INSULIN-LIKE GROWTH FACTOR-II (IGF-II) PREVENTS PRO-INFLAMMATORY CYTOKINE INDUCED APOPTOSIS AND SIGNIFICANTLY IMPROVES ISLET SURVIVAL POST-TRANSPLANTATION

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Abbreviations:

1way Anova, 1way analysis of variance; 7-AAD, 7-Aminoactinomycin D; Ad, Adenoviral; GSIS, Glucose stimulated insulin secretion; IBMIR, Instant blood mediated inflammatory reaction; IGF-II, Insulin-like Growth Factor-II; IGF-1R, Insulin-like Growth Factor-I receptor; IFN-γ, Interferon-γ; IL-1β, Interleukin-1β; MAPK, Mitogen-activated kinase, MOI, Multiplicity of infection; PI, Propidium Iodide; T1D, Type 1 Diabetes Mellitus; ELISA, enzyme-linked immunosorbent assay; GFP, green fluoresence protein; IR, insulin receptor, I.P, intra peritoneal; IEQ, islet equivalent; PI3K, phosphatidylinositol-3kinase; pAKT, phospho-akt; PVDF, polyvinyl difluoride; rIGF-II, recombinant IGF-II; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of the mean; SI, stimulation index; STZ, streptozotocin, VEGF, vascular endothelial growth factor.

Abstract:

Background: The early loss of functional islet mass (50-70%) due to apoptosis following clinical transplantation contributes to islet allograft failure. Insulin-like Growth Factor-II (IGF-II) is an anti-apoptotic protein that is highly expressed in β-cells during development, but rapidly decreases in post-natal life.

Methods: We used an Adenoviral (Ad) vector to over express IGF-II in isolated rat islets and investigated its anti-apoptotic action against exogenous cytokines IL-1β and IFN-γ induced islet cell death *in vitro*. Using an immunocompromised marginal mass islet transplant model, the ability of Ad-IGF-II transduced rat islets to restore euglycemia in NOD-SCID diabetic recipients was assessed.

Results: Ad-IGF-II transduction did not affect islet viability or function. Ad-IGF-II cytokine treated islets exhibited decreased cell death (40 ± 2.8%) vs. Ad-GFP and untransduced control islets (63.2 ± 2.5% and 53.6 ± 2.3%, respectively). Ad-IGF-II over expression during cytokine treatment resulted in a marked reduction in TUNEL positive apoptotic cells (8.3 ± 1.4%) vs. Ad-GFP control (41 ± 4.2%) and untransduced control islets (46.5 ± 6.2%). Western blot analysis confirmed that IGF-II inhibits apoptosis via activation of the PI3K/Akt signalling pathway. Transplantation of IGF-II over expressing islets under the kidney capsule of diabetic mice restored euglycemia in 77.8% of recipients, compared to 18.2% and 47.5% of Ad-GFP and untransduced control islet recipients, respectively ($p<0.05$ Log-rank (Mantel Cox)).

Conclusions: Anti-apoptotic IGF-II decreases apoptosis *in vitro* and significantly improved islet transplant outcomes *in vivo*. Anti-apoptotic gene transfer is a potentially powerful tool to improve islet survival post-transplantation.

Introduction

Islet transplantation is a promising therapeutic option for Type 1 Diabetes Mellitus (T1D), with the ability to improve glycometabolic control in T1D patients and in select cases achieve insulin independence (1, 2). While short term glucose stabilization and insulin independence rates continue to improve, long-term follow up reveals a marked reduction in islet graft function over time (3, 4). The reasons for the decline in insulin independence rates following islet transplantation are complex, beginning before pancreas procurement as brain death is associated with the production of islet toxic cytokines such as TNF-α, IL-1β and IL-6 (5-7).

Intraportally transplanted islets reside in the hostile environment of the liver, where they are exposed to the instant blood mediated inflammatory reaction (IBMIR) (8), alloimmunity (9), recurrence of islet specific autoimmunity (10), a highly toxic pro-inflammatory cytokine storm (e.g. IL-1β, IFN-α, IFN-γ and TNF-α) (11, 12) and hypoxia due to inadequate revascularization post-transplant (13, 14). All of these stresses contribute to islet apoptosis and promote failure of the islet allograft (15). Strategies to prevent early apoptosis are therefore highly desirable to enhance long term islet survival.

