Symbiosis

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ISSN Online: 2374-6890

Research Article | Journal of Endocrinology and Diabetes | Open Access

Exendin-4 Improves Yield and Function of Isolated Pre-Weaned Porcine Islets

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Received: August 21,2018; Accepted: August 28,2018; Published: September 04,2018

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Abstract

Background: orcine islets have been proposed as an alternative islet source for islet transplantation in patients with type 1 diabetes. Our focus on pre-weaned porcine islets (4-11 days old; PPIs) has shown that this model of porcine islets is a cost effective and viable mean to isolate and culture islets for transplantation. Exendin-4 (Ex-4) could improve islet viability, islet expansion, and insulin secretion. PPIs are immature after islet isolation and the addition of growth factors could accelerate their maturation. This study investigated the effects of exposure to Ex-4 on the maturation of freshly isolated PPIs during in vitro culture.

Methods: Pancreata from pre-weaned piglets (4-11 days old) were partially digested using low-dose collagenase and culture at 37°C and 5% CO2 for up to 3 days. 10nM of Ex-4 was added to culture media of freshly isolated PPIs. After 3-day culture, islets were assessed for islet yield, size, purity, membrane integrity, cellular viability and composition, and in vitro function.

Results: Islet count (IC) was significantly higher in the Ex-4 group than in the control group. Islets treated with Ex-4 were significantly smaller than islets from the control group. The percentages of major endocrine cells (β-cells, α-cells, and δ-cells) were similar in both groups. Ex-4 supplementation significantly increased insulin secretion in response to glucose challenge.

Conclusions: The addition of Ex-4 to culture media of freshly isolated PPIs could improve islet counts and produce islets with enhanced glucose-stimulated function. Culturing PPIs in Ex-4 could be beneficial to improve islet quality before transplantation.

Keywords: Pre-weaned porcine islets; exendin-4; glucagon like peptide-1; diabetes

Abbreviations: PPIs : Pre-weaned porcine islets; Ex-4: exendin-4; GLP-: 1Glucagon like peptide-1; NPIs : Neonatal porcine islets

Introduction

Diabetes mellitus type 1 (T1DM) is an autoimmune disorder characterized by the gradual destruction of insulin-producing β-cells in the Islets of Langerhans [1]. Transplantation of insulin-producing islets has been demonstrated to be a promising cure to T1DM; however, the lack of healthy human donor Pancreata and inconsistent yields due to variable donor conditions and isolation techniques severely hampered the widespread clinical application of allotransplantation [2-3]. As the demand for islet transplantation worldwide increases, it is crucial to search for a suitable, non-human islet donor.

Porcine islets have been extensively studied with a huge potential to be a viable alternative source of islets for transplantation [4]. Porcine islets are suitable donors due to the ease of husbandry, unlimited supply, and similar physiology to human islets. Even though adult porcine islets can have immediate response to glucose challenge, the isolation process is difficult and costly with low yield due to the increased islet fragility [5]. Our group has demonstrated that islet isolation using pre-weaned piglets (4-11 days old) is inexpensive without the need of elaborated equipment and requires substantially less breeding time [6]. Pancreata from pre-weaned piglets also have higher yield and immature exocrine content [6-7]. Pre-weaned porcine islets (PPIs) have been demonstrated to have both in vitro and in vivo functions [8]. Despite these advantages, PPIs require prolonged culture and their functions are often delayed. Thus, efforts must be taken to reduce the latent period from isolation to functional islets, both in vitro and in vivo.

