Author's Accepted Manuscript
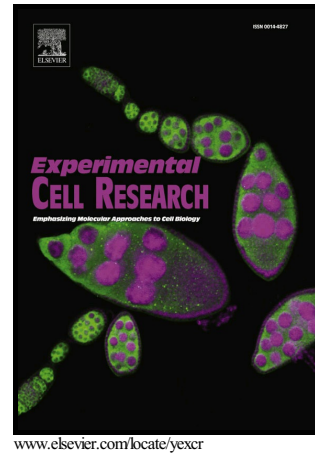
Functionality and antidiabetic utility of β-and l-cell containing pseudoislets

Alastair D Green, Srividya Vasu, Peter R Flatt

www.elsevier.com/locate/yexcr

Cite this article as: Alastair D Green, Srividya Vasu and Peter R Flatt, Functionality and antidiabetic utility of β-and l-cell containing pseudoislets, *Experimental Cell Research*, http://dx.doi.org/10.1016/j.yexcr.2016.04.007

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Functionality and antidiabetic utility of β- and L-cell containing pseudoislets

Alastair D Green, Srividya Vasu*, Peter R Flatt

SAAD Centre for Pharmacy & Diabetes, Ulster University, Coleraine, Northern Ireland, UK

* Corresponding author: Dr. Srividya Vasu, SAAD Centre for Pharmacy & Diabetes, University of Ulster, Cromore Road, Coleraine BT52 1SA, Northern Ireland, United Kingdom.

E-mail: s.vasu@ulster.ac.uk; s.vasu@outlook.com

Tel: +44 (0) 28 70124419

Short title: Insulin and GLP-1 pseudoislet therapy

Abstract

Unavailability of tissue and poor engraftment remain significant obstacles to clinical islet transplantation. Here, the therapeutic potential of pseudoislets generated from the insulin and GLP-1 releasing cell-lines MIN6 and GLUTag was investigated. Glucose and other secretagogues evoked 1.3–5.7 fold increases in insulin secretion from both pseudoislet types. Secretion expressed in relation to basal values did not greatly differ between configurations. Exposure of both types of pseudoislets to ninhydrin, H_2O_2, streptozotocin or cytokine cocktails decreased viability and increased apoptosis. However, combined pseudoislets exhibited enhanced resistance (1.2–1.7 fold increased LD_{50}, 1.2–1.4 fold decreased apoptosis). Implantation of pseudoislets into streptozotocin-diabetic SCID mice precipitated cell masses containing immunoreactive insulin and GLP-1. Implantation of both pseudoislet types was associated with significant reductions in blood glucose, increased plasma insulin, greater bodyweight, decreased polydipsia and improved glucose tolerance. These changes greatly exaggerated in MIN6 pseudoislet recipients, with mice becoming severely hypoglycaemic. In contract, combined pseudoislet recipients achieved tempered restoration of normoglycaemia and exhibited increased plasma GLP-1, decreased plasma and pancreatic glucagon, increased pancreatic insulin and enhancements in islet β:α cells and the ratio of Ki67:TUNEL positive β-cells. MIN6 pseudoislet implantation increased islet β:α cell ratio but did not affect β-cell proliferation or hormone content. Our observations highlight the potential of combining insulin and GLP-1 cell therapy using heterotypic pseudoislets.

Key words: Insulin, GLP-1, pseudoislets, MIN6, GLUTag, diabetes, cell therapy

Introduction

Islet transplantation (ITx) is attractive for the treatment of T1DM patients with poor glycaemic management but the limited availability of donor pancreata, poor graft function and survival remain significant barriers (Onaca *et al.* 2007, Barton *et al.* 2012, McCall & Shapiro, 2012). Consequently, there is significant motivation to find a more practically sustainable alternative for the replacement of lost β-cell mass in T1DM (Onaca *et al.* 2007, Barton *et al.* 2012, McCall & Shapiro 2012). The use of genetically engineered insulin-releasing β-cells is a possible alternative to the use of primary islets as it would allow for a theoretically unlimited quantity of insulin releasing cells to be generated *in vitro* for transplantation. Some studies implanting human β-cell lines in experimental animal models have already shown promising results (McCluskey *et al*. 2011, Scharfmann *et al.* 2014).

A number of strategies have been employed to improve the viability, function and survival of implanted islets, including exposure to GLP-1 (Buss *et al.* 2012, Wang *et al.* 2013). This potent incretin hormone has a wide range of beneficial actions on islet cells, such as regulation of glucose-induced insulin release, induction of insulin biosynthesis, inhibition of glucagon secretion, enhancement of β-cell proliferation and inhibition of β-cell apoptosis (Xu *et al.* 1999, Baggio *et al.* 2000, Skoglund *et al.* 2000, Stoffers *et al.* 2000, Campbell & Drucker, 2013). Injectable GLP-1 mimetics such as exenatide and liraglutide are widely used in the treatment of T2DM and their use has been investigated as a potential means of boosting islet function and survival post-transplantation in T1DM or preserving β-cell function in poorly controlled T2DM (Ghofaili *et al.* 2007, Faradji *et al.* 2008, Buss *et al.* 2012, Wang *et al.* 2013). Significant improvements in islet engraftment following treatments of T1DM recipients, or pre-transplant co-culture of islets with GLP-1 agonists have been reported by others (Ghofaili *et al.* 2007, Faradji *et al.* 2008, Gangemi *et al.* 2008, Merani *et al.* 2008,

3

Toso *et al.* 2010, McCall & Shapiro 2012).

Unfortunately, while GLP-1 mimetics can improve the outcomes of islet transplantation, chronic administration is required, and these agents are associated with a range of unpleasant side effects, particularly nausea and vomiting (Butler *et al.* 2013). Additionally, while improvements in the outcomes of islet transplantation following GLP-1R agonist based therapies have been shown in some studies, others have reported few beneficial effects, possibly reflecting relatively low circulating concentrations reaching the islets (King *et al.* 2005, Crutchlow *et al.* 2008). Consequently, more direct exposure of islets to GLP-1 agonists has been considered as a potentially more suitable approach. Implantation of islets encapsulated within various type of biological scaffolding impregnated with GLP-1 has been shown to considerably improve engraftment of implanted islets (Lin & Anseth 2009, Kizilel *et al.* 2010). Transfection of pancreatic α-cells with PC1/3 increased GLP-1 secretion has been shown to augment islet insulin secretion and cytoprotective mechanisms *in vitro* as well as improve the outcomes of islet transplants in insulin-deficient T1DM mice (Wideman *et al.* 2006).

Pancreatic islet cells (both primary and transformed) possess the capacity to reaggregate in suspension culture to form islet-like spheroids termed pseudoislets, which exhibit a remarkable enhancement in function compared to the combined responses of their individual constituent cells (Hauge-Evans *et al.* 1999, Kelly *et al.* 2011). This trait of islet cells also allows for the combination of more than one cell type to form heterotypic pseudoislets with various functional capabilities. To date such experimentation has primarily involved the use of combinations of β-cell lines, α-cell lines and/or δ-cell lines (Brereton *et al.* 2006, Brereton *et al.* 2007, Kelly *et al.* 2010b, Kelly *et al.* 2011). Considering the beneficial effects of

4

incorporating GLP-1 administration into islet transplantation procedures, the limited availability of primary human islets for transplantation, and the potential versatility of heterotypic pseudoislets, the creation of heterotypic pseudoislets incorporating both insulin-releasing cell lines and GLP-1 secreting cell lines represents an interesting line of investigation.

In the present study, the therapeutic potential of heterotypic mouse pseudoislets generated from MIN6 β-cells and the GLP-1 secreting enteroendocrine cell-line GLUTag were investigated. Specifically this involved comparing the function and cytoprotective mechanisms of heterotypic MIN6 and GLUTag pseudoislets and homotypic MIN6 pseudoislets *in vitro*; and a comparison of the anti-diabetic effects of both types of pseudoislets *in vivo* by implantation into streptozotocin diabetic SCID mice.