Insulin-like Growth Factor-II (IGF-II) is a potent growth factor, recognised for its anti-apoptotic ability in a wide variety of cell types including pancreatic islets (16-18). IGF-II binds to the Insulin-like Growth Factor-I receptor (IGF-1R) to mediate its anti-apoptotic function, resulting in activation of the phosphatidylinositol-3kinase (PI3K)/Akt pathway (19-21). IGF-II blocks proteolytic processing of the major executioner caspases, caspase-3 and caspase-7 (22, 23), preventing initiation of the apoptotic cascade (24).

In this study, the influence of local human IGF-II over expression on rat pancreatic islet cell survival *in vitro* and *in vivo* was examined. The human IGF-II protein has 85% sequence identity to the rat protein. Here we describe successful transduction of rat islets with an Adenoviral (Ad)-IGF-II based vector to over express IGF-II, without affecting islet viability or function. Over expression of IGF-II in islets resulted in enhanced islet survival *in vitro* and in an *in vivo* marginal mass islet transplant model, indicating that strategies to combat apoptosis may improve islet function post-transplantation.

Results

Transduction efficiency of Ad-GFP and Ad-IGF-II in isolated rat islets

Ad-GFP was used to transduce isolated rat islets *in vitro* at multiplicity of infection (MOI) 10, 100, 200, 500 and 1000, to assess the transduction efficiency of the Ad vector in rat islets. Green fluorescence protein (GFP) was included in the Ad-GFP vector to function as a transduction marker. At 48 h post-transduction, GFP expression was observed in transduced islets, although at different efficiencies depending on the MOI (Figure 1 A).

Ad-IGF-II transduced rat islets (MOI 100) constitutively produced and secreted 8.9 ± 1.3 ng human IGF-II into the cell culture supernatant at 24 h, this increased to 14.3 ng ± 2.4 at 48 h. The level of secreted IGF-II reached a maximum of 24.1 ng ± 1 on day 5 (Figure 2). The secretion of human IGF-II was observed only in the IGF-II transduced cells demonstrating that Ad-IGF-II mediated effective transduction and specific expression of IGF-II in transduced islet cells.

Effect of Ad-IGF-II transduction on islet function and viability

Islet viability was not adversely affected when transduced with Ad-IGF-II at MOI 100 (74.3% ± 8.2) compared to untransduced controls (69.2% ± 4.7). However, when rat islets were transduced with Ad-GFP there was minor cytotoxicity and as a result of this, islet viability was reduced to 58.2% ± 1.8 (Figure 1 B). To determine whether insulin secretion was adversely affected in Ad-IGF-II, Ad-GFP or untransduced islets, *in vitro* islet function was characterised by challenging islets with glucose. Transduction of islets with Ad-GFP or Ad-IGF-II (MOI 100) did not alter the insulin secretory function of islets (1.8 ± 0.4 and 2 ± 0.5

stimulation index (SI)) respectively, compared to untransduced islets (2 ± 0.5 SI) (Figure 1 C). Based on these findings MOI 100 was chosen as the MOI for further experiments.

Effect of Ad-IGF-II transduction on islet apoptosis *in vitro*

To investigate the anti-apoptotic effect of IGF-II, Ad-IGF-II transduced islets were incubated with a cocktail of pro-inflammatory cytokines interleukin-1β (IL-1β) and interferon-γ (IFN-γ) for 24 h to induce apoptosis. Following cytokine exposure, there was a marked reduction in the number of TUNEL positive (apoptotic) cells in the Ad-IGF-II transduced islets compared to Ad-GFP transduced islets (Figure 3 A – C). Specifically, 46.5% ± 6.2 and 41% ± 4.2 total islet cells in the untransduced and Ad-GFP transduced groups were TUNEL positive, respectively. However, when IGF-II was over expressed in islets during cytokine exposure a decrease in the number of TUNEL positive apoptotic cells was observed (8.3% ± 1.4), $p \leq 0.0001$ (1way ANOVA) (Figure 3 D).

The cytoprotective effect of Ad-IGF-II on islet cell death following cytokine exposure was investigated by Annexin V and PI staining. Following cytokine exposure, Ad-IGF-II transduced islets and recombinant (r)IGF-II treated islets displayed a decreased level of cytokine induced cell death (39.8% ± 2.8 and 41.9 ± 3.3%, respectively) when compared to control islets transduced with Ad-GFP (63.2% ± 2.5) or untransduced (53.6% ± 2.3) (Figure 3 E, $p<0.0001$), clearly demonstrating the anti-apoptotic effect of IGF-II *in vitro*.