Glucagon like peptide-1 (GLP-1) is a 30-amino acid peptide hormone secreted by the endocrine L-cells in the small intestine in response to meal intake [9]. The main functions of GLP-1 are to stimulate insulin secretion and inhibit glucagon secretion [10]. Short-term culture of fetal porcine islets in GLP-1 has been shown to improve both in vitro and in vivo glucose-stimulated insulin release and promote β-cell differentiation [11]. GLP-1 treatment prevented the gradual loss of insulin-positive cells and apoptosis, and enhanced in vitro response to glucose challenge in human islets [12]. Exendin-4, a long-lasting analog of GLP-1, offers similar functions to GLP-1 with a longer half-life [13]. Athymic nude rats treated with exendin-4 for 10 days after transplantation of human fetal islet-like cell clusters demonstrated an increased c-peptide level in response to glucose challenge and β-cell mass in explanted graft [14]. A 2-day culture of human islets after isolation in exendin-4 could restore β-cell function suppressed by Methylprednisolone treatment [15]. A recent study has shown that the addition of exendin-4 and other growth factors

to culture media of neonatal porcine islets (NPIs) could enhance the differentiation of endocrine cells, improve glucose stimulated insulin secretion and augment the ability to revert hyperglycemia in diabetic mice after 20 days of culture [16]. Whether PPIs are responsive to in vitro GLP-1/exendin-4 treatment has not been documented.

We hypothesized that PPIs are immature and their maturation into functional islets can be accelerated by in vitro treatment with exendin-4. The current study aimed to investigate the role of exendin-4 during short-term culture in the maturation of freshly isolated PPIs before transplantation.

Materials and Methods

Islet Isolation

Islets were isolated from 4-11-day-old, pre-weaned Yorkshire piglets (S&S Farms, Ramona, CA) as previously described [6]. All animal procedures were performed with approval from the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine. In brief, Pancreata were rapidly procured (≤10 mins) and placed in cold (4°C) HBSS (cat# 2402011, Gibco-Thermo Fisher Scientific, Waltham, MA) until enzymatic digestion. Cold ischemia was limited to less than 1 hour. Each pancreas was weighted and minced using 2 curved blunt/blunt 18cm Metzenbaum scissors (cat# 14019-18, Fine Science Tools Inc., Foster City, CA) into 1 mm3 pieces, while 3 washes using cold (4°C) HBSS were done in between to remove blood, connective tissue, fat, lymph nodes, etc. The minced tissues were digested using Sigma Type V Collagenase (2.5 mg/mL, dissolved in HBSS; cat# C8051, Sigma-Aldrich, St. Louis, MO) in 100 rpm shaking water bath at 37°C for 15 minutes. The digestion was stopped with HBSS supplemented with 1% porcine serum (cat# 26250084, Gibco-Thermo Fisher Scientific, Waltham, MA). The digested tissues were filtered through a 500μm metal mesh.

Islet Culture

Isolated islet tissue clusters were cultured in a novel maturation media supplemented with 10% porcine serum in T-150 untreated suspension flask (cat # CLS430825, Corning Inc., Corning, NY) at 37°C and 5% CO2 humidified incubator (cat # 3110, Thermo Forma Series II 3120 Water Jacketed CO2 Incubators, Carlsbad, CA) to allow islets to mature into complete islets. 10nM of exendin-4 (cat# E7144, Sigma-Aldrich, St. Louis, MO) were added to the culture media in the experimental group (n=8 pancreas) while the control group (n=12 pancreas) was cultured in media without exendin-4. Islets were cultured for 3 days. Full media change was performed on day 1 and 3. Islets were collected for assessment at the end of culture.

Islet assessment

Islet yield, size, purity, and membrane integrity

Islet count (IC) and islet equivalent (IEQ) were determined by staining an aliquot of approximately 100 IEQ with 1 mL dithizone (DTZ; cat# 150999, MP Biomedicals, Santa Ana, CA) for 5 minutes, and counted at 25x on a stereomicroscope (Max Erb, Santa Ynez, CA) with a 10x eyepiece graticule. The islet size ratio was calculated by dividing the IEQ (defined to be an islet with a diameter of 150 um) over the IC. The percentage of islet purity was calculated after DTZ staining by dividing the area of islets with positive DTZ staining over the total area of islets. Islet membrane integrity was analyzed by staining approximately 100 IEQ with calcein AM (cat# C1430, Invitrogen, Carlsbad, CA) and propidium iodide (PI; cat# P3566, Invitrogen, Carlsbad, CA) for 15 minutes. The stained islets were quantified using a Microplate reader (Infinite F200 Tecan, Männedorf, Switzerland). The membrane integrity percentage was calculated by the equation: calcein AM-positive cells/(calcein AM-positive cells + PI-positive cells) x 100.

