culture, islets cultured in IMM on day 7 and 14 of culture had a threefold and fourfold increase in the level of beta cells, respectively ($p < .01$, $p < .01$, respectively, Figure 4A). Similarly, the beta-cell composition of islets cultured in IMM on day 7 and 14 of culture was threefold and fourfold higher than control islets on day 7 of culture, respectively ($p < .01$, $p < .01$, respectively, Figure 4A). Control islets on day 14 of culture also had a twofold increase in the beta-cell composition compared to islets cultured in IMM on day 3 of culture, but was not significantly higher than islets cultured in IMM on day 7 of culture ($p < .01$, p = NS, respectively, Figure 4A).

Control islets on day 3 (1.66 ± 0.26%) of culture had a similar alpha-cell composition to those on day 7 (2.71 ± 0.35%) of culture (p = NS, Figure 4B). Culturing islets in control media for 14 (4.56 ± 0.71%) days resulted in a twofold increase in the level of alpha cells compared to those on day 3 of culture ($p < .05$, Figure 4B). There was no significant difference in the alpha-cell composition between control islets on day 7 and 14 of cultured (p = NS, Figure 4B). Culturing islets in IMM resulted in a twofold and sixfold increase in the level of alpha cells on day 7 (7.44 ± 0.69%) and 14 (16.90 ± 0.89%) of culture, respectively, compared to day 3 (2.71 ± 0.54%) of culture ($p < .01$, $p < .01$, respectively, Figure 4B). In comparison to control islets on day 3 of culture, a fourfold and tenfold increase in alpha-cell composition was observed in islets cultured on day 7 and 14 of culture, respectively ($p < .01$, $p < .01$, respectively, Figure 4B). On day 7 of culture, islets cultured in IMM had a significantly higher level of alpha cells compared to control islets ($p < .01$, Figure 4B). The alpha-cell composition of islets cultured in IMM on day 7 of culture was also significantly higher than control islets on day 14 of culture ($p < .05$, Figure 4B). After 14-day culture, islets cultured in IMM had a twofold increase in the alpha-cell composition compared to those on day 7 of culture ($p < .05$, Figure 4B). In comparison to control islets on day 7 and 14 of culture, islets cultured in IMM on day 14 of culture had a sixfold and threefold increase in the alpha-cell composition, respectively ($p < .01$, $p < .01$, respectively, Figure 4B).

The delta-cell composition of control islets on day 3 (0.48 ± 0.06%) of culture was similar to those on day 7 (1.42 ± 0.23%) of culture (p = NS, Figure 4C). Control islets on day 14 (2.08 ± 0.21%) of culture had a fourfold increase in the level of delta cells compared to islets on day 3 of culture, but was not significantly different than islets on day 7 of culture ($p < .01$, p = NS, respectively, Figure 4C). On day 7 (2.66 ± 0.29%) of culture, the delta-cell composition of

islets cultured in IMM was twofold higher than islets on day 3 (1.25 ± 0.14%) of culture ($p < .05$, Figure 4C). Islets cultured in IMM on day 14 (8.84 ± 0.62%) of culture had a sevenfold and threefold increase in the level of delta cells compared to islets on day 3 and 7 of culture, respectively ($p < .01$, $p < .01$, respectively, Figure 4C). In comparison to control islets on day 3 of culture, the delta-cell composition of islets cultured in IMM on day 7 and 14 of culture was fivefold and eighteenfold higher, respectively ($p < .01$, $p < .01$, respectively, Figure 4C). Islets cultured in IMM on day 7 and 14 of culture also had a twofold and sixfold increase in the alpha-cell composition compared to control islets on day 7 of culture ($p < .05$, $p < .01$, respectively, Figure 4C). After 14-day culture, islets cultured in IMM had a threefold and fourfold increase in the alpha-cell composition in comparison to those on day 7 of culture and control islets on day 14 of culture, respectively ($p < .01$, $p < .01$, respectively, Figure 4C).

Effects of IMM on pancreatic endocrine cell differentiation of PPIs

Flow cytometry was utilized to assess the percentage of Ngn3-positive and Nkx6.1-positive endocrine progenitor cells. Ngn3 is a transcription factor that facilitates the differentiation of pancreatic precursor cells to endocrine progenitor cells.[30] The level of Ngn3-positive cells in control islets had no significant difference on day 3 (89.58 ± 2.78%), 7 (90.87 ± 1.73%), and 14 (83.43 ± 1.88%) of culture (p = NS, Figure 5A). In comparison to islets cultured in IMM on day 3 (80.50 ± 7.43%) of culture, islets on day 7 (61.20 ± 3.68%) and 14 (28.20 ± 4.13%) of culture had a significant decrease in the percentage of Ngn3-positive cells ($p < .05$, $p < .01$, respectively, Figure 5A). After 14 days of culture, islets cultured in IMM had a twofold decrease in the level of Ngn3-positive cells compared to those on day 7 of culture ($p < .05$, Figure 5A). Culturing islets in IMM resulted in a significant

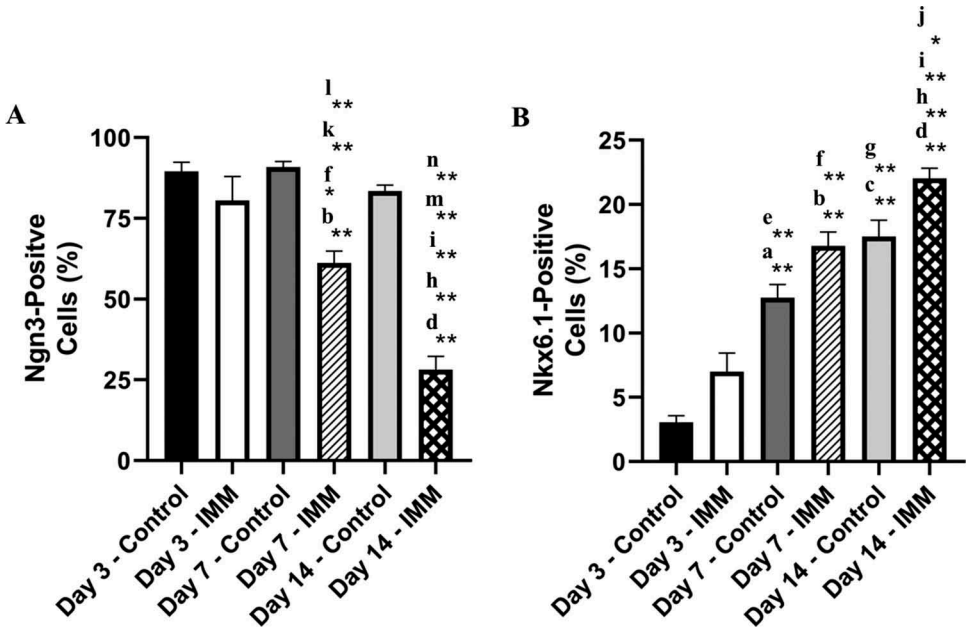

Figure 5. Flow cytometric analysis of islet endocrine cell differentiation after 3, 7, and 14 days of culture in either control media or IMM. 3000 islet equivalents were dissociated using Accutase, stained with Ngn3 and Nkx6.1 antibodies, and analyzed by flow cytometry. (A) The percentage of Ngn3-positive cells. (B) The percentage of Nkx6.1-positive cells. n = 5 for each group. *p <.05. **p <.01. Data expressed as mean ± SEM. a. Day 3 – Control vs. Day 7 – Control; b. Day 3 – Control vs. Day 7 – IMM; c. Day 3 – Control vs. Day 14 – Control; d. Day 3 – Control vs. Day 14 – IMM; e. Day 3 – IMM vs. Day 7 – Control; f. Day 3 – IMM vs. Day 7 – IMM; g. Day 3 – IMM vs. Day 14 – Control; h. Day 3 – IMM vs. Day 14 – IMM; i. Day 7 – Control vs. Day 14 – IMM; j. Day 7 – IMM vs. Day 14 – IMM; k. Day 7 – Control vs. Day 7 – IMM; l. Day 7 – IMM vs. Day 14 – Ham; m. Day 7 – IMM vs. Day 14 – IMM; n. Day 14 – Ham vs. Day 14 – IMM.