Materials and Methods

Cell culture and pseudoislet formation: The creation and characteristics of MIN6 and GLUTag cells have been described previously (Miyazaki *et al.* 1990, Lee *et al.* 1992, Drucker *et al.* 1992). MIN6 cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose and 2 mM L-glutamine supplemented with 10 % (v/v) FCS and antibiotics (100 U/ml penicillin and 0.1 g/l streptomycin). GLUTag cells were routinely cultured in DMEM containing 5.5 mM glucose and with the same supplements. MIN6 cells were used in experiments to a maximum passage number of 35, while GLUTag cells were used up to a passage number of 25. To form pseudoislets, MIN6 cells or a 1:1 combination of MIN6 and GLUTag cells were seeded at a density of 1 x 10^5 cells per well into 6 well ultra-low attachment, flat bottomed tissue culture plated (Corning Inc., NY, USA). Pseudoislets were maintained in the same culture medium as GLUTag cells.

5

Acute insulin release assays: Insulin release from pseudoislets was determined as described previously (McCluskey *et al.* 2011, Guo-Parke *et al.* 2012). For acute tests, groups of 5 pseudoislets were first preincubated for 40 mins at 37 °C in Krebs-Ringer bicarbonate buffer (KRBB) (115 mmol/l NaCl, 4.7 mmol/l KCl, 1.28 mmol/l CaCl$_2$, 1.2 mmol/l MgSO$_4$ 10 mmol/l NaHCO$_3$, 20 mmol/l Hepes) containing 1.1 mmol/l glucose supplemented with 0.1% w/v bovine serum albumin (BSA) (Gibco® Invitrogen, Paisley, UK), before being incubated for a further 60 min in KRBB supplemented with (v/v) 0.1% BSA and a range of concentrations of glucose and modulators of insulin secretion as described in the Figures. Following acute tests, supernatants were stored at -20 °C until insulin analysis by radioimmunoassay (Flatt & Bailey, 1981). Cells were extracted using acid ethanol overnight to determine hormone content (Vasu *et al.* 2013a).

Cytotoxin treatments: To investigate the relative cytoprotective capabilities of MIN6 cell pseudoislets and pseudoislets formed from MIN6 cells and GLUTag cells, pseudoislets were dispersed using enzyme free cell-dissociation buffer and cells were incubated for 2 h at 37 °C with cytotoxins at the concentrations indicated in the Figures. The cytotoxic agents studied were ninhydrin, H$_2$O$_2$, streptozotocin (STZ), and cocktails of the proinflammatory cytokines containing IL1-β, IFNγ and TNFα.

Assessment of cell metabolic viability: Metabolic competency of cell suspensions derived from pseudoislets was determined following incubation with a range of cytotoxic agents usingy the colorimetric 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mossman 1983).

Measurement of cellular apoptosis: Apoptosis was assessed by staining with acridine orange

and ethidium bromide. Pseudoislets were dispersed, exposed to cytotoxins, harvested and then resuspended in phosphate buffered saline (PBS) at a density of 5 x 10^5 cells/ml. The cell suspension was then stained with solutions of acridine orange and ethidium bromide (20 µg/ml each) for 5 min at room temperature. Cells were mounted with antifade mounting medium and viewed under 488- and 594-nm filters using a fluorescent microscope (Olympus System microscope, BX51) and photographed using the DP70 camera adapter system. The apoptotic state of cells was categorized as follows: bright green nuclei—healthy cells; dense green nuclei (evidence of chromatin condensation)—early apoptosis; bright yellow nuclei and yellow cytoplasm—late apoptosis; and orange/red nuclei, orange cytoplasm—late apoptosis/ necrosis. Membrane blebs and apoptotic bodies were also taken into account for analysis. Approximately 100 cells per replicate (n=4) were analyzed using ImageJ software.

Animal and surgical procedures: Adult female SCID mice (15 – 20 weeks) were bred and maintained under specific pathogen-free conditions in the Biomedical and Behavioral Research Unit (BBRU) at University of Ulster, Coleraine. Food and water were provided *ad libatum* unless specified otherwise. Animals were rendered diabetic by intraperitoneal administration of streptozotocin (165 mg/kg) after an 8 h fast. Animals were provided with 5 % sucrose water to prevent overnight hypoglycaemia. Prior to implantation procedures mice were anaesthetized by intraperitoneal injection with 10 mg/kg fluanisone, 0.3 mg/kg fentanyl citrate (Hypnorm) (VetaPharma Ltd, Leeds, UK) and midazolam 5 mg/kg (Hypnovel) (Roche, Welwyn, UK). For pseudoislet implantation, harvested pseudoislets were resuspended at a density of 1000 pseudoislets per ml and 500 µl were injected into the subscapular region using an 18-G needle. Food intake, water intake and body weight were monitored daily while blood glucose was measured once every 3 days using Ascensia contour glucose strips (Bayar, Uxbridge, UK). Glucose tolerance tests were carried out at the end of

each study. Animals were fasted for 8 h blood and plasma samples were collected in heparin coated tubes immediately before (time 0) and 15, 30, 60, 90 and 120 min after intraperitoneal injection of 18 mmol/kg glucose. On day 28, terminal blood, implants and pancreatic tissues were collected for further analyses. For measurement of hormone contents tissues were extracted overnight at 4°C using acid ethanol (Moffett *et al.* 2014). All animal procedures were performed in adherence to the UK home office regulations (UK Animal Scientific Procedures Act 1986) and 'Principles of laboratory animal care' (NIH Publication no 86-23, revised 1985).

Glucose and hormone determination: Insulin was determined by radioimmunoassay (Flatt & Bailey, 1981). Total GLP-1 was determined using specific enzyme linked immunoassay, (GLP-1 Total ELISA, EZGLP-1T-36K, Millipore, MA, USA) and glucagon was determined using specific glucagon chemiluminescent assay (EZGLU-30K, Millipore, MA, USA). Glucose in plasma samples was determined using an Analox GM9 glucose analyser (Analox, London, UK). Protein contents were determined by Bradford assay.

Immunohistochemistry: Tissues were fixed in 4% paraformaldehyde and processed using an automated tissue processor (Leica TP1020, Leica Microsystems, Nussloch, Germany). After embedding, tissues were sectioned (Shandon Finesse 325, Thermo Scientific, UK) at a thickness of 7 μm at intervals of ten sections. Sections were dewaxed using Histoclear II (National Diagnostics, UK) and rehydrated with a gradient of ethanol concentrations. For haematoxylin and eosin staining, dewaxed and rehydrated sections of implants were processed as described previously (Vasu *et al.* 2013c). Slides were viewed using an Olympus IX51 inverted microscope and images were captured using a SPOT RT-Ke camera (Diagnostic Instruments Inc, Sterling Heights, MI).

For peroxidase immunostaining, dewaxed and rehydrated sections were blocked in 0.3 % (v/v) H_2O_2 in 50 % (v/v) methanol to quench endogenous peroxidase activity, before incubation at 90 °C in citrate buffer (pH 6.0) for antigen retrieval. After cooling, sections were incubated at 4 °C with primary antibodies overnight (Mouse monoclonal anti-insulin antibody, ab6995, 1:1000, Abcam, UK; rabbit anti-GLP-1 antibody (XJIC8, 1:200; raised in-house, specific for total GLP-1), before the addition of the corresponding ImmPRESS peroxidase reagent and 3, 30-Diaminobenzidine substrate. Lastly, sections were counterstained with haematoxylin at 60 °C, and slides were cleared with Histo-clear II and mounted with Histomount mounting medium. Slides were viewed and images captured as described above.