Islet cellular composition

The cellular composition of islets was determined by flow cytometry [17]. 5000 IEQ were washed twice with DPBS (cat# 14190250, Thermo Fisher Scientific, Waltham, MA) and dissociated into a single cell suspension by incubating in Accutase (cat# AT104-500, Innovative Cell Technologies, San Diego, CA) for 15 minutes in a 37°C, 100 rpm shaking water bath. The cell suspension was filtered through a 40μm filter (cat # 10199-654, VWR, Visalia, CA) and stained with 7-aminoactinomycin D (7-AAD; cat# A1310, Invitrogen, Carlsbad, CA) for 30 minutes on ice to detect live and dead cells. The cells were fixed in 4% paraformaldehyde for 10 minutes and permeabilized using Intracellular Staining Permeabilization Wash Buffer (cat# 421002, BioLegend, San Diego, CA) for 15 minutes on ice. Permeabilized cells were incubated for 30 minutes on ice with Protein Block (cat# ab64226, Abcam, Burlingame, CA) to reduce nonspecific binding followed by staining with fluorescently conjugated antibodies for intracellular markers in Intracellular Staining Permeabilization Wash Buffer (cat#421002, BioLegend, San Diego, CA) supplemented with 1% bovine serum albumin (BSA; cat# BAL62-0500, Equitech-Bio, Inc., Kerrville, TX) for 30 minutes on ice. PE conjugated anti-insulin (Anti-insulin-PE; cat# 8508, CST, Danvers, MA) was used as a marker for β-cells, APC conjugated anti-glucagon (Anti-glucagon-APC; cat# NBP2-21803AF647, Novus Biological, Littleton, CO) was used as a marker for α-cells, and PE conjugated anti-somatostatin (Anti-somatostatin-PE; cat# NBP2-37447PE, Novus Biological, Littleton, CO) was used as a marker for δ-cells. Cell populations were quantified using the NovoCyte 3000VYB Flow Cytometer (ACEA Biosciences, Inc., San Diego, CA) and analyzed using FlowJo software (FlowJo, Ashland, OR). An unstained, single-stained and matching isotype control were used as controls.

Islet function

Islet function was determined using glucose-stimulated insulin release assay [6]. 3 samples of 100 IEQ per isolation were incubated for 1 hour at 37°C and 5% CO2 in media of low glucose (2.8 mm; L1), high glucose (28 mm; H), high glucose plus 3-isobutyl-1-methylxanthine (28mm + 0.1mm IBMX; H+), and again in low glucose (2.8 mm; L2). Insulin concentration was measured using a standard porcine insulin enzyme-linked immunosorbent assay (Porcine Insulin ELISA; cat# 10-1200-01, Mercodia, Winston Salem, NC) and the absorbance was measured using a Microplate reader (Infinite F200, Tecan and Magellan V7,

Männedorf, Switzerland). Secreted insulin concentration was normalized to ieqs. The stimulation index (SI) was calculated as the ratio of insulin concentration secreted in high glucose over the insulin concentration secreted in the first low glucose.

Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM). An unpaired t-test was performed to determine statistical significance. A p-value less than 0.05 was considered to be statistically significant and a P value less than 0.01 was considered to be highly statistically significant.

Results

Effects of exendin-4 treatment on PPI yield, size, purity, and membrane integrity

PPIs cultured in plain media without exendin-4 (control group) or media supplemented with exendin-4 (Ex-4) after islet isolation were stained with dithizone (DTZ) and counted on day 3 of culture. The islet counts (IC) per gram of pancreas tissue in the Ex-4 group were significantly higher than the control group (Ex-4 = 17613 ± 1867 IC/g, control = 11126 ± 1568 IC/g; $p<0.05$) (Table 1). The islet equivalents (IEQ) per gram of pancreas tissue were not significantly different between the two groups (Ex-4 = 11651 ± 1375 IEQ/g, control = 14753 ± 2123 IEQ/g; $p>0.05$) (Table 1). Islets from the Ex-4 group was significantly smaller than islets in the control group as indicated by the ratio of IEQ/IC (Ex-4 = 0.76 ± 0.11, control = 1.60 ± 0.2; $p<0.01$) (Table 1). The purity and membrane integrity measured by calcein AM-propidium iodide staining were not significantly different between the two groups (purity: Ex-4 = 78.4 ± 4.1%, control = 80.2 ± 2%; $p>0.05$; membrane integrity: Ex-4 = 86.6 ± 4.5%, control = 92.3 ± 1.7%; $p>0.05$) (Table 1).