decrease in the number of Ngn3-positive cells on day 7 and 14 of culture compared to control islets on day 3 of culture ($p < .01$, $p < .01$, respectively, Figure 5A). Similarly, islets cultured in IMM had a significant reduction in the level of Ngn3-positive cells in comparison to control islets on day 7 of culture ($p < .01$, $p < .01$, respectively, Figure 5A). On day 7 and 14 of culture, the percentage of Ngn3-positive cells from islets cultured in IMM was significantly lower than control islets on day 14 of culture ($p < .05$, $p < .01$, respectively, Figure 5A).

The homeodomain transcription factor Nkx6.1 is required for the development of mature beta cells from pancreatic endocrine progenitor cells.[30] In comparison to control islets on day 3 (3.06 ± 0.51%) of culture, control islets on day 7 (12.75 ± 1.03%) and 14 (17.50 ± 1.28%) of culture had a fourfold and sixfold increase in the number of Nkx6.1-positive cells, respectively ($p < .01$, $p < .01$, respectively, Figure 5B). Similarly, islets cultured in IMM on day 7 (16.78 ± 1.09%) and 14 (22.03 ± 0.81%) of culture had a twofold and threefold increase in the level of Nkx6.1-positive cells compared to those on day 3 (7.01 ± 1.44%) of culture, respectively ($p < .01$, $p < .01$, respectively, Figure 5B). The percentage of Nkx6.1-positive cells in islets cultured in IMM on day 14 was also significantly higher than those on day 7 of culture ($p < .05$, Figure 5B). In comparison to control islets on day 3 of culture, islets cultured in IMM on day 7 and 14 of culture had a fivefold and sevenfold increase in the level of Nkx6.1-positive cells ($p < .01$, $p < .01$, respectively, Figure 5B). On day 3 of culture, the number of Nkx6.1-positive cells from islets cultured in IMM was significantly lower than control islets on day 7 and 14 of culture ($p < .05$, $p < .01$, respectively, Figure 5B). The level of Nkx6.1-positive cells from control islets on day 7 of culture remained similar to those on day 14 of culture, but was significantly lower than islets cultured in IMM on day 14 of culture (p = NS, $p < .01$, respectively, Figure 5B).

Effects of IMM on function of PPIs

To assess islet function after culturing in either control media or IMM, both static and dynamic glucose stimulated insulin release (GSIR) assays were performed on day 3, 7, and 14 of culture. During static GSIR assays, islets from each sample were incubated for one hour in the following concentration and order of glucose media: 2.8 mM (L1), 28 mM (H), 2.8 mM (L2), and 28 mM + 0.1 mM IBMX (H+). The insulin concentration released per hour in each glucose media was normalized to the DNA content in each sample. There was no significant difference in the amount of insulin secreted by control islets in response to glucose challenge on day 3 (L1 = 0.29 ± 0.10 pg/ng DNA/h, H = 0.52 ± 0.13 pg/ng DNA/h, L2 = 0.38 ± 0.12 pg/ng DNA/h, H+ = 0.97 ± 0.35 pg/ng DNA/h), 7 (L1 = 0.29 ± 0.07 pg/ng DNA/h, H = 0.64 ± 0.25 pg/ng DNA/h, L2 = 0.44 ± 0.22 pg/ng DNA/h, H+ = 0.97 ± 0.20 pg/ng DNA/h), and 14 (L1 = 0.30 ± 0.05 pg/ng DNA/h, H = 0.48 ± 0.11 pg/ng DNA/h, L2 = 0.27 ± 0.03 pg/ng DNA/h, H+ = 0.41 ± 0.08 pg/ng DNA/h) (p = NS, Figure 6A). In islets cultured in IMM, the insulin secretion on day 3 (L1 = 0.34 ± 0.05 pg/ng DNA/h, H = 0.57 ± 0.08 pg/ng DNA/h, L2 = 0.39 ± 0.04 pg/ng DNA/h, H+ = 1.12 ± 0.21 pg/ng DNA/h) of culture in each corresponding glucose media was similar to day 7 (L1 = 0.40 ± 0.07 pg/ng DNA/h, H = 0.89 ± 0.14 pg/ng DNA/h, L2 = 0.48 ± 0.04 pg/ng DNA/h, H + = 1.31 ± 0.28 pg/ng DNA/h) of culture (p = NS, Figure 6A). However, islets cultured in IMM on day 14 (L1 = 0.59 ± 0.04 pg/ng DNA/h, H = 1.05 ± 0.07 pg/ng DNA/h, L2 = 0.67 ± 0.04 pg/ng DNA/h, H+ = 1.67 ± 0.13 pg/ng DNA/h) of culture had a significant increase in the amount of secreted insulin in L1, H, and L2 but not H+ glucose media when compared to those on day 3 of culture ($p < .05$, $p < .05$, $p < .05$, p = NS, respectively, Figure 6A). There was no significant difference in the insulin secretion between islets cultured in IMM on day 7 and 14 of culture (p = NS, Figure 6A).

In comparison to control islets on day 3 of culture, islets cultured in IMM on day 14 had a twofold increase in the amount of secreted insulin in L1, H, and L2 glucose media ($p < .05$, $p < .05$, $p < .05$, respectively, Figure 6A). The insulin secretion in L1 media was significantly higher in islets cultured in IMM on day 14 of culture compared to control islets on day 7 of culture ($p < .05$, Figure 6A). On day 14 of culture, the amount of insulin secreted from islets cultured in IMM in L1, H, L2, and H+ glucose media