For immunofluorescence staining, following dewaxing, rehydration, antigen retrieval with citrate buffer and blocking with BSA solution, sections with incubated at 4 °C overnight with primary antibodies (Mouse anti-insulin antibody, ab6995, 1:1000, Abcam; guineapig anti-glucagon antibody, PCA2/4, 1:200, raised in-house; Rabbit anti-Ki67 antibody, 1:200, ab15580, Abcam, UK) prior to incubation at 37 °C for 45 min with secondary antibody (Alexa Fluor 488 or 594, Invitrogen, UK). For TUNEL staining, slides were incubated with working TUNEL reagent (In situ cell death detection kit, Fluorescein, Roche, UK). Finally, slides were mounted with anti-fade mounting medium and viewed under green FITC filter (488 nm) or red TRITC filter (594 nm) using a fluorescent microscope (Olympus, model BX51) and photographed using a connected DP70 camera adapter system.

Image analysis: Cell$^\wedge$F image analysis software (Olympus Soft Imaging Solutions, GmbH) was used to analyze islet parameters including islet area, alpha cell area and beta cell area using closed polygon tool. All islets were analyzed regardless of insulin staining. Pancreatic

9

area was also calculated from 10 x images and used to calculate the number of islets per mm^2 of pancreas in a blinded fashion. For analysis of islet size distribution, islets smaller than 10000 μm^2 were considered small, those larger than 10000 μm^2 but smaller than 25000 μm^2 were considered medium and those larger than 25000 μm^2 were considered large. In order to assess islet cell proliferation and apoptosis, pancreatic sections were double stained for insulin and either Ki67 (proliferation) or TUNEL (apoptosis). Beta cell proliferation or apoptosis frequencies were expressed as a percentage of the total number of insulin positive cells observed. Approximately 1000 β-cells were analyzed per replicate.

Statistics: Results are expressed as mean ± S.E.M. Groups of data were compared using Student's unpaired t-test with two-tailed p-values. Groups were considered significant where $p < 0.05$.

Results

Morphology of homotypic and heterotypic pseudoislets: Both MIN6 cells and a combination of MIN6 and GLUTag cells grew into islet-like spheroids within 4-7 days of seeding in ultra-low attachment plates. Both types of pseudoislets had a mean diameter of around 150 μm and comprised approximately 4500 cells per pseudoislet. The insulin content of MIN6 and heterotypic pseudoislets was 2164 ± 289 and 292 ± 35 ng/mg protein ($p < 0.001$). The latter also contained GLP-1 (61 ± 4 pg/mg protein).

Insulin secretion from homotypic and heterotypic pseudoislets: Glucose, amino acids, elevated K^+ and Ca^{2+}, IBMX and forskolin all evoked significant increases in insulin secretion from both MIN6 pseudoislets (1.3–4.8 fold increases, $p < 0.05$, $p < 0.01$, Figure 1A) and heterotypic pseudoislets (1.3–5.7 fold increases, $p < 0.05$, $p < 0.01$, Figure 1B). When

values were presented as stimulation index relative to corresponding glucose controls, the secretory responses were similar in (Figure 1C). However, in terms of ~~absolute~~ secretion as a percentage of insulin content however, responses were largely augmented in heterotypic pseudoislets compared to MIN6 cell pseudoislets.

Responses of homotypic and heterotypic pseudoislets to cytotoxicity: Both homotypic and heterotypic pseudoislets showed dose-dependent step-wise decreases in cell metabolic viability in response to increasing doses of cytotoxic agents. Combined cell pseudoislets exhibited significantly enhanced cytoprotective mechanisms against ninhydrin, H_2O_2, STZ and pro-inflammatory cytokines (1.4 – 1.8 fold increases in metabolic viability, $p<0.05$, $p<0.01$, $p<0.001$, Figure 2) compared to pseudoislets formed from MIN6 cells alone (Figure 2). This change in viability was accompanied by alterations of acridine orange/ ethidium bromide staining (Figure 3). Exposure to all agents tested evoke significant ($p<0.05$, $p<0.01$, $p<0.001$, Figure 3) increases in apoptosis in both homotypic and heterotypic pseudoislets compared to untreated controls. The frequency of apoptosis was significantly decreased in MIN6 and GLUTag pseudoislets compared to homotypic pseudoislets following exposure to ninhydrin, H_2O_2 and STZ (1.2 – 1.4 fold decreases, $p<0.05$, $p<0.001$). Apoptotic responses to a cocktail of proinflammatory cytokines did not differ significantly between the two test groups.

Effects of pseudoislet implantation on food and fluid intake, body weight and blood glucose in STZ diabetic SCID mice: Implantation of MIN6 pseudoislets and combined cell pseudoislets had small inhibitory effects on daily food intake during the study (Figure 4A). MIN6 and particularly MIN6 plus GLUTag pseudoislet implant recipients exhibited significantly ($p<0.05$, $p<0.001$, Figure 4B) decreased fluid intake compared to diabetic

11

controls from day 9 of the study. As shown in Figure 4C, both MIN6 pseudoislet and combined cell pseudoislet recipients displayed significantly ($p<0.05$, $p<0.01$, $p<0.001$, Figure 5C) greater body weights compared to diabetic controls. While body weight began to rise within 3 days of implantation in MIN6 pseudoislet recipients, this was delayed to around 12 days for combined cell pseudoislet recipients. Body weights were significantly lower in combined cell pseudoislet recipients than MIN6 pseudoislet recipients from 3 – 12 days after implantation ($p<0.05$, Figure 4C).

STZ caused an increase in mean blood glucose from <5 mmol/l to >20 mmol/l within 6 days of administration (Figure 4D). Mice receiving homotypic MIN6 pseudoislets reverted to normoglycaemic levels within 3 days of implantation, but began to exhibit hypoglycaemia after 6 days and were culled on day 21 of the study due to very low blood glucose concentrations. Implantation of heterotypic MIN6 and GLUTag pseudoislets evoked a gradual restoration of normoglycaemia, with blood glucose gradually decreasing from 9 days onwards ($p<0.001$, Figure 4D) compared to diabetic controls. Homotypic and heterotypic pseudoislet recipients both showed significant decreases (1.6–5.8 fold decreases, $p<0.001$, Figure 4E) in area under the curve values compared to diabetic controls, and MIN6 pseudoislets values were 3.5 fold lower than those of MIN6 and GLUTag pseudoislet recipients ($p<0.001$, Figure 4E).

Effects of pseudoislet implantation on glucose tolerance in STZ diabetic SCID mice: Following an 8 h fast and intraperitoneal glucose administration, plasma glucose levels of both MIN6 pseudoislet and combined cell pseudoislet recipients were significantly ($p<0.001$, Figure 5F) lower than those of diabetic control animals at all the time points observed. Additionally, MIN6 pseudoislet recipients exhibited significantly lower plasma glucose than

12

control mice or combined cell implantees throughout (p<0.05, Figure 4F). Implantation of heterotypic pseudoisletes resulted in normalization of glucose tolerance (Figure 4F).

Effects of pseudoislet implantation on terminal plasma and pancreatic hormone content in STZ diabetic SCID mice: Compared with diabetic controls, terminal plasma insulin was significantly (p<0.01, Figure 5A) raised in mice implanted with combined cell or particularly MIN6 pseudoislets, with levels in MIN6 pseudoislet recipients compared to combined cell pseudoislet recipients (p<0.05, Figure 5A). Plasma glucagon levels were significantly decreased (25 % decrease, p<0.05, Figure 5B) compared to diabetic controls in mice receiving combined cell pseudoislets but not in those receiving homotypic MIN6 pseudoislet implants (Figure 5B). Plasma GLP-1 levels were increased (p<0.05, Figure 5C) with combined cell pseudoislets and decreased (p<0.01, Figure 5C) with homotypic MIN6 pseudoislets compared to diabetic controls. MIN6 pseudoislets had no significant effect on pancreatic insulin or glucagon content (Figure 5D, E). Combined cell pseudoislets significantly (p<0.05, Figure 5D, E) increased pancreatic insulin content while decreasing pancreatic glucagon content.