To investigate the pathway through which IGF-II works to inhibit apoptosis, rat islets were pre-treated with wortmannin, a potent and specific PI3K inhibitor (25) prior to Ad-IGF-II transduction. Ad-IGF-II transduced islets displayed increased levels of phospho-Akt (pAkt) compared to the control groups and pre-treament of Ad-IGF-II with wortmannin abolished

IGF-II stimulated Akt phosphorylation (Figure 4). Thus, indicating that IGF-II inhibits islet cell apoptosis through activation of the PI3K/Akt pathway.

Effect of Ad-IGF-II islet survival and function after transplantation

To investigate the effect of Ad-IGF-II transduction in an islet transplant setting, a marginal mass of islets (50 islet equivalents (IEQ)) were transduced with Ad-GFP, Ad-IGF-II or untransduced and transplanted under the kidney capsule of NOD-SCID STZ-induced diabetic mice. The *in vivo* study was limited to 28 days as it is estimated that 50–70% of islets are destroyed in the immediate post transplant period by apoptosis (26, 27).

Transplantation of Ad-IGF-II islets resulted in a return to euglycemia in 77.8% of recipients (n=9), compared to 18.2% of Ad-GFP and 45.5% of untransduced islet recipients (both n=11) (Figure. 5 A), $p<0.05$ (Log-rank (Mantel Cox) Test. Ad-IGF-II recipient mice stabilized and slightly increased their weight following transplantation, while this was not the case for mice receiving untransduced or Ad-GFP transduced islet grafts, $p<0.0001$ (1way ANOVA) (Figure 5 B). While nephrectomy of the graft bearing kidney was not performed due to ethical guidelines, the diabetic state was confirmed by staining the mice pancreata for endogenous insulin (data not shown).

Discussion

Apoptosis is a major cause of islet cell loss in the early post-transplant period (28, 29). The insulin-like Growth Factors (IGF-I and IGF-II) are mitogenic peptides involved in the regulation of apoptosis (23). IGF-II is produced in the pancreas during fetal and neonatal life (30), where it is highly expressed in different islet cell populations (including β-cells) (31-33). Recently, Hills and colleagues (34) have described IGF-II as a more effective anti-apoptotic survival factor than IGF-I in human placental villous cytotrophoblasts. Moreover, Ad-mediated IGF-I over expression was unable to protect isolated human islets from apoptosis induced by a cocktail of pro-inflammatory cytokines, including IL-1β and IFN-γ (35). Therefore, in this study, we evaluated the effect of IGF-II over expression on rat pancreatic islet cell survival via an *ex vivo* gene transfer approach.

Firstly, Ad-IGF-II efficiently transduced rat islets and IGF-II over expression did not affect islet viability or physiological function. Ad-GFP islets displayed decreased cell viability compared to untransduced control islets. This may be due to the potential cell toxicity associated with GFP (36-38). In the same set of experiments, Ad-IGF-II transduced islets displayed increased viability compared to control cells, likely due to the anti-apoptotic properties of IGF-II. The utility of Ad mediated IGF-II over expression is that IGF-II mediates its anti-apoptotic effect via autocrine and paracrine mechanisms, which negates the need for every islet cell to express the therapeutic gene.

In this study, Ad-IGF-II transduced rat islets were protected from apoptosis and cell death induced by pro-inflammatory cytokine exposure *in vitro* and enhanced islet survival *in vivo*. Western blot confirmed that IGF-II inhibits islet apoptosis via activation of the PI3K/Akt

intracellular signalling pathway. This result is supported by the well-documented ability of IGF-II to prevent apoptosis via activation of the IGF-1R (16, 17, 39).

Jourdan and colleagues (40) co-encapsulated islets with bio-engineered TM4 cells producing IGF-II and found *in vitro* islet survival and post-transplant outcomes to be improved. However, encapsulated islets are unable to revascularize following transplantation, exacerbating islet hypoxia and subsequent β-cell death (41, 42). Moreover, the bio-engineered TM4 cells permanently over express the IGF-II gene, raising concern as elevated and persistent concentrations of IGF-II have been associated with malignancy (43, 44).

Ilieva and colleagues (45) have shown that incubation of hamster islets with pancreatic duct conditioned medium containing 34 ng/ml IGF-II, successfully protects islets from apoptosis and necrosis that occurs following the islet isolation procedure. Furthermore, the supplementation of minimally nutritive (serum-free) medium with 500 ng/ml IGF-II improves *in vitro* islet viability and allows successful transplantation using a smaller number of microencapsulated rat islets (46). This beneficial effect is likely a result of the exogenous IGF-II improving islet survival following isolation and encapsulation, resulting in a larger mass of islets being transplanted in the IGF treatment group. Due to the limited biological half life of growth factors, is unlikely that any residual IGF-II transplanted with the encapsulated islets would have any significant effect *in vivo*.