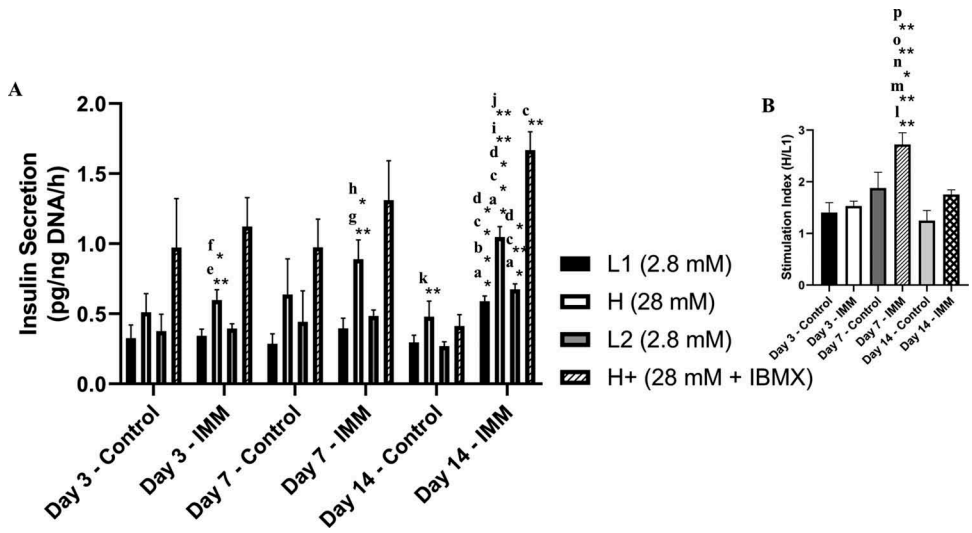

Figure 6. Static glucose stimulated islet insulin secretion after 3, 7, and 14 days of culture in either control media or IMM. Islet function was evaluated using static glucose stimulated insulin release assay. A triplicate of 100 islet equivalents was incubated for one hour in the following concentration and order of glucose media: 2.8 mM (L1), 28 mM (H), 2.8 mM (L2), and 28 mM + 0.1 mM IBMX (H+). The amount of insulin secreted by islets in each media was quantified using standard porcine insulin ELISA and normalized to the DNA content in each sample. (A) Insulin secretion in glucose media of varying concentration. (B) Stimulation index – calculated as the amount of insulin secreted in H media over L1 media. n = 5 for each group. *p <.05. **p <.01. Data expressed as mean ± SEM. a. Day 3 – IMM vs. Day 14 – IMM; b. Day 7 – Control vs. Day 14 – IMM; c. Day 14 – Control vs. Day 14 – IMM; d. Day 3 – Control vs. Day 14 – IMM; e. Day 3 – IMM – H vs. Day 3 – IMM – L1; f. Day 3 – IMM – H vs. Day 3 – IMM – L2; g. Day 7 – IMM – H vs. Day 7 – IMM – L1; h. Day 7 – IMM – H vs. Day 7 – IMM – L2; i. Day 14 – IMM – H vs. Day 14 – IMM – L1; j. Day 14 – IMM – H vs. Day 14 – IMM – L2; k. Day 14 – Control – H vs. Day 14 – Control – L2; l. Day 3 – Control vs. Day 7 – IMM; m. Day 3 – IMM vs. Day 7 – IMM; n. Day 7 – Control vs. Day 7 – IMM; o. Day 7 – IMM vs. Day 14 – Control; p. Day 7 – IMM vs. Day 14 – IMM.

was significantly higher than control islets ($p < .05$, $p < .05$, $p < .01$, $p < .01$, respectively, Figure 6A). Islets cultured in IMM on day 3, 7, and 14 of culture had significantly higher insulin secretion in H glucose media compared to L1 and L2 glucose media (Day 3: $p < .01$, $p < .05$; Day 7: $p < .01$, $p < .05$; Day 14: $p < .01$, $p < .01$, respectively, Figure 6A). In control islets, only those from day 14 of culture had a significantly higher amount of insulin secreted in H glucose media compared to L2 glucose media ($p < .01$, Figure 6A).

There was no significant difference in the stimulation indices of control islets on day 3 (1.41 ± 0.19), 7 (1.88 ± 0.30), and 14 (1.25 ± 0.19) of culture (p = NS, Figure 6B). Islets cultured in IMM on day 7 (2.72 ± 0.22) of culture had a significantly higher stimulation index compared to those on day 3 (1.53 ± 0.09) and 14 (1.75 ± 0.09) of culture ($p < .01$, $p < .01$, respectively, Figure 6B). Similarly, the stimulation indices of control islets on day 3, 7, and 14 of culture were significantly lower than islets cultured in IMM on day 7 of culture ($p < .01$, $p < .05$, $p < .01$, respectively, Figure 6B).

During dynamic GSIR perifusion assays, 200 islet equivalents from each group were sequentially exposed for 15 minutes in each glucose media: 2.8 mM, 28 mM, and 2.8 mM. The perifusate was collected every 5 minutes for the insulin content analysis. On day 3 of culture, islets cultured in IMM () secreted significantly more insulin at 30 minutes in 28 mM glucose media and at 40 and 45 minutes in 2.8 mM glucose media compared to control islets () ($p < .05$, $p < .01$, $p < .01$, respectively, Figure 7A). In comparison to control islets, the insulin secretion of islets cultured in IMM on day 7 of culture was significantly higher at 35, 40, and 45 minutes in 2.8 mM glucose media ($p < .01$, $p < .05$, $p < .01$, respectively, Figure 7B). The amount of insulin secreted throughout 45 minutes of dynamic perifusion assay from islets cultured in IMM was significantly higher than control islets on day 14 of culture (5 to 25 minutes: $p < .01$, 30 to 45 minutes: $p < .05$, Figure 7C). The total insulin secretion over 45 minutes calculated as the area under the curve (AUC) of islets cultured in IMM (961.72 ± 30.41 (mU/L)*minutes) on day 14 of culture was significantly higher than