Characteristics of excised implants: MIN6 and combined cell pseudoislets grew into very small masses over the course of the study. These masses were not visible from examinations prior to autopsy. MIN6 pseudoislet derived masses showed particularly intense peroxidase staining for insulin (Figure 6A). Combined cell pseudoislet derived cell masses stained positively for both insulin and GLP-1 (Figure 6B). For heterotypic growths, whole sections exhibited greater hormone staining than the negative controls. Haematoxylin and eosin staining showed that the cell masses were vascularized and encapsulated (Figure 6 A, B). The insulin contents of MIN6 and combined cell masses were 3706 ± 423 and 510 ± 19 ng/mg

13

protein (p<0.001). The latter also contained GLP-1 (111 ± 4 pg/mg protein).

Effects of pseudoislet implantation on pancreatic islets of STZ diabetic SCID mice: Induction of diabetes by administration of STZ was associated with significant decreases in islet area, beta cell area, β to α cell ratio and number of islets (Figure 7A – E). Both MIN6 pseudoislet implantation and combined cell pseudoislet implantation were associated with an increase (p<0.05) in islet β-cell to α-cell ratio, though this effect was significantly (p<0.05) greater for combined cell pseudoislet implants (Figure 7E). Pseudoislet implants did not significantly affect islet number but combined cell pseudoislets significantly (p<0.05) increased islet number compared to diabetic controls due to greater proportion of medium sized islets (Figure 7E, F). Administration of streptozotocin was associated with a significantly decreased frequency of β-cell proliferation and increased frequency of β-cell apoptosis (Figure 7G, H). MIN6 pseudoislet implantation did not significantly affect the proliferative or apoptotic status of pancreatic β-cells. However, combined cell pseudoislet implantation was associated with a significant (p<0.01, Figure 7G) increase in β-cell proliferation and a decrease in β-cell apoptosis compared to diabetic controls. Ki67/TUNEL ratio was significantly higher in combined cell pseudoislet implant group, thus favoring beta cell proliferation (Figure 7I).

Discussion

The therapeutic utility of combined insulin and GLP-1 treatment delivered by cell therapy using heterotypic pseudoislets was investigated using pseudoislets formed from the widely adopted mouse model insulin and GLP-1 secreting cell lines, MIN6 and GLUTag (Ishihara *et al.* 1993, Brubaker *et al.* 1998). Agents tested that work through diverse β-cell signaling pathways evoked significant increases in insulin secretion in a pattern similar to previous studies (Ishihara *et al.* 1993, Landa Jr. *et al.* 2005, Kelly *et al.* 2010b). Pseudoislets formed

14

from MIN6 and GLUTag cells did not exhibit enhanced insulin responses compared to homotypic cells, but absolute amounts released were considerably enhanced. This supports an important effect of GLP-1 on insulin biosynthesis but indicates that intra-islet production of GLP-1 as recently observed in alpha cells under conditions of islet stress (Thyssen *et al.* 2006, Ellingsgaard *et al.* 2011, Hansen *et al.* 2011, Whalley *et al.* 2011, Moffett *et al.* 2014, Vasu *et al.* 2014), might not markedly affect insulin secretion. The difference in cellular insulin content between the two types of pseudoislets was not due simply to dilution of beta cells. Thus although we estimate that implanted heterotypic pseudoislets most likely contained 2:1 ratio of GLUTag:MIN6 cells, due to faster growth rate of former cell type, this difference is small compared to the difference in cellular insulin content.

Ninhydrin, H_2O_2, STZ and pro-inflammatory cytokines are used frequently to evaluate cytoprotective mechanisms related to pancreatic β-cells in T1DM (Liu *et al.* 2008, Prause *et al.* 2014, Vasu *et al.* 2014). These agents decreased mitochondrial viability and increased the frequency of cellular apoptosis in both homotypic and heterotypic pseudoislets, mirroring previous findings in clonal β-cells (Liu *et al.* 2008, Prause *et al.* 2014, Vasu *et al.* 2014). Consistent with the cytoprotective effects of GLP-1 on pancreatic β-cells (Cornu *et al.* 2009, Garber *et al.* 2011, Vasu *et al.* 2013c), MIN6 and GLUTag pseudoislets showed significantly enhanced cytoprotective responses (significantly increased LD_{50}) to ninhydrin, H_2O_2, STZ and higher concentrations of cytokines. These results are supported by significant protection against apoptosis and by other findings showing the enhancement of cytoprotective effects of GLP-1 (Wideman *et al.* 2006, Faradji *et al.* 2008, Cornu *et al.* 2009, Toso *et al.* 2010). The frequency of apoptotic cells was not altered by the cytokine cocktail, perhaps due to a lower strength of cocktail being used for this experiment. However, the overall contrast between positive effects of heterotypic pseudoislet formation on β-cell protection versus lack of effect

on insulin secretory responses is interesting and may reflect divergence between the signal-transduction pathways governing the effects of GLP-1 on these processes in pancreatic β-cells (Holst 2007, Campbell & Drucker, 2013).

Implantation of homotypic MIN6 pseudoislets rapidly reversed hyperglycaemia and restored glucose tolerance in STZ treated SCID mice. This effect was not associated with significant alterations in protection against depletion of pancreatic insulin, increase of cellular glucagon or disruption of islet morphology, but a small increase in islet β-cell to α-cell ratio was observed. Since this was not associated with any alterations in pancreatic β-cell proliferation or apoptosis, it might reflect changes in pancreatic α-cells but further work is needed to clarify this possibility. Although MIN6 pseudoislets quickly rescued diabetes by provision of bioactive insulin, their prolonged actions lead to hyperinsulinaemia without correction of hyperglucagonaemia and the consequent hypoglycaemia necessitated termination of the mice. This presumably reflects the high insulin content of MIN6 cell transplants, combined with loss of the normal tight mechanisms which regulate insulin secretion. In contrast, reversal of hyperglycaemia in STZ diabetic SCID mice by heterotypic pseudoislets incorporating GLUTag cells was progressive and considerably better controlled. Blood glucose levels normalized much more gradually and mice exhibited normal plasma glucagon concentrations and did not become hypoglycaemic during the study. Such differences may also be due to differences in insulin contents between homotypic and heterotypic pseudoislets. Nevertheless combined implantation enhances functionality in addition to cytoprotection together with significant restorative effects on the endocrine pancreas, specifically, increased insulin and decreased glucagon plus increases in islet area and β to α cell ratio, stimulation of β-cell proliferation, and inhibition of β-cell apoptosis. This is consistent with restorative effects of GLP-1 and its agonists on pancreatic islets and implantation of GLUTag cells into insulin-

16

treated STZ diabetic SCID mice (Cornu *et al.* 2009, Garber *et al.* 2011, Vasu *et al.* 2013c).

Both homotypic and heterotypic pseudoislets grew into small cell masses with no signs of metastatsis in either group. *In vitro* studies have previously shown limited proliferation rates in pseudoislets formed from various β-cell-lines compared with corresponding monolayers (Lock *et al.* 2011, Reers *et al.* 2011, Guo-Parke *et al.* 2012, Spelios *et al.* 2013). Indeed, our present and previous *in vitro* work with MIN6 pseudoislets indicates that they tend to reach a maximum size at which point their growth ceases (Kelly *et al.* 2010a, Kelly *et al.* 2010b). This observed arrest in MIN6 pseudoislet growth size has previously been attributed to the self-regulation of cluster size by the modulation of proliferation and apoptotic pathways via gap junction proteins (Kelly *et al.* 2011). However, it is also possible that an important contributor to size limiting factor of pseudoislets was central hypoxia (Lock *et al.* 2011). Indeed, our results indicate that pseudoislets reached a maximum size *in vitro,* but when implanted *in vivo* acquired a vasculature which permitted growth into small cell masses without evidence of central necrosis.