Table 1: Islet yield, purity, and viability of pre-weaned porcine islets after 3 days of culture in media with or without Exendin-4

	IC/g	IEQ/g	IEQ/IC	Purity(%)	Membrane Integrity(%)
Control	11126±1568	14753±2123	1.60±0.2	80.2±2	92.3±1.7
EX-4	17613±1867*	11651±1375	0.76±0.11**	78.4±4.1	86.6±4.5

IC/g: Islet count per gram of pancreas tissue. IEQ/g: Islet equivalent per gram of tissue. Ex-4: Exendin-4. *p<.05 versus control group. **p<.01 versus control group. Values represent mean ± SEM.

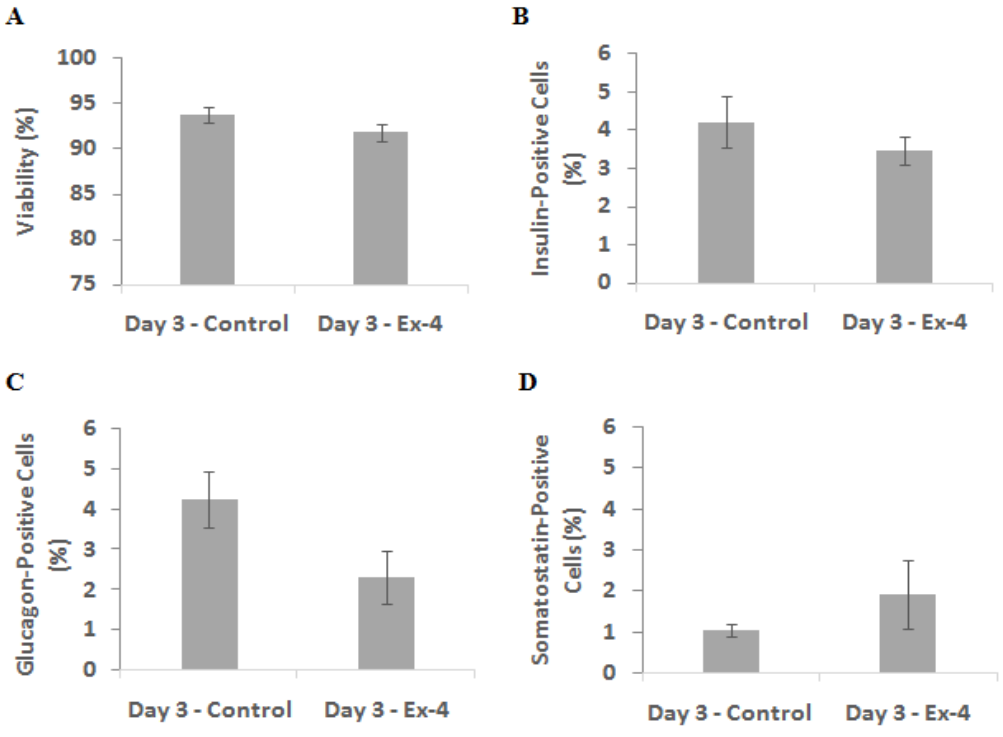

Figure 1: Flow cytometric analysis of pre-weaned porcine islets after 3 days of culture in media with or without Exendin-4. Islets were dissociated on day 3 of culture, stained with 7-AAD viability dye, anti-insulin, anti-glucagon, and anti-somatostatin antibodies, and analyzed by flow cytometry. Insulin, glucagon, and somatostatin gates were drawn after dead cells were excluded by 7-AAD viability staining. (A) Percent viability of pre-weaned porcine islet cells on day 3 of culture. (B) Percent of positive-insulin pre-weaned porcine islet cells on day 3 of culture. (C) Percent of positive-glucagon pre-weaned porcine islet cells on day 3 of culture. (D) Percent of positive-somatostatin pre-weaned porcine islet cells on day 3 of culture. Ex-4: Exendin-4. *p<0.05 versus control group. **p<0.01 versus control group. Data shown as mean ± SEM.