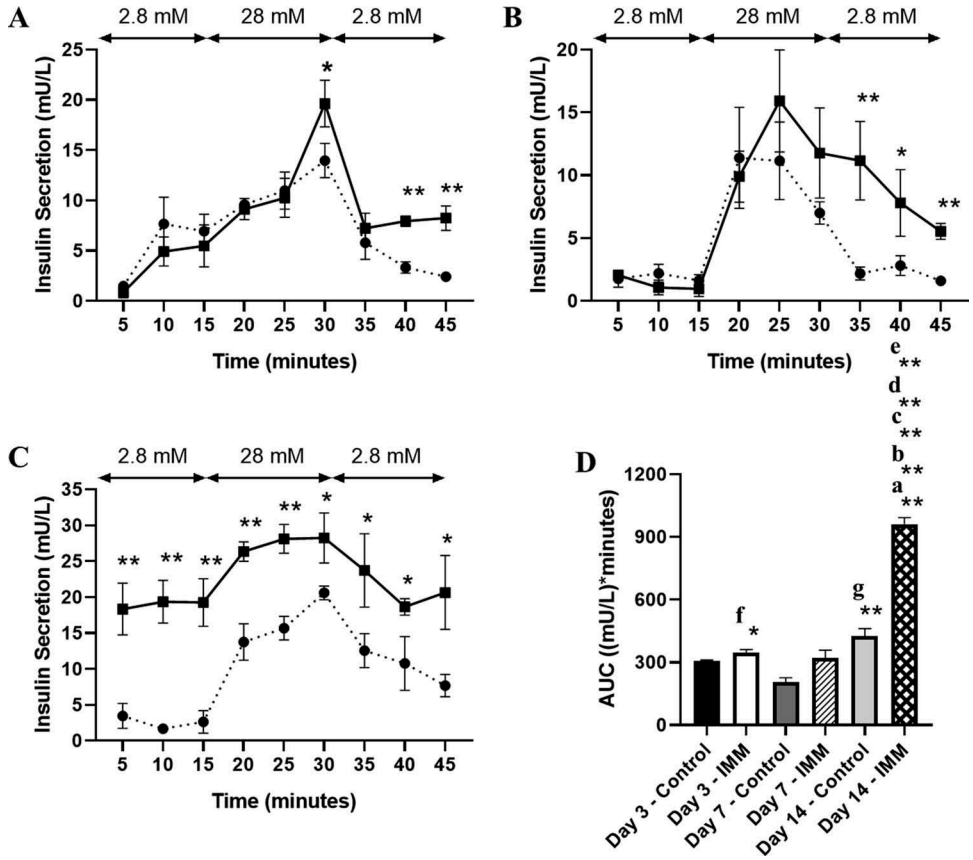

Figure 7. Dynamic glucose stimulated islet insulin secretion after 3, 7, and 14 days of culture in either control media or IMM. 200 islet equivalents were assessed for insulin secretion by dynamic glucose stimulated insulin release perifusion assay. Islets cultured in either control media (black circle, dotted line) or IMM (black square, solid line) were sequentially exposed for 15 minutes in each glucose media: 2.8 mM, 28 mM, and 2.8 mM. Perifusate was collected every 5 minutes and analyzed for insulin content using standard porcine insulin ELISA. (A) Islet insulin secretion on day 3 of culture. (B) Islet insulin secretion on day 7 of culture. (C) Islet insulin secretion on day 14 of culture. (D) Total insulin secretion after perifusion assay expressed as AUC. n = 3 for each group. *p <.05. **p <.01. Data expressed as mean ± SEM. a. Day 3 – Control vs. Day 14 – IMM; b. Day 3 – IMM vs. Day 14 – IMM; c. Day 7 – Control vs. Day 14 – IMM; d. Day 7 – IMM vs. Day 14 – IMM; e. Day 14 – Control vs. Day 14 – IMM; f. Day 3 – IMM vs. Day 7 – Control; g. Day 7 – Control vs. Day 14 – Control.

those on day 3 (348.26 ± 13.41 (mU/L)*minutes) and 7 (321.81 ± 63.33 (mU/L)*minutes) of culture as well as control islets on day 3 (305.07 ± 11.81 (mU/L)*minutes), 7 (209.99 ± 37.53 (mU/L)*minutes), and 14 (424.89 ± 63.05 (mU/L)*minutes) of culture ($p < .01$, Figure 7D).

Discussion

Optimizing the culture media of PPIs is crucial to improve the islet quantity and quality before transplantation. While a differentiation media has been developed and shown to accelerate the development of endocrine cells and improve insulin secretion capacity of neonatal porcine islets in comparison to Ham's F-10 media, no studies have described an improved culture media for PPIs.[31] In the present study, we examined the effects of culturing PPIs in IMM compared to control Ham's F-10 media on the islet yield, insulin content, development of endocrine cells, and insulin secretion over 14 days of culture. Culturing PPIs in IMM significantly improved islet yield, isolation index, viability, insulin content, endocrine cellular composition, differentiation of endocrine progenitor cells, and insulin response to glucose challenge.

Our findings that the yield of PPIs cultured in control media decreased throughout the 14-day culture was consistent with previous findings, which showed a significant reduction in the islet equivalent of neonatal porcine islets over 27 days of culture.[32] While IMM did not prevented the islet loss throughout the culture period, islets cultured in IMM had significantly higher islet count

on day 3 and islet equivalent on day 7 and 14 of culture compared to control islets. This twofold increase in the islet equivalent on day 7 and 14 of culture could be attributed to the addition of the serine-protease inhibitor, Pefabloc, to IMM that has been demonstrated to increase islet yield in both animal and human islet isolation.[27,33-36] The addition of Pefabloc to the digestion solution doubled the recovery of purified adult porcine islets.[34] After overnight culture, the supplementation of a serine-protease inhibitor to the culture medium significantly improved the recovery of impure human islets.[37] The higher yield from islets cultured in IMM compared to control islets could also be due to the higher isolation index, indicating less islet fragmentation. This finding supports a previous study, showing islets with a lower isolation index that were isolated from adult porcine pancreata after 7 hours of cold storage had significantly lower islet yield than freshly procured pancreata.[38] As multiple studies have demonstrated that higher islet yield is a crucial factor in a successful islet transplantation, the improvement in the yield of PPIs after culture in IMM may potentially contribute to more favorable outcomes following xenogeneic islet transplantation.[39-41]

Using two viability assays, the findings of the present study demonstrated that only control islets, but not islets cultured in IMM, on day 3 of culture had lower viability than control islets and islets cultured in IMM on day 7 and 14 of culture. Flow cytometric analysis of islet cellular viability after dissociation also showed that control islets on day 14 of culture were less viable than islets cultured in IMM on day 7 and 14 of culture. This discrepancy in the islet viability measured by two different viability assays may be because flow cytometric analysis of dissociated islet cells have been suggested to be more sensitive than microfluorometric viability assay.[42] The effect of culturing islets in IMM on maintaining islet viability throughout prolonged culture could be due to the use of glutathione. In murine islets, the addition of glutathione to the collagenase solution improved islet viability, fractional beta-cell viability, and transplant outcomes.[20] Culturing human islets in standard media supplemented with glutathione for 24 hours decreased the rate of apoptosis, leading to the maintenance of islet viability.[20]

Our finding that the insulin content of PPIs cultured in control media was similar throughout 14 days of culture is in line with a previously published study. Jimenez-Vera et al. reported that neonatal islets cultured in Ham's F-10 media had no significant changes in islet insulin content over a 27-day culture period.[32] When neonatal islets were cultured in differentiated media for 20 days, the higher beta-cell content was associated with a marked increase in the recovery of total cellular insulin compared to islets cultured in control Ham's F-10 media.[31] Thus, the increase in the insulin content of islets cultured in IMM is most likely the result of the significant expansion of beta-cell population on both day 7 and 14 of culture. As young porcine islets contain a major stem-cell like population that maintains multipotency in culture, this acceleration in the differentiation of beta cells in islets cultured in IMM could be due to the supplementation of ITS mixture and nicotinamide that have been used to differentiate human labia minora dermis-derived fibroblasts into insulin-producing cells.[21] The NK homeobox factor Nkx6.1 is a transcription factor that activates a major pathway of beta-cell neogenesis.[43] Based on the higher level of Nkx6.1-positive progenitor cells in islets cultured in IMM, it is possible that the increased beta-cell proportion of islets cultured in IMM is also due to the improved differentiation of pancreatic progenitor cells. This finding is consistent with a previously published report, which showed that the expression level of Nkx6.1 increased as progenitor cells differentiated into hormone-expressing cells during *in vitro* culture.[44] Ngn3 is a transcription factor that induces the development of endocrine progenitor cells from pancreatic precursor cells and is suppressed after the maturation of endocrine cells.[45,46] In correlation with the increase in the proportion of endocrine cells in islets cultured in IMM, the decrease in the level of Ngn3-positive cells throughout 14 days of culture indicates the differentiation of endocrine progenitor cells toward mature endocrine cells. This result is consistent with previous findings, which showed that the differentiation of human embryonic stem cells into mature endocrine cells after 24 days of culture was associated with the downregulation in the expression of Ngn3.[44]

The increase in the insulin content and composition of endocrine cells in islets cultured in IMM most likely leads to the significant increase in insulin secretion in response to glucose challenge. Hassouna et al. have reported that the higher proportion of beta cells and insulin content in neonatal porcine islets cultured in differentiated media were correlated with increased insulin secretion during glucose stimulation, which further supports our findings.[31] The increase in insulin release could also be due to the combination of ITS mixture and nicotinamide in IMM, which has been shown to improve insulin secretion of differentiated insulin-producing cells derived from human labia minora dermis-derived fibroblasts in high glucose (16.7 mM) media by nearly 4 times compared to undifferentiated cells.[21] Furthermore, the use of trolox to promote the differentiation of embryonic stem cell-derived beta-like cells over a 5–7 days culture period has been reported to increase the amount of insulin secreted during glucose stimulation by 2 times in comparison to untreated cells.[47] The addition of trolox to IMM potentially contributes to the improved insulin secretion of islets cultured in IMM.