Transplanted islets are vulnerable to stress induced apoptosis prior to engraftment and revascularization (26, 27), therefore a major advantage of the present approach is that islets are exposed to a constitutively produced supply of anti-apoptotic IGF-II prior to

transplantation and during the immediate post-transplant period. Our data confirms the ability of IGF-II to promote islet survival *in vitro* and this was achieved with a 40-fold lower IGF-II concentration than that previously used by Robitaille et al (46). These results suggest that there is enhanced anti-apoptotic function when IGF-II is over expressed within the local islet microenvironment.

Rat islets exhibit a highly controlled and robust insulin secretory response not present in mouse islets (47). For this reason, a minimal mass islet transplant of only 50 IEQ was sufficient to induce euglycemia in a minimal number of diabetic recipients. Although this study has shown a statistically significant improvement in transplant outcomes in Ad-IGF-II recipient mice, the small number of transplanted islets prevented post-transplant validation of the islet grafts. Despite this, these findings indicate that anti-apoptotic IGF-II decreases apoptosis *in vitro* and improves islet transplant outcomes *in vivo*.

One of the limitations of our anti-apoptotic strategy is that it targets only apoptosis as a cause of islet loss in the early post-transplant period. In addition to being protected against apoptotic insults, islets need to be able to resist alloimmunity, recurrent autoimmunity and effectively revascularize following transplantation. Multigene vectors that co-express two or three transgenes under the direction of a single promoter (48-52) provide a promising alternative to overcome this issue. In conclusion, IGF-II over expression in the early post-transplant period is a potentially effective anti-apoptotic strategy to improve islet survival and function following transplantation.

Materials and methods

Islet isolation and culture

Female Albino Wistar rats (250 – 300g) were obtained from the Animal Resources Centre (Perth, Australia) and were housed in an approved animal care facility (Institute of Medical and Veterinary Science, SA Pathology, Adelaide). Animal experiments were conducted in accordance with the animal ethics committees of the University of Adelaide and the IMVS guidelines. The pancreatic duct was cannulated and the pancreas was inflated with 1 x Hank's Balanced Salt Solution (Invitrogen, Grand Island, NY, USA). The pancreas was digested with collagenase X1 (Sigma-Aldrich, St. Louis, MO, USA). The islets were separated from the digested pancreas using a Histopaque-1077 (Invitrogen, Grand Island, NY, USA) density gradient. Purified islets were handpicked under a microscope and then cultured for 24 h in RPMI 1640 (Invitrogen, Grand Island, NY, USA) medium supplemented with 10% fetal bovine serum (FBS) (Gibbco, Grand Island, NY, USA) at 37°C and 5% CO_2. Islets were quantified by dithizone staining using an islet diameter of 150 μm as one IEQ.

Ad vectors and transduction of rat islets

Replication-deficient Ad vectors expressing GFP or human IGF-II were purchased from Welgen, Inc., Worcester, MA, USA. The gene(s) of interest were controlled by the constitutive human cytomegalovirus CMV promoter. The titers of Ad-GFP and Ad-IGF-II were both 2 x 10^{10} particle forming units (pfu)/ml. Islets (50 IEQ) were transduced with Ad vectors in serum-free RPMI 1640 (Invitrogen, Grand Island, NY, USA) for 4 h at 37°C. Following transduction islets were cultured for 48 h in complete RPMI 1640 (Invitrogen, Grand Island, NY, USA) containing 10% FBS (Gibbco, Grand Island, NY, USA) prior to

experimental assays. Untransduced islets (50 IEQ) underwent the same procedure but were not transduced with virus. MOI was calculated based on the assumption that one IEQ contains on average 2000 cells (53, 54).

Analysis of Ad transduction efficiency and islet viability following Ad transduction

GFP reporter gene expression after Ad-GFP transduction in islets was determined by flow cytometry. Viability of dissociated islets following transduction was assessed by staining with 7-Aminoactinomycin D (7-AAD) (Invitrogen, Grand Island, NY, USA) (5 μg/ml), and determined by flow cytometric analysis using a FACS CANTO II (Becton Dickson, San Jose, CA, USA).