Effects of exendin-4 treatment on the cellular viability and composition of PPIs

Cellular viability and composition were analyzed by FACS after islet dissociation. Similar to calcein AM-propidium iodide staining, 7-Aminoactinomycin D staining of islet cells show no significant differences in the viability between the control group and Ex-4 group (Ex-4 = 91.8 ± 1%, control = 93.7 ± 0.8%; p>0.05) (Figure 1A). PPIs were dissociated into single cells and analyzed by flow cytometry to determine the effect of Ex-4 on the proliferation/differentiation of major endocrine cells after 3 days of culture. There were no significant differences in the percentages of insulin-, glucagon-, and somatostatin-positive cells between the control group and the Ex-4 group (insulin-positive cells: Ex-4 = 3.5 ± 0.4%, control = 4.2 ± 0.7%; p>0.05; glucagon-positive cells: Ex-4 = 2.3 ± 0.7%, control = 4.3 ± 0.7%; p>0.05; somatostatin-positive cells: Ex-4 = 1.9 ± 0.8%, control = 1.1 ± 0.1%; p>0.05;) (Figure 1B, C, and D, respectively).

Effects of exendin-4 treatment on the function of PPIs in response to glucose challenge

Glucose stimulated insulin release assay was performed to evaluate the function of PPIs after 3-day culture. Islets were incubated in low glucose media (L1, 2.8mM), followed by high glucose media (H, 28mM), then high glucose media with 0.1mM 3-isobutyl-1-methylxanthine (H+, 28mM + 0.1mM IBMX), and finally to low glucose media again (L2) to determine whether islets can respond to glucose challenge in a physiological manner. Although the insulin concentration per IEQ released in L1 media was significantly lower in the Ex-4 group compared to the control group, islets from the Ex-4 group released significantly more insulin in both H and H+ media (L1 media: Ex-4 = 0.00080 ± 0.00013 ug/IEQ, control = 0.0022 ± 0.00057 ug/IEQ; H media: Ex-4 = 0.0056 ± 0.0011 ug/IEQ, control = 0.0027 ± 0.00041 ug/IEQ; H+ media: Ex-4 = 0.011 ± 0.0020 ug/IEQ, control = 0.0054 ± 0.0012 ug/IEQ; p<0.05) (Figure 2A). However, no significant difference was found in the insulin concentration released in L2 media between the two groups (L2 media: Ex-4 = 0.0085 ± 0.0028 ug/IEQ, control = 0.0036 ± 0.00075 ug/IEQ; p>0.05) (Figure 2A). Islets treated with exendin-4 also had significantly higher response to glucose challenge as measured by the stimulation index ration (Ex-4 = 8.53 ± 1.96, control = 1.66 + 0.11; p<0.01) (Figure 2B).