Strikingly, only islets cultured in IMM media had a significantly higher insulin output after incubation in 28 mM glucose media compared to the first and second 2.8 mM glucose media throughout 14 days of culture. This suggests a return to the basal rate of insulin secretion and enhanced insulin response to glucose stimulation in islets cultured in IMM. These findings are in line with a published report by Littman et al., showing that incubating murine islets in media supplemented with glutathione enhanced insulin secretion during glucose stimulation and restored the basal rate of insulin secretion.[48] Jimenez-Vera et al. has reported that neonatal porcine islets cultured in Ham's F-10 media had similar stimulation indices throughout a 27-day culture period, which supports our findings that prolonged culture did not improve the stimulation index of control islets.[32] While differentiated neonatal porcine islets secreted more insulin in response to glucose challenge, there was no significant difference in the stimulation index compared to undifferentiated islets after 21-day prolonged culture.[31] In accordance with these previous findings, we found that culturing islets in IMM for 14 days could only increase the amount of insulin secreted but not the stimulation index. However, islets cultured in IMM on day 7 of culture had significantly higher stimulation index than islets on day 14 of culture but secreted similar amount of insulin in response to glucose stimulation. This finding suggests that culturing PPIs for 7 days may be the optimal culture time to minimize islet loss while also having comparable function to islets cultured for 14 days. In consistent with our findings, multiple studies have indicated that neonatal porcine islets are only cultured for 7–9 days prior to transplantation.[22,49,50] Thus, future studies should be conducted to determine whether culturing PPIs for over 7 days can translate to enhanced in vivo function and improved diabetes reversal in animal models of human Type 1 diabetes.

To our knowledge, this is the first study to develop a novel IMM for PPIs and assess its effect on islet development over a 14-day culture period. Taken together, PPIs cultured in IMM had higher islet yield, isolation index, viability, insulin content, endocrine cellular composition, differentiation of endocrine progenitor cells toward beta cells, and insulin secretion than control islets after a 14-day culture period. The current findings corroborate that the addition of cytoprotective agents and growth factors in culture media improves the quality and quantity of PPIs. Future studies that examine the use of novel pharmacological agents during in vitro culture will augment the development of PPIs prior to transplantation and assist to advance the outcomes of clinical islet xenotransplantation.

Materials and methods

Islet isolation

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine. PPIs were isolated from from 8-15-day-old, pre-weaned Yorkshire piglets (S&S Farms, Ramona, CA) as previously described.[13] Briefly, pancreata were rapidly procured in less than 10 minutes and stored in cold HBSS (cat# 2402011, Gibco-Thermo Fisher Scientific) until digestion. The cold ischemic time was restricted to less than 1 hour. Digestion was performed by mincing each pancreas into 1 mm^3 pieces and digested with Sigma Type V Collagenase (2.5 mg/

mL, dissolved in HBSS; cat# C8051, Sigma-Aldrich) in a 37°C, 100 rpm shaking water bath for 15 minutes. HBSS supplemented with 1% porcine serum (cat# 26250084, Gibco-Thermo Fisher Scientific) was used to quench the digestion. Digested tissues were filtered through a 500 μm metal mesh before culture.

Islet culture

After islet isolation, PPIs were divided into 2 groups for culture in Ham's F-10 media (control, n = 5; cat# 11550043, Gibco-Invitrogen) as previously described or islet maturation media (IMM, n = 5), composed of 50% Ham's F-12 medium (cat# 10–080, Corning Inc.), 50% medium 199 (cat# 50-051-PB, Corning Inc.), 10 mM HEPES (cat# H3375, Sigma-Aldrich), 5 mM L-glutathione (cat# G4251, Sigma-Aldrich), 0.6 mL/L ITS+3 (cat# I2771, Sigma-Aldrich), 10 mM nicotinamide (cat# N5535, Sigma-Aldrich), 100 ug/mL gentamicin sulfate (cat# 30-005-CR, Corning Inc.), 10 uM trolox (cat# 238813, Sigma-Aldrich), 200 U/L heparin (cat# 400–10, Sagent Pharmaceuticals), 0.1 mM pefabloc (cat# sc-202041B, Santa Cruz Biotechnology), 2 mM L-glutamine (cat# 56-85-9, Alfa Aesar), 2.5 mM calcium chloride dihydrate (cat#C79-3, Fisher Scientific), and 1000 U/L DNase (cat#D4263, Sigma-Aldrich). The media in both groups were supplemented with antibiotic/antimycotic solution (cat# 30-004-CI, Corning Inc.) and 10% porcine serum (cat# 26250084, Gibco-Thermo Fisher Scientific). All islets were cultured in T-150 untreated suspension flasks (cat # CLS430825, Corning Inc., Corning, NY) in a 37°C and 5% CO^2 humidified incubator (cat# 3110, Thermo Forma Series II 3120 Water Jacketed CO^2 Incubators, Carlsbad, CA) for 14 days.[31] A full media change was performed on day 1, and a half media change was performed on day 3, 5, 7, 9, 11, and 13. Islet samples were collected for assessment on day 3, 7, and 14 of culture.

Islet yield, isolation index, and beta-cell recovery

Islet count (IC) and islet equivalent (IEQ) was obtained by staining a 100 uL aliquot of islets in culture with 1 mL dithizone (DTZ; cat# 150999, MP Biomedicals) for 5 minutes.[13] The stained islets were counted on a standard stereomicroscope (Max Erb, Santa Ynez, CA) with a 10x eyepiece graticule. The islet yield was expressed as both islet count and islet equivalent per gram of pancreatic tissue (IC/g and IEQ/g) and per pancreas (IC/pancreas and IEQ/pancreas).[51] Islet fragmentation was estimated using the isolation index, which was calculated as the ratio of IEQ divided by IC as previously described.[38] The beta-cell recovery per pancreas was calculated from the proportion of islet insulin-positive beta cells and the total cell number per pancreas as described in a previous study.[31] The total cell number per pancreas was determined from the DNA content per islet and the amount of DNA per young porcine islet cell, which have been estimated to be 7.1 pg/cell.[22]

Islet viability

100 IEQ were stained with calcein AM (CalAM; cat# C1430, Invitrogen) and propidium iodide (PI; cat# P3566, Invitrogen) for 30 minutes. Stained islets were analyzed through fluorescence with a microplate reader (Tecan Infinite F200; Tecan, Mannedorf, Switzerland).[18] The percentage of viable cells was calculated by the equation: CalAM-positive cells/(CalAM-positive cells+PI-positive cells) × 100.