The development of strategies to provide reversibly immortalized β-cell and GLP-1 secreting L-cell lines of human lineage would provide a potential means of exploiting the observed beneficial effects of combined insulin and GLP-1 delivery by cell therapy in a sustainable manner. Some studies have shown promising results generating large numbers of human insulin releasing β-cells by incorporating a selectively activated oncogene which can be turned off by addition of cre-recombinase, or by the generating large numbers of insulin-releasing cells by differentiation of human pluripotent stem cells (Ravassard *et al.* 2011, Scharfmann *et al.* 2014, Pagliuca *et al.* 2014). As yet data is sparse regarding the potential for generating large numbers of non-tumorigenic intestinal L-cells, but it is possible that some of

the techniques described for the generation of β-cells could be transferrable to such efforts. In addition, much progress is being made currently regarding development of small implantation devices that contain, nurture and protect transplanted cells in potentially hostile *in vivo* environment (Kirk *et al.* 2014, An *et al.* 2015, Fotino *et al.* 2015).

To conclude, addition of GLP-1 releasing GLUTag cells to MIN6 pseudoislets conferred significant enhancements in functionality and cytoprotective mechanisms to the pseudoislets *in vitro*, and offered restorative effects on pancreatic islets and better regulated reversal of hyperglycaemia when implanted in diabetic mice compared to homotypic MIN6 pseudoislets. Further research will be necessary to elucidate the potential of using pseudoislets formed from mixed human cell-lines, and to circumvent the potential problems associated with implanting immortalized cell-lines. This work however, provided proof of concept that combined insulin and GLP-1 cell therapy using pseudoislets holds therapeutic potential, contributing important new knowledge to the fields of islet transplantation and β-cell replacement therapy.

Declaration of interest

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

Author contributions

PRF designed the study. ADG and SV conducted the experimental work and data analysis. PRF, ADG and SV wrote the manuscript and all authors approved the final version submitted for publication.

Acknowledgements

References

1) Onaca N, Naziruddin B, Matsumoto S, Noguchi H, Klintmalm GB & Levy MF 2007 Pancreatic islet cell transplantation: update and new developments. *Nutr Clin Pract.* **22** 485–493.

2) Barton FB, Rickels MR, Alejandro R, Hering BJ, Wease S, Naziruddin B et al. 2012 Improvement in outcomes of clinical islet transplantation: 1999-2010. *Diabetes Care*. **35** 1436 – 1445.

3) McCall M & Shapiro AM 2012 Update on islet transplantation. *Cold Spring Harb Perspect Med.* **2** a007823.

4) McCluskey JT, Hamid M, Guo-Parke H, McClenaghan NH, Gomis R & Flatt PR 2011 Development and functional characterization of insulin-releasing human pancreatic beta cell lines produced by electrofusion. *J Biol Chem.* **286** 21982–21992.

5) Scharfmann R, Pechberty S, Hazhouz Y, von Bülow M, Bricout-Neveu E, Grenier-Godard M, Guez F, Rachdi L, Lohmann M, Czernichow P et al. 2014 Development of a conditionally immortalized human pancreatic β cell line. *J Clin Invest*. **124** 2087–2098.

6) Buss JL, Rajab A, Essig ED, Bergdall VK, Wang J & Osei K. 2012 Exenatide pretreatment improved graft function in nonhuman primate islet recipients compared to treatment after transplant only. *J Transplant*. doi: 10.1155/2012/382518

7) Wang Y, Qi M, McGarrigle JJ, Rady B, Davis ME, Vaca P & Oberholzer J 2013 Use of glucagon-like peptide-1 agonists to improve islet graft performance. *Curr Diab Rep*. **13** 723 – 732.

19

8) Xu G, Stoffers DA, Habener JF & Bonner-Weir S. 1999 Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes.* **48** 2270–2276.

9) Baggio L, Kieffer TJ & Drucker DJ. 2000 Glucagon-like peptide-1, but not glucose-dependent insulinotropic peptide, regulates fasting glycemia and nonenteral glucose clearance in mice. *Endocrinology*. **141** 3703–09.

10) Skoglund G, Hussain MA & Holz GG. 2000 Glucagon-like peptide 1 stimulates insulin gene promoter activity by protein kinase A-independent activation of the rat insulin I gene cAMP response element. *Diabetes.* **49** 1156–64.

11) Stoffers DA, Kieffer TJ, Hussain MA, Drucker DJ, Bonner-Weir S, Habener JF & Egan JM 2000 Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. *Diabetes*. **49** 741–48.

12) Campbell JE & Drucker DJ**.** 2013 Pharmacology, physiology, and mechanisms of incretin hormone action. *Cell Metab.* **17** 819 – 37.

13) Ghofaili KA, Fung M, Ao Z, Meloche M, Shapiro RJ, Warnock GL, Elahi D, Meneilly GS & Thompson DM 2007 Effect of exenatide on β cell function after islet transplantation in type 1 diabetes. *Transplantation*. **83** 24–28.

14) Faradji RN, Tharavanij T, Messinger S, Froud T, Pileggi A, Monroy K, Mineo D, Baidal DA, Cure P, Ponte G et al. 2008 Long-term insulin independence and improvement in insulin secretion after supplemental islet infusion under exenatide and etanercept. *Transplantation.* **86** 1658–1665.

15) Gangemi A, Salehi P, Hatipoglu B, Martellotto J, Barbaro B, Kuechle JB, Qi M, Wang Y, Pallan P, Owens C et al. 2008 Islet transplantation for brittle type 1 diabetes: The UIC protocol. *Am J Transplant.* **8** 1250–61.

16) Merani S, Truong W, Emamaullee JA, Toso C, Knudsen LB & Shapiro AM 2008 Liraglutide, a long-acting human glucagon-like peptide 1 analog, improves glucose homeostasis in marginal mass islet transplantation in mice. *Endocrinology*. **149** 4322–28.

17) Toso C, McCall M, Emamaullee J, Merani S, Davis J, Edgar R, Pawlick R, Kin T, Knudsen LB & Shapiro AM 2010 Liraglutide, a long-acting human glucagon-like peptide 1 analogue, improves human islet survival in culture. *Transpl Int*. **23** 259–265.

18) Butler PC, Elashoff M, Elashoff R & Gale EA 2013 A critical analysis of the clinical use of incretin-based therapies: Are the GLP-1 therapies safe? *Diabetes Care*. **36** 2118–25.

19) King A, Lock J, Xu G, Bonner-Weir S & Weir GC 2005 Islet transplantation outcomes in mice are better with fresh islets and exendin-4 treatment. *Diabetologia*. **48** 2074–79.

20) Crutchlow MF, Yu M, Bae YS, Deng S & Stoffers DA 2008 Exendin-4 does not promote Beta-cell proliferation or survival during the early post-islet transplant period in mice. *Transplant Proc*. **40** 1650–1657.

21) Lin CC & Anseth KS. 2009 Glucagon-like peptide-1 functionalized PEG hydrogels promote survival and function of encapsulated pancreatic beta-cells. *Biomacromolecules*. **10** 2460–67.

22) Kizilel S, Scavone A, Liu X, Nothias JM, Ostrega D, Witkowski P, & Millis M 2010 Encapsulation of pancreatic islets within nano-thin functional polyethylene glycol coatings for enhanced insulin secretion. *Tissue Eng Part A*. **16** 2217–2228.

23) Wideman RD, Yu IL, Webber TD, Verchere CB, Johnson JD, Cheung AT & Kieffer TJ 2006 Improving function and survival of pancreatic islets by endogenous production of glucagon-like peptide 1 (GLP-1). *Proc Natl Acad Sci*. **103** 13468–13473.