Glucose Stimulated Insulin Secretion

To determine the effect of Ad transduction and/or IGF-II over expression on insulin secretion in response to glucose, a static glucose stimulation test was performed with rat islets. Ad-GFP, Ad-IGF-II or untransduced islets (50 IEQ) were incubated in either 1.2 ml of low (2.8 mM) or high (25 mM) glucose RPMI 1640 (Invitrogen, Grand Island, NY, USA) at 37°C for 2 h. Supernatant (1 ml) was collected for insulin analysis and stored at -20°C. Samples were diluted 1:10 and then insulin concentrations were measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). The glucose SI of islets was calculated by dividing the insulin response to high glucose incubation by the insulin response to low glucose incubation.

In vitro determination of IGF-II secretion from Ad-IGF-II transduced islets

Rat islets (50 IEQ) were divided into three groups comprising 1) untransduced islets, 2) Ad-GFP transduced islets and 3) Ad-IGF-II transduced islets. To measure IGF-II secretion, rat islet supernatant was collected each day for five days and samples were stored at -20°C. Samples were diluted 1:10 and then the concentration of IGF-II was analysed using a human IGF-II ELISA kit (Life Research Pty Ltd, Victoria, Australia).

Measuring islet cell death

To evaluate the effect of IGF-II expression on islet cell death, rat islets (50 IEQ) were untransduced, transduced with Ad-GFP, Ad-IGF-II or pre-treated for 2 h with 500 ng/ml rIGF-II, human (R&D, Minneapolis, MN, USA) and then treated with pro-inflammatory cytokines IL-1β (ProSpecbio, Ness-Ziona, Israel) (35ng/ml) and IFN-γ (Sigma-Aldrich, St. Louis, MO, USA) (40ng/ml) for 24 h. Islet cell death was assessed using flow cytometry. The islets were dispersed and stained using Annexin V (Invitrogen, Grand Island, NY, USA) (5 µl) for 15 min at RT. 2 µl Propidium Iodide (PI) (Invitrogen, Grand Island, NY, USA) at 250 µg/ml was then added to the cell suspension which was then analysed using a FACS CANTO II. Apoptosis was determined on islet cytospin preparations using an *In Situ* Cell Death Detection Kit as per manufacturer's instructions (Roche, Indianapolis, IN, USA). As a minimum, each sample was stained in triplicate for six different rat islet preparations and the images were produced using a Nikon C1-Z Confocal Microscope. The number of TUNEL-positive cells (red) within each islet were counted and compared with the total number of DAPI-positive nuclei (blue) within each islet to determine percent apoptosis.

Western blotting analysis

Rat islets (50 IEQ) were untransduced, transduced with Ad-GFP, Ad-IGF-II or pre-treated for 1 h with 200 mM wortmannin (Sigma-Aldrich, St. Louis, MO, USA) prior to Ad-IGF-II transduction for 48 h. Following transduction, all experimental groups were treated with pro-inflammatory cytokines IL-1β (35ng/ml) and IFN-γ (40ng/ml) for 24 h. Islets were harvested for western blotting analysis. The protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyl difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The blots were incubated with rabbit polyclonal pAkt (Ser473) antibody (1:1000) and rabbit polyclonal Akt antibody (1:1000). Blots were then incubated in anti-rabbit IgG, HRP-linked secondary antibody (1:2000). All antibodies were purchased from Cell Signalling Technology, Inc., Danvers, MA, USA. The bands were detected using a Novex® ECL Chemiluminescent Substrate Reagent Kit (Invitrogen, Grand Island, NY, USA).

Diabetes induction

Female NOD-SCID mice (9 – 12 weeks) were obtained from the Animal Resources Centre (Perth, Australia) and housed in a pathogen-free environment (IMVS, SA Pathology, Adelaide). Diabetes was induced in mice by a single intra peritoneal (i.p) injection of 170 – 180 mg/kg streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA), freshly dissolved in 0.1M citrate buffer (pH 4.5). Tail blood was taken from non-fasting animals and blood glucose concentrations were measured with an Optium Xceed^{TM} glucometer (Medisense, Victoria, Australia). Diabetes was confirmed when blood glucose levels were ≥16.6 mmol/l for two consecutive days (55, 56).

Marginal mass islet transplantation

Diabetic recipients received a marginal mass islet transplant (50 IEQ) of Ad-GFP (MOI 100), Ad-IGF-II (MOI 100) or untransduced islets under the left kidney subcapsular space. Reversal of diabetes was defined as blood glucose ≤11.2 mmol/l for two consecutive days (56).

Statistical analysis

Values were given as data ± standard error of the mean (SEM) and compared using one-way analysis of variance (1way ANOVA) followed by Bonferroni's multiple comparison test, with *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001, where appropriate (GraphPad Prism software 5, Inc, San Diego, CA). Densitometry of western blots was performed using ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). Log-rank (Mantel Cox) analysis was used in the minimal islet mass model (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP). For all comparisons, $P \leq 0.05$ was considered to be statistically significant.