Islet insulin content

150 IEQ were lysed by incubating in cell lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, pH 8) and sonicated (Sonics VibraCell Ultrasonic Processor Model VC70 T; Sonics & Materials, Inc, Newtown, CT) for 30 seconds on ice as previously described.[33,52] After centrifugation at 4°C and 1400 g for 15 minutes, the supernatant was analyzed using a standard porcine insulin enzyme-linked immunosorbent assay kit (Porcine Insulin ELISA; cat# 10-1200-01, Mercodia). The insulin content was quantified using a microplate reader (Infinite F200, Tecan and Magellan V7, Männedorf, Switzerland) and normalized to the DNA content of each sample. The islet insulin content was expressed as pg of insulin/ng of DNA.

Islet cellular viability, composition, and differentiation

A single islet cell suspension was obtained by dissociating 3000 IEQ in Accutase (cat# AT104-500,

Innovative Cell Technologies) at 37°C in a 100 rpm shaking water as described.[42] The dissociated islet cells were filtered through a 40 μm filter (VMR, Visalia, CA) to remove any debris and undissociated islet clusters. Filtered cell samples were stained with 7-AAD viability dye (7-aminoactinomycin D; cat# A1310, Invitrogen) for 30 minutes on ice. After viability staining, cells were fixed in 4% paraformaldehyde for 10 minutes and permeabilized using Intracellular Staining Permeabilization Wash Buffer (cat# 421002, BioLegend) for 15 minutes on ice. Permeabilized cells were incubated in Protein Block (cat# ab64226, Abcam) for 30 minutes on ice to reduce nonspecific binding. Cells were then stained with fluorescently conjugated antibodies in Intracellular Staining Permeabilization Wash Buffer (cat# 421002, BioLegend) supplemented with 0.5% bovine serum albumin (cat# BAL62-0500, Equitech-Bio, Inc.) for 30 minutes on ice. PE-conjugated anti insulin (Anti-insulin-PE, 1:50; cat# 8508, CST) was used as a marker for beta-cells. APC-conjugated anti-glucagon (Anti-glucagon-APC, 1:100; cat# NBP2-21803AF647, Novus Biological) was used as a marker for alpha cells. PE-conjugated anti-somatostatin (Anti-somatostatin-PE, 1:100; cat# NBP2-37447PE, Novus Biological) was used as a marker for delta cells. FITC-conjugated anti-neurogenin 3 (Anti-neurogenin 3-FITC, 1:133; cat# bs-0922 R, Bioss) was used as a marker for pancreatic endocrine progenitor cells.[44] APC-conjugated anti-Nkx6.1 (Anti-Nkx6.1-APC, 1:33; cat# 563338, BD Pharmingen) was used as a marker for the development of pancreatic endocrine progenitor cells to mature beta cells.[53] Stained cells were analyzed using a NovoCyte 3000VYB Flow Cytometer (ACEA Biosciences, Inc., San Diego, CA). Cell populations were quantified using FlowJo software (FlowJo, Ashland, OR). An unstained, single-stained, fluorescence minus one, and matching isotype control were used as gating controls.

Static glucose stimulated insulin release assay

Glucose-stimulated insulin release assay (GSIR) was used to determine the in vitro islet function.[13] A triplicate of 100 IEQ per sample was incubated at 37°C and 5% CO^2 for 1 hour in the following concentration and order of glucose media: low glucose #1 (2.8 mM; L1), high glucose (28 mM; H), low glucose #2 (2.8 mM; L2), and high glucose plus 3-isobutyl-1-methylxanthine (28 mM + 0.1 mm IBMX; H+). The supernatant from each sample was collected for storage at −20° C until analysis. The amount of secreted insulin was analyzed using a standard porcine insulin enzyme-linked immunosorbent assay (Porcine Insulin ELISA; cat# 10-1200-01, Mercodia) and quantified using a microplate reader (Infinite F200, Tecan and Magellan V7, Männedorf, Switzerland). The insulin concentration was normalized to the DNA content in each sample and expressed as pg of insulin/ng of DNA/h. The stimulation index (SI) was calculated by dividing the insulin concentration in the high glucose media (H) by the insulin concentration in the first low glucose media (L1).

Dynamic glucose stimulated insulin release perifusion assay

Dynamic perifusion assay was adapted from previous studies.[54,55] After overnight culture in 2.8 mM glucose media, 200 islet equivalents were placed in a perifusion chamber at 37°C connected to a syringe containing the perifusion solutions. Glucose media was circulated using a syringe pump at 0.2 mL/minutes. After 45 minutes of washing with 2.8 mM glucose media for stabilization, islets were exposed to the following sequence of glucose media: 15 minutes of 2.8 mM glucose, 15 minutes of 28 mM glucose, and 15 minutes of 2.8 mM glucose. Serial perifusate samples were collected every 5 minutes for storage at −20°C until analysis. Insulin concentration was measured using a standard porcine insulin enzyme-linked immunosorbent assay (Porcine Insulin ELISA; cat# 10-1200-01, Mercodia). The total insulin secretion from each glucose media condition was calculated as the area under the curve (AUC).[56]

Islet DNA content

Islets were lysed with cell lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, pH 8) and sonicated (Sonics VibraCell Ultrasonic Processor Model VC70 T; Sonics & Materials, Inc, Newtown,

CT) for 30 seconds on ice. After centrifugation at 4°C and 1400 g for 15 minutes, the supernatant was analyzed for DNA content with a fluorescent DNA stain (Quant-iT PicoGreen dsDNA kit; cat# Q32850, Molecular Probes) and quantified on a microplate reader (Infinite F200, Tecan and Magellan V7, Männedorf, Switzerland).[52]

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). An unpaired Student's t-test was used to determine the statistical significance of the IC/g, IEQ/g, isolation index, and amount of insulin secreted in the high glucose media (H) compared to the first low glucose media (L1) or the second low glucose media (L2) of islets cultured in control media or IMM on day 3, 7, and 14 of culture. A one-way ANOVA followed by a post hoc Tukey's HSD test was performed for the statistical analysis of the IC/g, IEQ/g, and isolation index of islets cultured in the same media after 3, 7 and 14 days of culture. Statistical significance of the islet viability, insulin content, cellular viability, composition, differentiation, and function between islets cultured in control media or IMM throughout 14-day culture was also calculated by a one-way ANOVA followed by a post hoc Tukey's HSD test. Statistical significance of differences was considered for p-values < .05. Statistical analysis was performed using GraphPad Prism (GraphPad Software 8.0.1, San Diego, CA).