24) Hauge-Evans AC, Squires PE, Persaud SJ & Jones PM 1999 Pancreatic beta-cell-to-beta-cell interactions are required for integrated responses to nutrient stimuli: enhanced Ca2+ and insulin secretory responses of MIN6 pseudoislets. *Diabetes*. **48** 1402 – 1408.

25) Kelly C, McClenaghan NH & Flatt PR. 2011 Role of islet structure and cellular interactions in the control of insulin secretion. *Islets*. **3** 41–47.

26) Brereton HC, Carvell MJ, Asare-Anane H, Roberts G, Christie MR, Persaud SJ & Jones PM. 2006 Homotypic cell contact enhances insulin but not glucagon secretion. Biochem Biophys Res Commun. **344** 995–1000.

27) Brereton H, Carvell MJ, Persaud SJ & Jones PM. 2007 Islet alpha-cells do not influence insulin secretion from beta-cells through cell-cell contact. *Endocrine*. **31** 61–65.

28) Kelly C, Guo-Parke H, McCluskey JT, Flatt PR & McClenaghan NH 2010b The role of glucagon- and somatostatin-secreting cells in the regulation of insulin release and beta-cell function in heterotypic pseudoislets. *Diabetes Metab Res Rev*. **26** 525–533.

29) Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y & Yamamura K 1990 Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology*. **127** 126–132.

30) Lee YC, Asa SL & Drucker DJ. 1992 Glucagon gene 5'-flanking sequences direct expression of simian virus 40 large T antigen to the intestine, producing carcinoma of the large bowel in transgenic mice. *J Biol Chem*. **267** 10705–10708.

31) Drucker DJ, Lee YC, Asa SL & Brubaker PL. 1992 Inhibition of pancreatic glucagon gene expression in mice bearing a subcutaneous glucagon-producing GLUTag transplantable tumor. *Mol Endocrinol*. **6** 2175–2184.

32) Guo-Parke H, McCluskey JT, Kelly C, Hamid M, McClenaghan NH & Flatt PR 2012 Configuration of electrofusion-derived human insulin-secreting cell line as pseudoislets enhances functionality and therapeutic utility. *J Endocrinol*. **214** 257–65.

33) Flatt PR & Bailey CJ. 1981 Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. *Diabetologia*. **42** 670–674.

34) Moffett RC, Vasu S, McClenaghan NH & Flatt PR. 2014 Incretin receptor null mice reveal key role for GLP-1 but not GIP in pancreatic beta cell adaptation to pregnancy. *PLOS One*. **9** e96863.

35) Mossman T. 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunolog Meth*. **65** 55–63.

36) Vasu S, Moffett RC, Thorens B & Flatt PR 2014 Role of endogenous GLP-1 and GIP in beta cell compensatory responses to insulin resistance and cellular stress. *PLOS One* **9** e101005.

37) Ishihara H, Asano T, Tsukuda K, Katagiri H, Inukai K, Anai M, Kikuchi M, Yazaki Y, Miyazaki JI & Oka Y 1993 Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia*. **36** 1139–45.

38) Brubaker PL, Schloos J & Drucker DJ. 1998 Regulation of glucagon-like peptide-1 synthesis and secretion in the GLUTag enteroendocrine cell line. *Endocrinology*. **139** 4108 – 4114.

39) Landa LR Jr, Harbeck M, Kaihara K, Chepurny O, Kitiphongspattana K, Graf O, Nikolaev VO, Lohse MJ, Holz GG & Roe MW 2005 Interplay of Ca^{2+} and cAMP signaling in the insulin-secreting MIN6 beta-cell line. *J Biol Chem*. **280** 31294–31302.

40) Thyssen S, Arany E & Hill DJ. 2006 Ontogeny of regeneration of beta-cells in the neonatal rat after treatment with streptozotocin. Endocrinology. **147** 2346 – 2356.

41) Ellingsgaard H, Hauselmann I, Schuler B, Habib AM, Baggio LL, Meier DT, Eppler E, Bouzakri K, Wueest S, Muller YD et al. 2011 Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. Nat Med. **17** 1481 – 1489.

42) Hansen AM, Bödvarsdottir TB, Nordestgaard DN, Heller RS, Gotfredsen CF, Maedler K, Fels JJ, Holst JJ & Karlsen AE. 2011 Upregulation of alpha cell glucagon-like peptide 1 (GLP-1) in Psammomys obesus--an adaptive response to hyperglycaemia? *Diabetologia*. **54** 1379 – 1387.

43) Whalley NM, Pritchard LE, Smith DM & White A. 2011 Processing of proglucagon to GLP-1 in pancreatic α-cells: is this a paracrine mechanism enabling GLP-1 to act on β-cells? *J Endocrinol*. **211** 99 – 106.

44) Moffett RC, Vasu S, McClenaghan NH & Flatt PR 2014 Incretin receptor null mice reveal key role for GLP-1 but not GIP in pancreatic beta cell adaptation to pregnancy. *PLOS One*. **9** e96863.

45) Liu HK, McCluskey JT, McClenghan NH & Flatt PR. 2008 Iterative exposure of clonal BRIN-BD11 cells to ninhydrin enables selection of robust toxin-resistant cells but with decreased gene expression of insulin secretory function. *Pancreas*. **36** 294–301.

46) Vasu S, McClenaghan NH, McCluskey JT & Flatt PR. 2014 Mechanisms of toxicity by proinflammatory cytokines in a novel human pancreatic beta cell line, 1.1B4. *Biochim Biophys Acta.* **1840** 136–145.

47) Prause M, Christensen DP, Billestrup N & Mandrup-Poulsen T. 2014 JNK1 Protects against Glucolipotoxicity-Mediated Beta-Cell Apoptosis. *PLOS One*. **24** e87067.

48) Cornu M, Yang JY, Jaccard E, Poussin C, Widmann C & Thorens B. 2009 Glucagon-like peptide-1 protects beta-cells against apoptosis by increasing the activity of an IGF-2/IGF-1 receptor autocrine loop. *Diabetes*. **58** 1816–1825.

49) Garber AJ. 2011 Incretin effects on β-cell function, replication, and mass: the human perspective. *Diabetes Care*. **34** S258–S263.

50) Vasu S, Moffett RC, McCluskey JT, Hamid MH, Irwin N & Flatt PR. 2013c Beneficial effects of parenteral GLP-1 delivery by cell therapy in insulin-deficient streptozotocin diabetic mice. *Gene Ther*. **20** 1077–1084.

51) Holst JJ. 2007 The physiology of glucagon-like peptide 1. *Physiol Rev*. **87** 1409 – 1439.

52) Lock LT, Laychock SG & Tzanakakis ES. 2011 Pseudoislets in stirred-suspension culture exhibit enhanced cell survival, propagation and insulin secretion. *J Biotechnol.* **151** 278–286.

53) Reers C, Hauge-Evans AC, Morgan NG, Wilcox A, Persaud SJ & Jones PM. 2011 Down-regulation of proliferation does not affect the secretory function of transformed β-cell lines regardless of their anatomical configuration. *Islets*. **3** 80–8.

54) Spelios MG, Kenna LA, Wall B & Akirav EM. 2013 In vitro formation of β cell pseudoislets using islet-derived endothelial cells. *PLOS One*. **8** e72260.

55) Kelly C, Guo-Parke H, McCluskey JT, Flatt PR & McClenaghan NH. 2010a Evaluation of role of islet cell paracrine interactions in regulation of insulin secretion using a new heterotypicpseudoislet model. *Regulatory peptides.* **164** 33.