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Figure. 1. Evaluation of Ad transduction on rat islets *In Vitro*. (A) Rat islets were transduced with Ad-GFP at MOI 10, 100, 200, 500 and 1000 or left untransduced (control). Flow cytometry was used to determine percent GFP expression in dispersed islet cells. Data are the mean ± SEM of three independent experiments. There was no significant difference in GFP expression amongst the groups transduced with Ad-GFP ($p=0.06$) (1way ANOVA). (B) Rat islets were transduced with Ad-GFP (MOI 100), Ad-IGF-II (MOI 100) or left untransduced (control). Flow cytometry 7-AAD staining was used to determine the viability in dispersed islet cells. Data are the mean ± SEM of three (Ad-IGF-II) or two (Ad-GFP) independent experiments, $p=0.322$ (1way ANOVA). (C) Rat islets were transduced with Ad-GFP (MOI 100), Ad-IGF-II (MOI 100) or untransduced (control) and then subject to GSIS. Insulin ELISA was used to determine the stimulation index in experimental samples. Data are the mean ± SEM of five independent experiments, $p=0.903$ (1way ANOVA).

Figure. 2. Time course of human IGF-II secretion from Ad-IGF-II transduced rat islets *in vitro*. Rat islets were transduced with Ad-GFP MOI 100, Ad-IGF-II MOI 100 or untransduced (control). Secreted IGF-II levels in the medium were measured using a human IGF-II ELISA. Error bars are the data ± SEM for three (days 3, 4 and 5) or nine (days 1 and 2) replicate experiments, ****$p<0.0001$ (1wayANOVA). Bonferroni Multiple Comparison Test: **$p<0.01$ Ad-GFP transduced islets vs. Ad-IGF-II Day 1 transduced islets, ****$p<0.0001$ Ad-GFP transduced islets vs. Ad-IGF-II day 2-5 transduced islets.

Figure. 3. Anti-apoptotic investigation of Ad-IGF-II transduced islets following pro-inflammatory cytokine exposure. Islets were left untreated or transduced at MOI 100 with Ad-GFP, Ad-IGF-II or untransduced 48 h before addition of IL-1β (35ng/ml) and IFN-γ (40