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References

1. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. Lancet. 2014;383(9911):69–82. doi:10.1016/S0140-6736(13)60591-7.
2. Group TDCaCTR. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med. 1993;329(14):977–986. doi:10.1056/NEJM199309303291401.
3. Selam J-L. Evolution of diabetes insulin delivery devices. J Diabetes Sci Technol. 2010;4(3):505–513. doi:10.1177/193229681000400302.
4. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JRT, Shapiro AMJ. Five-year follow-up after clinical islet transplantation. Diabetes. 2005;54(7):2060. doi:10.2337/diabetes.54.7.2060.
5. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med. 2000;343(4):230–238. doi:10.1056/NEJM200007273430401.
6. Shapiro AMJ. Strategies toward single-donor islets of Langerhans transplantation. Curr Opin Organ Transplant. 2011;16(6):627–631. doi:10.1097/MOT.0b013e32834cfb84.
7. Ichii H, Ricordi C. Current status of islet cell transplantation. J Hepatobiliary Pancreat Surg. 2009;16(2):101–112. doi:10.1007/s00534-008-0021-2.
8. Ekser B, Bottino R, Cooper DKC. Clinical islet xenotransplantation: a step forward. EBioMedicine. 2016;12:22–23. doi:10.1016/j.ebiom.2016.09.023.
9. Kemter E, Wolf E. Recent progress in porcine islet isolation, culture and engraftment strategies for xenotransplantation. Curr Opin Organ Transplant. 2018;23(6):633–641. doi:10.1097/MOT.0000000000000579.
10. Dufrane D, D'Hoore W, Goebbels RM, Saliez A, Guiot Y, Gianello P. Parameters favouring successful adult pig islet isolations for xenotransplantation in pig-to-primate models. Xenotransplantation. 2006;13(3):204–214. doi:10.1111/j.1399-3089.2006.00275.x.
11. Vanderschelden R, Sathialingam M, Alexander M, Lakey JRT. Cost and scalability analysis of porcine islet isolation for islet transplantation: comparison of

juvenile, neonatal and adult pigs. Cell Transplant. 2019;28(7):967–972. doi:10.1177/0963689719847460.
12. Ellis C, Lyon JG, Korbutt GS. Optimization and scale-up isolation and culture of neonatal porcine islets: potential for clinical application. Cell Transplant. 2016;25(3):539–547. doi:10.3727/096368915X689451.
13. Lamb M, Laugenour K, Liang O, Alexander M, Foster CE, Lakey JR. In vitro maturation of viable islets from partially digested young pig pancreas. Cell Transplant. 2014;23(3):263–272. doi:10.3727/096368912X662372.
14. Krishnan R, Truong N, Gerges M, Stiewig M, Neel N, Ho-Nguyen K, Kummerfeld C, Alexander M, Spizzo T, Martin M, et al. Impact of donor age and weaning status on pancreatic exocrine and endocrine tissue maturation in pigs. Xenotransplantation. 2015;22(5):356–367. doi:10.1111/xen.12184.
15. van der Windt DJ, Bottino R, Kumar G, Wijkstrom M, Hara H, Ezzelarab M, Ekser B, Phelps C, Murase N, Casu A, et al. Clinical islet xenotransplantation: how close are we? Diabetes. 2012;61(12):3046–3055. doi:10.2337/db12-0033.
16. Smith KE, Purvis WG, Davis MA, Min CG, Cooksey AM, Weber CS, Jandova J, Price ND, Molano DS, Stanton JB, et al. In vitro characterization of neonatal, juvenile, and adult porcine islet oxygen demand, β-cell function, and transcriptomes. Xenotransplantation. 2018;25(6):e12432. doi:10.1111/xen.12432.
17. Lau H, Corrales N, Lee S, Lee S, Heng J, Zhang K, Alexander M. Exendin-4 Improves Yield and Function of Isolated Pre- Weaned Porcine Islets. J Endocrinol Diabetes. 2018;5(5):1–7. doi:10.15226/2374-6890/5/5/001115.
18. Lau H, Corrales N, Alexander M, Mohammadi MR, Li S, Smink AM, de Vos P, Lakey JR. Necrostatin-1 supplementation enhances young porcine islet maturation and in vitro function. Xenotransplantation. 2020 Jan;27(1):e12555.
19. Davalli AM, Bertuzzi F, Socci C, SCAGLIA L, GAVAZZI F, FRESCHI M, DICARLO V, PONTIROLI AE, POZZA G. Paradoxical release of insulin by adult pig islets in vitro. Recovery after culture in a defined tissue culture medium. Transplantation. 1993;56(1):148–154. doi:10.1097/00007890-199307000-00028.
20. Do Amaral AS, Pawlick RL, Rodrigues E, Costal F, Pepper A, Ferreira Galvão FH, Correa-Giannella ML, Shapiro AMJ. Glutathione ethyl ester supplementation during pancreatic islet isolation improves viability and transplant outcomes in a murine marginal islet mass model. PLoS One. 2013;8(2):e55288. doi:10.1371/journal.pone.0055288.
21. Kim B, Yoon BS, Moon J-H, Kim J, Jun EK, Lee JH, Kim JS, Baik CS, Kim A, Whang KY, et al. Differentiation of human labia minora dermis-derived fibroblasts into insulin-producing cells. Exp Mol Med. 2012;44(1):26–35. doi:10.3858/emm.2012.44.1.002.
22. Korbutt GS, Elliott JF, Ao Z, Smith DK, Warnock GL, Rajotte RV. Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest. 1996;97(9):2119–2129. doi:10.1172/JCI118649.
23. Ching CD, Harland RC, Collins BH, Kendall W, Hobbs H, Opara EC. A reliable method for isolation of viable porcine islet cells. JAMA Surg. 2001;136:276–279.
24. Koh A, Senior P, Salam A, Kin T, Imes S, Dinyari P, Malcolm A, Toso C, Nilsson B, Korsgren O, et al. Insulin-heparin infusions peritransplant substantially improve single-donor clinical islet transplant success. Transplantation. 2010;89(4):465–471. doi:10.1097/TP.0b013e3181c478fd.
25. Cabric S, Sanchez J, Lundgren T, Foss A, Felldin M, Källen R, Salmela K, Tibell A, Tufveson G, Larsson R, et al. Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. Diabetes. 2007;56(8):2008–2015. doi:10.2337/db07-0358.
26. Rose NL, Palcic MM, Helms LM, Lakey JR. Evaluation of Pefabloc as a serine protease inhibitor during human-islet isolation. Transplantation. 2003;75(4):462–466. doi:10.1097/01.TP.0000046537.47139.CE.
27. Matsumoto S, Rigley TH, Reems JA, Kuroda Y, Stevens RB. Improved islet yields from Macaca nemestrina and marginal human pancreata after two-layer method preservation and endogenous trypsin inhibition. Am J Trans. 2003;3(1):53–63. doi:10.1034/j.1600-6143.2003.30110.x.
28. Brandhorst H, Duan Y, Iken M, Bretzel RG, Brandhorst D. Effect of stable glutamine compounds on porcine islet culture. Transplant Proc. 2005;37(8):3519–3520. doi:10.1016/j.transproceed.2005.09.043.
29. Brandhorst H, Theisinger B, Guenther B, Johnson PR, Brandhorst D. Pancreatic L-Glutamine administration protects pig islets from cold ischemic injury and increases resistance toward inflammatory mediators. Cell Transplant. 2016;25(3):531–538. doi:10.3727/096368915X688623.
30. Chakrabarti SK, Mirmira RG. Transcription factors direct the development and function of pancreatic beta cells. Trends Endocrinol Metab. 2003;14(2):78–84. doi:10.1016/S1043-2760(02)00039-5.
31. Hassouna T, Seeberger KL, Salama B, Korbutt GS. Functional maturation and in vitro differentiation of neonatal porcine islet grafts. Transplantation. 2018;102(10):e413–e423. doi:10.1097/TP.0000000000002354.
32. Jimenez-Vera E, Davies S, Phillips P, O'Connell PJ, Hawthorne WJ. Long-term cultured neonatal islet cell clusters demonstrate better outcomes for reversal of diabetes: in vivo and molecular profiles. Xenotransplantation. 2015;22(2):114–123. doi:10.1111/xen.12151.