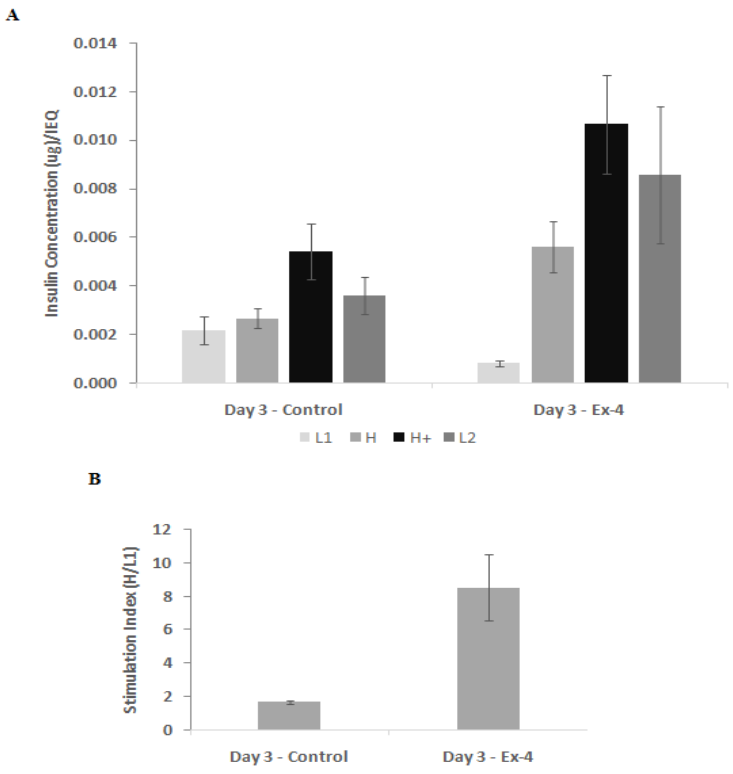

Figure 2: In vitro function of pre-weaned porcine islets after 3 days of culture in media with or without Exendin-4. In vitro function of islets was analyzed using glucose stimulated insulin release assay. Islets were incubated for 1 hour in media with the corresponding order of glucose concentration: 2.8mM (L1), 28mM (H), 28mM + 0.1mM IBMX (H+), and 2.8mM (L2) glucose media. The concentration of secreted insulin was measured by ELISA. Stimulation index (SI) was calculated as the ratio of H:L1. (A) Insulin concentration per IEQ (ug/IEQ) released by pre-weaned porcine islets cultured in media with or without Exendin-4 after incubation in varying concentration of glucose media. (B) Stimulation index of pre-weaned porcine islets cultured in media with or without Exendin-4. Ex-4: Exendin-4. *p<0.05 versus control group. **p<0.01 versus control group. Data shown as mean ± SEM.

Discussion

A major hurdle in the application of young porcine islets for xenotransplantation is the prolonged culture required for islets to exhibit glucose-stimulated function after isolation and the dormant period from transplantation to in vivo function. The reason for this has been suggested to be that young porcine islets are immature and often require a minimum of 7 days to have function [6]. Jimenez-Vera et al. have suggested that a 12-day culture would be best for NPI function [18]. Lamb et al. and Krishnan et al. have reported that PPIs have improved glucose responsiveness and could revert hyperglycemia in diabetic mice after 7-day culture [6,8]. Even though prolonged culture allows islets to mature as evidenced by the increase in insulin-positive cells and function, the number of islets recovered is markedly lowered [18]. Therefore, identifying potential treatments that could accelerate the maturation and function of young porcine islets would advance their application in clinical settings. Our results demonstrated the addition of exendin-4 to culture media of freshly isolated PPIs significantly improves islet yield and produces smaller islets with enhanced function after 3 days of culture.

Islets experience considerable damages during cold ischemia and enzymatic digestion that could impact islet yield, viability, and function [19-21]. Exendin-4 has been demonstrated to have anti-apoptotic and improve islet survival after isolation [22]. Atsushi et al. have shown that culturing human islets with methylprednisolone and exendin-4 for 48 hours after isolation could improve viable beta cell mass [15]. Even though our results showed that exendin-4 treated PPIs have similar viability to control islets, which might be due to the high viability in both groups (>90% viable), exendin-4 treatment significantly improved the amount of islet recovered as indicated by the higher islet count. This effect could be due to the anti-apoptotic property of exendin-4; however, the exact mechanism remains to be investigated.

Exendin-4/GLP-1 treatment has been shown to have beneficial effects on the function of murine, rat, human and fetal porcine islets [11-12, 15, 23-24]. Human islets treated with exendin-4 for 48-hour during pre-transplant culture could improve insulin secretion compared to control islets or islets treated with methylprednisolone [15]. Farilla et al. have shown that the addition of GLP-1 to culture media of freshly isolated human islets has the most significant improvement in glucose induced insulin secretion after 3 days of culture [12]. Consistent with previous studies, our data confirmed that treatment of exendin-4 during 3-day culture enhanced the insulin secretory response of PPIs to glucose challenge.