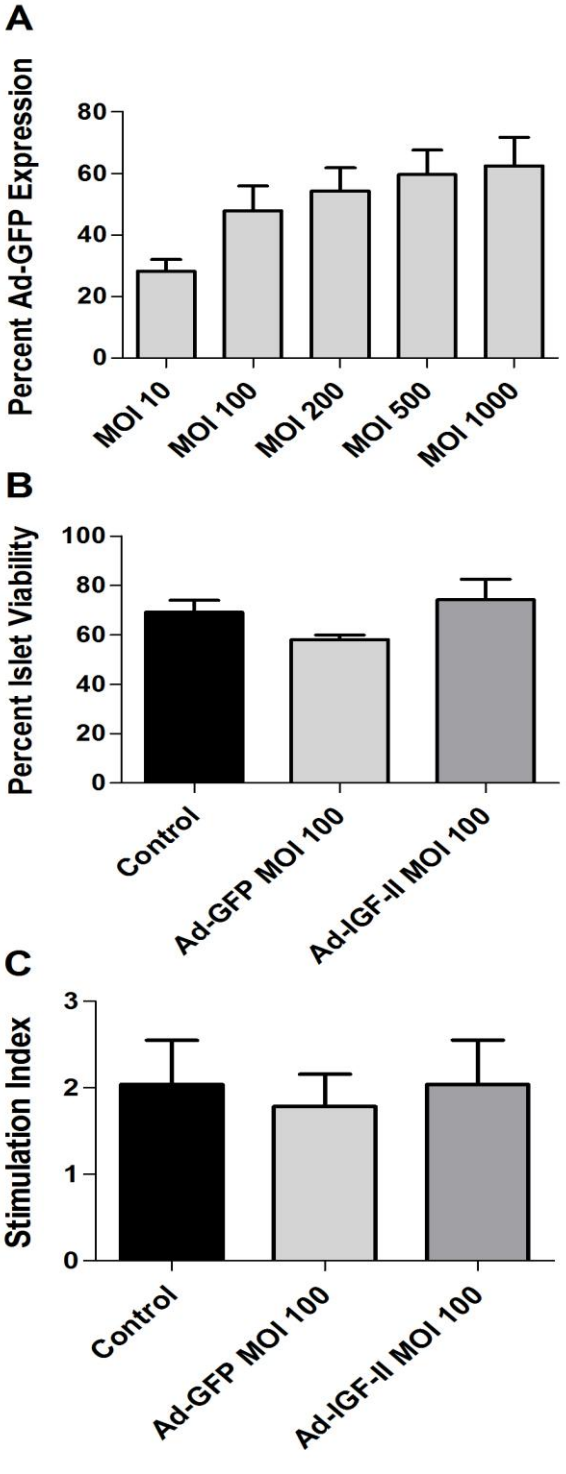
ng/ml) for an additional 24 h, or islets were pre-treated for 2 h with rIGF-II (500 ng/ml) before addition of pro-inflammatory cytokines. (A-D) TUNEL staining was used to determine islet cell apoptosis. Representative images of Untransduced, Ad-GFP and Ad-IGF-II TUNEL stained islets are depicted in A – C respectively. TUNEL positive cells (red) in the fluorescent images correspond to apoptotic cells. Blue = DAPI nuclear stain. Images were taken at 20 x magnification, Scale bar = 50μm. (D) A total of 25 untreated islets, 22 untransduced islets, and 32 Ad-GFP or Ad-IGF-II transduced islets from six different rat islet preparations were counted to quantify the number of apoptotic cells. The percentage of apoptotic islets was calculated by dividing the number of TUNEL positive islets by total islet cells. Data are mean ± SEM, ****P<0.0001 (1way ANOVA). Bonferroni Multiple Comparison Test: ****p<0.0001 Untreated islets vs. Untransduced islets, ****p<0.0001 Untreated islets vs. Ad-IGF-II transduced islets, ****p<0.0001 Untransduced vs. Ad-IGF-II transduced islets, ****p<0.0001 Ad-GFP transduced islets vs. Ad-IGF-II transduced islets. (E) Flow cytometry was used to determine percent islet cell death of dispersed islet cells using Annexin V and PI staining. The data is expressed as ± SEM of five independent experiments, ***p<0.0001 (1wayANOVA). Bonferroni Multiple Comparison Test: ***p<0.001 Untreated islets vs. Untransduced islets, ****p<0.0001 Untreated islets vs. Ad-GFP transduced islets, *p<0.05 Untransduced vs. Ad-IGF-II transduced islets, ***p<0.001 Ad-GFP transduced islets vs. 500 ng/ml rIGF-II islets, ***p<0.001 Ad-GFP transduced islets vs. Ad-IGF-II transduced islets.

Figure. 4. IGF-II signals through the PI3K/Akt pathway to inhibit apoptosis. Western blot of islets untreated, untransduced or transduced at MOI 100 with Ad-GFP or Ad-IGF-II for 48 h, before addition of IL-1β (35 ng/ml) and IFN-γ (40 ng/ml) for an additional 24 h. Islets were also treated with wortmannin (200 mM) for 1 h prior to Ad-IGF-II transduction

and cytokine treatment. Phospho-Akt (pAKT) levels were determined by densitometry (above pAKT blot) and corrected for total Akt levels relative to the untransduced islet control lane. Wortmannin treatment of Ad-IGF-II transduced islets reduced the phosphorylation of Akt, indicating that IGF-II activates the PI3K/Akt pathway to inhibit apoptosis.

Figure. 5. Return to euglycemia in STZ-induced diabetic recipients of a marginal mass islet graft. Diabetes was induced in immunocompromised NOD-SCID mice by STZ injection. Diabetic mice were transplanted with Ad-IGF-II (n=9), Ad-GFP (n=11) or untransduced (n=11) marginal mass islet grafts and followed for a maximum of 28 days. (A) Return to euglycemia post-transplantation was defined as blood glucose ≤11.2 mmol/l for two consecutive days and was achieved in 77.8% of recipients receiving Ad-IGF-II transduced islets (7/9) (solid line) compared with 18.2% (2/11) and 45.5% (5/11) of control animals receiving Ad-GFP (broken line) or untransduced islets (dotted line), respectively. $p<0.05$ (Log-rank (Mantel Cox) Test. Bonferroni Multiple Comparison Test: *$p<0.05$ Ad-GFP vs. Ad-IGF-II. (B) Mice were weighed prior to STZ injection (SW= starting weight) and then daily for 28 days following their transplant. Data are the mean ± SEM. ****$p<0.0001$ (1way ANOVA). Bonferroni Multiple Comparison Test: ****$p<0.0001$ Control vs. Ad-IGF-II, ****$p<0.0001$ Ad-GFP vs. Ad-IGF-II.