33. Bai RX, Fujimori K, Koja S, Sekiguchi S, Doi H, Tsukamoto S, Satake M, Ohkohchi N, Satomi S. Effect of prophylactic administration of trypsin inhibitors in porcine pancreas islet isolation. Transplant Proc. 1998;30(2):349–352. doi:10.1016/S0041-1345(97)01300-6.
34. Basir I, van der Burg MP, Scheringa M, Tons A, Bouwman E. Improved outcome of pig islet isolation by Pefabloc inhibition of trypsin. Transplant Proc. 1997;29(4):1939–1941. doi:10.1016/S0041-1345(97)00168-1.
35. Heiser A, Ulrichs K, Muller-Ruchholtz W. Isolation of porcine pancreatic islets: low trypsin activity during the isolation procedure guarantees reproducible high islet yields. J Clin Lab Anal. 1994;8(6):407–411. doi:10.1002/jcla.1860080611.
36. Lakey JR, Helms LM, Kin T, Korbutt GS, Rajotte RV, James Shapiro AM, Warnock GL. Serine-protease inhibition during islet isolation increases islet yield from human pancreases with prolonged ischemia. Transplantation. 2001;72(4):565–570. doi:10.1097/00007890-200108270-00003.
37. Loganathan G, Dawra RK, Pugazhenthi S, Wiseman AC, Sanders MA, Saluja AK, Sutherland DER, Hering BJ, Balamurugan AN. Culture of impure human islet fractions in the presence of alpha-1 antitrypsin prevents insulin cleavage and improves islet recovery. Transplant Proc. 2010;42(6):2055–2057. doi:10.1016/j.transproceed.2010.05.119.
38. Brandhorst D, Iken M, Brendel MD, Bretzel RG, Brandhorst H. Successful pancreas preservation by a perfluorocarbon-based one-layer method for subsequent pig islet isolation. Transplantation. 2005;79(4):433–437. doi:10.1097/01.TP.0000151765.96118.1B.
39. Ahmad SA, Lowy AM, Wray CJ, D'Alessio D, Choe KA, James LE, Gelrud A, Matthews JB, Rilo HLR. Factors associated with insulin and narcotic independence after islet autotransplantation in patients with severe chronic pancreatitis. J Am Coll Surg. 2005;201(5):680–687. doi:10.1016/j.jamcollsurg.2005.06.268.
40. Nano R, Clissi B, Melzi R, Calori G, Maffi P, Antonioli B, Marzorati S, Aldrighetti L, Freschi M, Grochowiecki T, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. Diabetologia. 2005;48(5):906–912. doi:10.1007/s00125-005-1725-3.
41. Trinh KV, Smith KD, Gardner TB. Patient and procedural factors associated with increased islet cell yield in total pancreatectomy with islet autotransplantation. Pancreas. 2018;47(8):985–989. doi:10.1097/MPA.0000000000001116.
42. Ichii H, Inverardi L, Pileggi A, Molano RD, Cabrera O, Caicedo A, Messinger S, Kuroda Y, Berggren P-O, Ricordi C, et al. A novel method for the assessment of cellular composition and beta-cell viability in human islet preparations. Am J Trans. 2005;5(7):1635–1645. doi:10.1111/j.1600-6143.2005.00913.x.
43. Sander M, Sussel L, Conners J, Scheel D, Kalamaras J, Dela Cruz F, Schwitzgebel V, Hayes-Jordan A, German M. Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. Development. 2000;127:5533–5540.
44. Cai Q, Bonfanti P, Sambathkumar R, Vanuytsel K, Vanhove J, Gysemans C, Debiec-Rychter M, Raitano S, Heimberg H, Ordovas L, et al. Prospectively isolated NGN3-expressing progenitors from human embryonic stem cells give rise to pancreatic endocrine cells. Stem Cells Transl Med. 2014;3(4):489–499. doi:10.5966/sctm.2013-0078.
45. Schwitzgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, Anderson DJ, Sussel L, Johnson JD, German MS. Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development. 2000;127:3533–3542.
46. Gradwohl G, Dierich A, LeMeur M, Guillemot F. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A. 2000;97(4):1607–1611. doi:10.1073/pnas.97.4.1607.
47. Massumi M, Pourasgari F, Nalla A, Batchuluun B, Nagy K, Neely E, Gull R, Nagy A, Wheeler MB. An abbreviated protocol for in vitro generation of functional human embryonic stem cell-derived beta-like cells. PLoS One. 2016;11(10):e0164457. doi:10.1371/journal.pone.0164457.
48. Littman ED, Opara EC, Akwari OE. Glutathione-mediated preservation and enhancement of isolated perifused islet function. J Surg Res. 1995;59(6):694–698. doi:10.1006/jsre.1995.1225.
49. Yoon KH, Quickel RR, Tatarkiewicz K, Ulrich TR, Hollister-Lock J, Trivedi N, Bonner-Weir S, Weir GC. Differentiation and expansion of beta cell mass in porcine neonatal pancreatic cell clusters transplanted into nude mice. Cell Transplant. 1999;8(6):673–689. doi:10.1177/096368979900800613.
50. Vizzardelli C, Molano RD, Pileggi A, Berney T, Cattan + P, Fenjves ES, Peel A, Fraker C, Ricordi C, Inverardi L, et al. Neonatal porcine pancreatic cell clusters as a potential source for transplantation in humans: characterization of proliferation, apoptosis, xenoantigen expression and gene delivery with recombinant AAV. Xenotransplantation. 2002;9(1):14–24. doi:10.1034/j.1399-3089.2002.0o128.x.
51. Ricordi C, Gray DW, Hering BJ, Kaufman DB, Warnock GL, Kneteman NM, Lake SP, London NJM, Socci C, Alejandro R, et al. Islet isolation assessment in man and large animals. Acta Diabetol Lat. 1990;27(3):185–195. doi:10.1007/BF02581331.
52. Qi M, Bilbao S, Forouhar E, Kandeel F, Al-Abdullah IH. Encompassing ATP, DNA, insulin, and protein content for quantification and assessment of human pancreatic islets. Cell Tissue Bank. 2018;19(1):77–85. doi:10.1007/s10561-017-9659-9.

53. Nelson SB, Schaffer AE, Sander M. The transcription factors Nkx6.1 and Nkx6.2 possess equivalent activities in promoting beta-cell fate specification in Pdx1+ pancreatic progenitor cells. Development. 2007;134 (13):2491–2500. doi:10.1242/dev.002691.
54. Buchwald P, Tamayo-Garcia A, Manzoli V, Tomei AA, Stabler CL. Glucose-stimulated insulin release: parallel perifusion studies of free and hydrogel encapsulated human pancreatic islets. Biotechnol Bioeng. 2018;115 (1):232–245. doi:10.1002/bit.26442.
55. Henquin J-C, Nenquin M, Kulkarni R. Dynamics and regulation of insulin secretion in pancreatic islets from normal young children. PLoS One. 2016;11(11):e0165961. doi:10.1371/journal.pone.0165961.
56. Hui H, Khoury N, Zhao X, Balkir L, D'Amico E, Bullotta A, Nguyen ED, Gambotto A, Perfetti R. Adenovirus-mediated XIAP gene transfer reverses the negative effects of immunosuppressive drugs on insulin secretion and cell viability of isolated human islets. Diabetes. 2005;54(2):424. doi:10.2337/diabetes.54.2.424.