56) Ravassard P, Hazhouz Y, Pechberty S, Bricout-Neveu E, Armanet M, Czernichow P & Scharfmann R. 2011 A genetically engineered human pancreatic β cell line exhibiting glucose-inducible insulin secretion. *J Clin Invest.* **121** 3589–3597.

57) Pagliuca FW, Millman JR, Gürtler M, Segel M, Van Dervort A, Ryu JH, Peterson QP, Greiner D & Melton DA 2014 Generation of Functional Human Pancreatic β Cells In Vitro. *Cell.* **159** 428–39.

58) Kirk K, Hao E, Lahmy R & Itkin-Ansari P 2014 Human embryonic stem cell derived islet progenitors mature inside an encapsulation device without evidence of increased biomass or cell escape. *Stem Cell Research*. **12** 807 – 814.

59) An D, Ji Y, Chiu A, Lu YC, Song W, Zhai L, Qi L, Luo D & Ma M. 2015 Developing robust, hydrogel-based, nanofiber-enabled encapsulation devices (NEEDs) for cell therapies. *Biomaterials*. **37** 40 – 8.

60) Fotino N, Fotino C & Pileggi A. 2015 Re-engineering islet cell transplantation. *Pharmacological Research.* **98** 76 – 85.

Figure Legends

Figure 1 Insulin secretory responses of the pseudoislets A Secretory responses of MIN6 pseudoislets. **B** Secretory responses of MIN6 + GLUTag pseudoislets. **C** shows comparison of responses from the two types of pseudoislets with results expressed as a ratio of stimulated insulin secretion to that of cells incubated in corresponding glucose containing buffer alone. Values are mean ± S.E.M. (n=4). *p<0.05, **p<0.01 compared with 5.6 mM glucose alone. $^{\Delta}$p<0.05 and $^{\Delta\Delta}$p<0.01 compared with 16.7 mM glucose alone.

Figure 2 Viability of cells derived from MIN6 and GLUTag pseudoislets following 2 h exposure to cytotoxins. The viability of MIN6 / MIN6 + GLUTag psedoislets was assessed by MTT assay following 2 h incubation with a range of doses of various cytotoxic agents. Cytokine cocktail compositions were as follows: A - 100 U.ml^{-1} IL1β, 20 U.ml^{-1} IFNγ, 200 U.ml^{-1} TNFα; B -200 U.ml^{-1} IL1β, 30 U.ml^{-1} IFNγ, 250 U.ml^{-1} TNFα; C. 300 U.ml^{-1} IL1β, 40 U.ml^{-1} IFNγ, 300 U.ml^{-1} TNFα; D. 400 U.ml^{-1} IL1β, 50 U.ml^{-1} IFNγ, 350 U.ml^{-1} TNFα; E 500 U.ml^{-1} IL1β, 60 U.ml^{-1} IFNγ, 400 U.ml^{-1} TNFα. Values are mean ± S.E.M. (n=4) *p<0.05, **p<0.01 and ***p<0.001 compared with untreated controls. $^{\Delta}$p<0.05 and $^{\Delta\Delta}$p<0.01 compared with corresponding results in MIN6 pseudoislets.

Figure 3 Assessment of apoptosis in cells derived from MIN6 and GLUTag pseudoislets following 2 h exposure to a range of cytotoxins. Percentages of apoptotic cells counted are shown. Values are mean ± SEM. (n=4) **p<0.01 and ***p<0.001 compared to appropriate untreated controls. $^{\Delta}$p<0.05 and $^{\Delta\Delta\Delta}$p<0.001 compared with corresponding results in MIN6 pseudoislets. Cytokine cocktail composition was 300 U.ml-1 IL1β, 40 U.ml-1 IFNγ and 300 U.ml-1 TNFα.

27

Figure 4 Metabolic effects of implanting pseudoislets formed from MIN6 and GLUTag cells into streptozotocin diabetic SCID mice. SCID mice were rendered diabetic by a single intraperitoneal injection of streptozotocin on day 0. On day 3, pseudoislets or blank injection vehicles were administered subcutaneously to the subscapular region. Food intake (A), fluid intake (B), body weights (C) and blood glucose (D) and were measured every 3 days. E Area under the curve, expressed as mmol/l/day. At the end of the study, terminal intraperitoneal glucose tolerance was also measured (F). Values are mean ± S.E.M (n=5). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to diabetic controls. $^{\Delta}p<0.05$, $^{\Delta\Delta}p<0.01$ and $^{\Delta\Delta\Delta}p<0.001$ compared to mice implanted with homotypic MIN6 pseudoislets.

Figure 5 Effects of pseudoislet implantation on hormone contents of plasma and pancreatic tissue. Terminal plasma insulin (A), glucagon (B) and GLP-1 (C), as well as pancreatic insulin (D) and glucagon (E) contents of mice receiving pseudoislet implants were assessed at the end of the study. Values are mean ± S.E.M. (n=5). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to diabetic controls. $^{\Delta}p<0.05$ compared to mice implanted with homotypic MIN6 pseudoislets.

Figure 6 Histological examination of excised implants. Representative images of tissue sections of cell masses formed from MIN6 pseudoislets (**A**) and combined MIN6 and GLUTag cell pseudoislets (**B**) immunostained for hormones with methyl green counterstaining or stained with haematoxylin and eosin. BV = blood vessel. EN = encapsulated edges. Scale bars represent 100 µm.

Figure 7 Effects of pseudoislet implantation on pancreatic islet morphology. Islet area (**A**), β-cell area (**B**), α-cell area (**C**), β to α cell ratio (**D**), number of islets (**E**), islet size

distribution (**F**) were determined by quantitative histological analysis using cell^F software.

Frequency of β-cell proliferation (**G**) and apoptosis (**H**) and ratio of Ki67 to TUNEL positive

β-cells (**I**) were determined by quantitative histological analysis. Values are mean ± S.E.M.

(n=5). *p<0.05, **p<0.01 compared to diabetic control. $^{\Delta}$p<0.05, $^{\Delta\Delta}$p<0.01 compared to
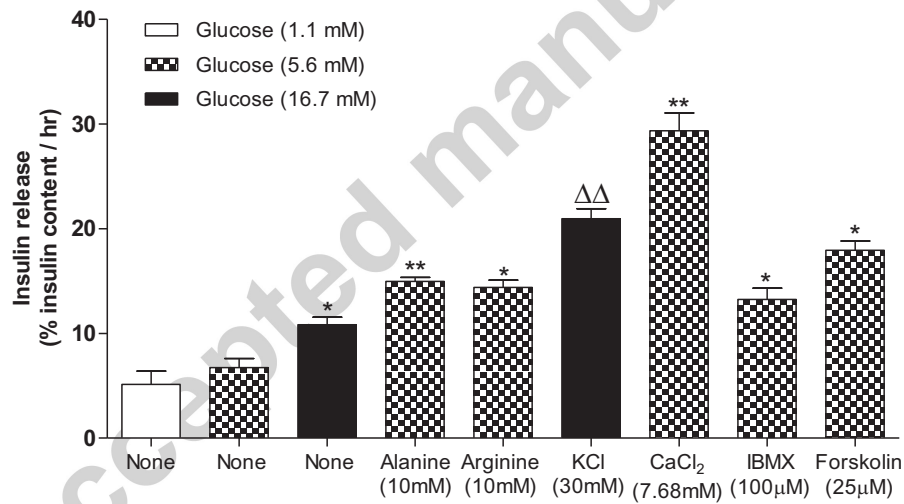
MIN6 pseudoislet group.