Islet size can have a profound influence on islet function [25-26]. Small rat islets (diameter <125 um) are capable of releasing up to twice as much insulin in comparison to large islets (diameter >150 um) [26]. In addition, transplantation of large rat islets could not revert hyperglycemia while small rat islets have an 80% cure rate at the same IEQ [27]. Similarly, Lehmann et al. have shown that smaller human islets (diameter <150um) produced approximately twice as much insulin during static and dynamic glucose challenge compared to large islets (>150um) [28]. The positive effect of exendin-4 on the improved islet function could partially be attributed to the smaller islet size as indicated by the smaller IEQ/IC ratio.

Exposure of fetal porcine islets to GLP-1 could induce beta cell differentiation and proliferation, potentially account for the increase in glucose-stimulated insulin secretion and reversal of hyperglycemia in diabetic mice [11]. We found that exendin-4 supplementation in culture media did not induce endocrine cells in PPIs to differentiate or proliferate after 3 days of culture as the numbers of endocrine cells remained similar in both groups. Therefore, the increase in the ability of PPIs to response to glucose challenge could not be attributed to the proliferation or differentiation of beta cells. Movassat et al. have shown that exendin-4 treatment at a similar dosage does not improve the numbers of insulin-positive cells after 4 days of culture, even though the PDX1- positive cells significantly increase [14]. Atsushi et al. have also reported that beta cell proliferation was not observed after freshly isolated human islets were cultured in exendin-4 for 2 days [15]. Thus, the absence of beta cell growth after treatment of exendin-4 could be due to the short culture time. In clinical settings, patients will potentially be receiving GLP-1 receptor agonist treatment after islet transplantation. Oral administration of exendin-4 in diabetic mice transplanted with murine islet-like cell clusters could induce the growth of insulin-positive cells and enhance the graft function [24]. Therefore, exendin-4 could potentially induce in vivo proliferation and/or differentiation of PPIs and improve islet graft function.

Our previous report suggested that PPIs should be cultured for at least 7 days to have adequate function, exendin-4 treated PPIs could secrete insulin in a glucose-dependent manner only after 3 days of culture [8]. The results indicated that shortening the culture time to 3 days could improve the islet recovery by 2 folds [6]. Our in vitro function assay showed that control PPIs have a similar stimulation index to NPIs cultured in DMEM-F12 differentiation media containing exendin-4 and growth factors (1.66 vs. 1.6, respectively) [16]. However, exendin-4 treated PPIs had comparable insulin secretion to adult porcine islets (the stimulation indices of both groups = 8.5), suggesting that exendin-4 treatment could augment the maturation of PPIs into functional islets [29].

Conclusion

In summary, our data have provided evidence to support the beneficial effects of in vitro exposure of PPIs to exendin-4, which could improve islet yield and produce smaller islets with enhanced insulin secretion in response to glucose challenge. These findings suggest that the addition of exendin-4 to PPI culture media may be useful during islet preparation before transplantation. Future studies evaluating the in vivo effects of exendin-4 on the proliferation/differentiation and graft function of PPIs in diabetic animals will help to advance the clinical applications of exendin-4 and PPIs in islet transplantation.

Acknowledgements

This study was funded by the Juvenile Diabetes Research Foundation (JDRF Grant #17-2013-288) and the Department of

Surgery, University of California, Irvine, CA. The author would like to acknowledge the support from the Sue and Bill Gross Stem Cell Center, a California Institute of Regenerative Medicine (CIRM) supported facility, the Flow Cytometry Core - Institute for Immunology at UC Irvine, the Brownstein Family Foundation, and the PADRE Foundation.

Declarations

Authorship

HL and NC performed experiments, collated data, and wrote the manuscript. SL, JH, KZ performed experiments for the manuscript. MA and JL designed the study, reviewed data, and consented to final version of the manuscript.

Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

Ethical Approval

All animal procedures were performed under approved protocols from the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine, #AUP-17-129.

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