Figure 1

A

B

C

30
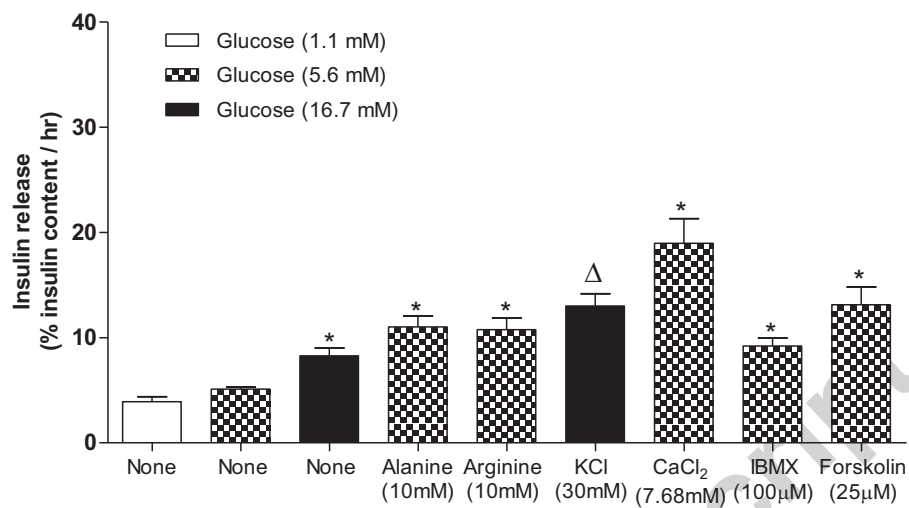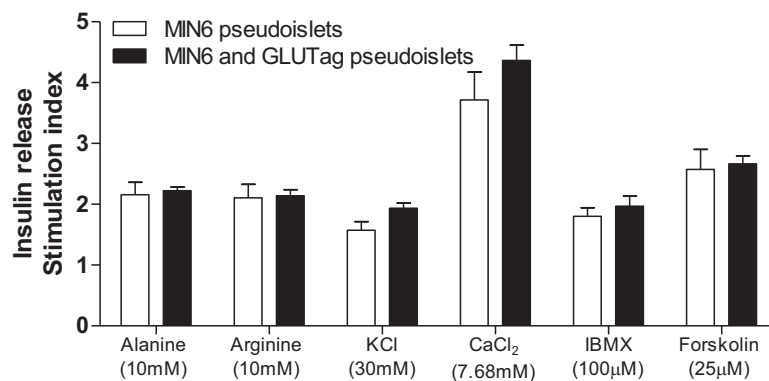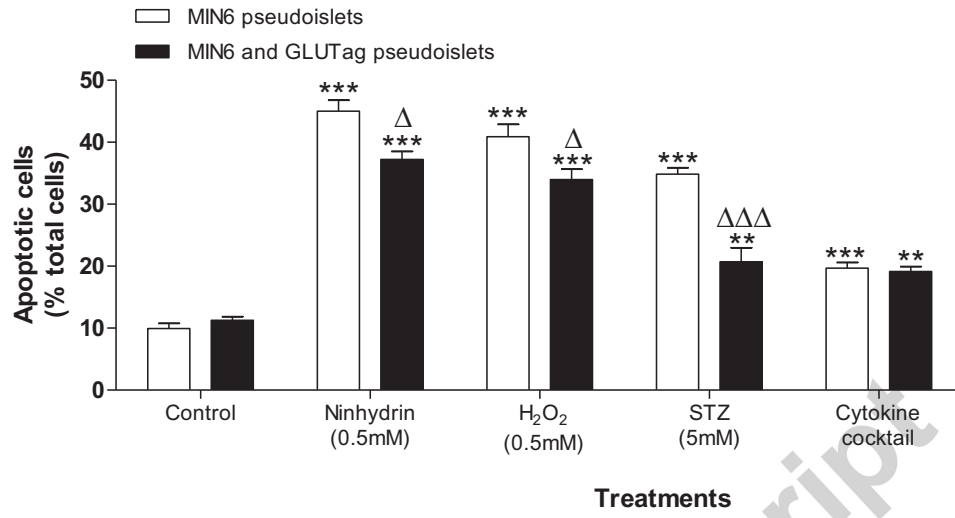
Figure 2
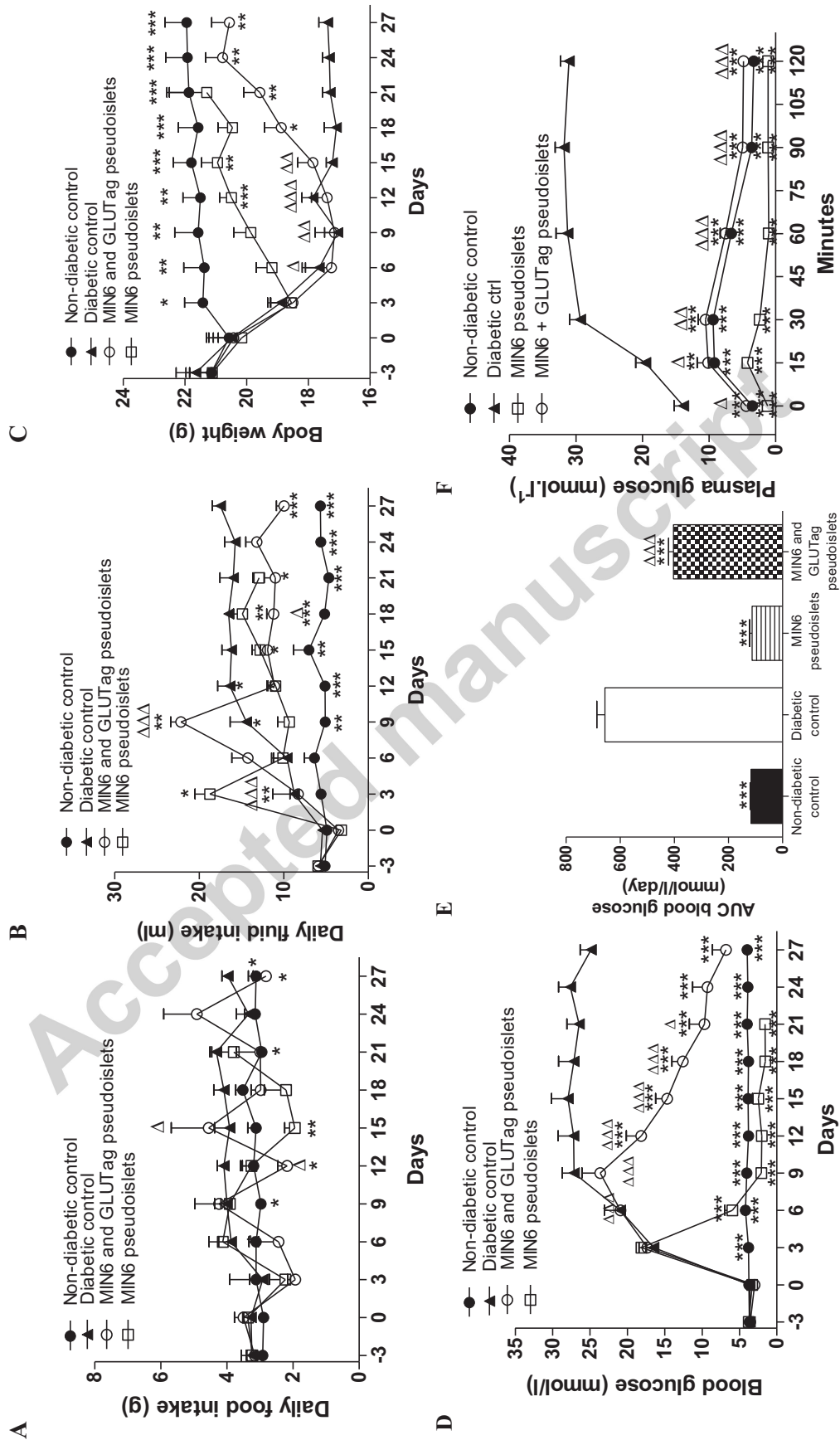
Figure 3

Figure 4

Figure 5

Figure 6

Figure 7