Fig. 1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20
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MOI 10
MOI 100
MOI 200
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MOI 1000
B
Percent Islet Viability
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80
60
40
20
0
Control
Ad-GFP MOI 100
Ad-IGF-II MOI 100
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1
0
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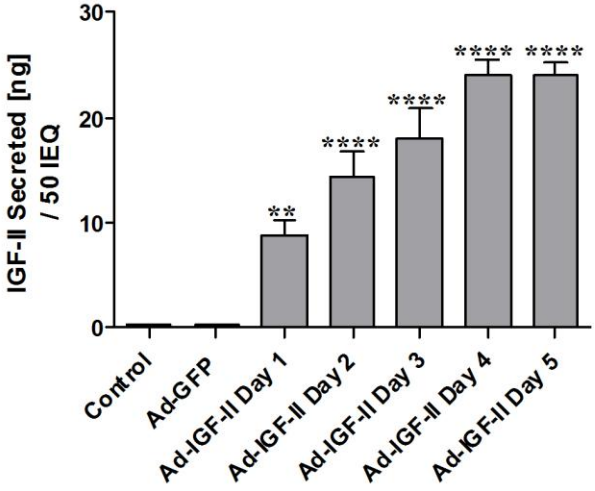
Ad-GFP MOI 100
Ad-IGF-II MOI 100

Fig. 2


IGF-II Secreted [ng] / 50 IEQ
30
20
10
0
**
****
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****
****
Control
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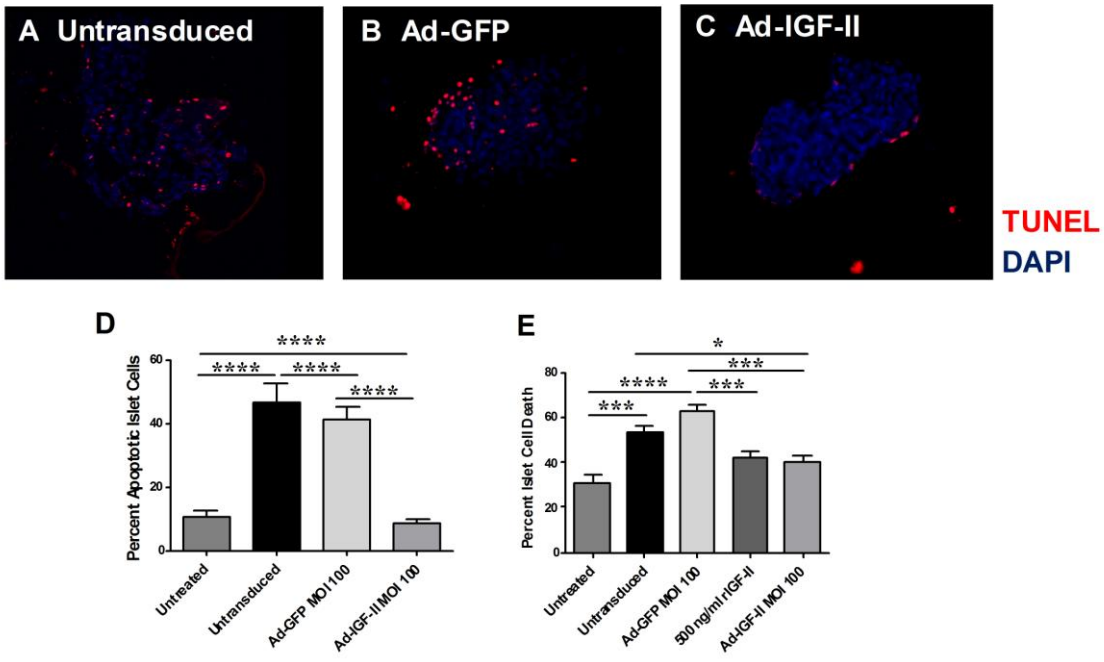
Ad-IGF-II Day 2
Ad-IGF-II Day 3
Ad-IGF-II Day 4
Ad-IGF-II Day 5

Fig. 3


A Untransduced
B Ad-GFP
C Ad-IGF-II
TUNEL
DAPI
D
Percent Apoptotic Islet Cells
60
40
20
0
****
****
****
****
Untreated
Untransduced
Ad-GFP MOI 100
Ad-IGF-II MOI 100
E
Percent Islet Cell Death
80
60
40
20
0
*
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Untreated
Untransduced
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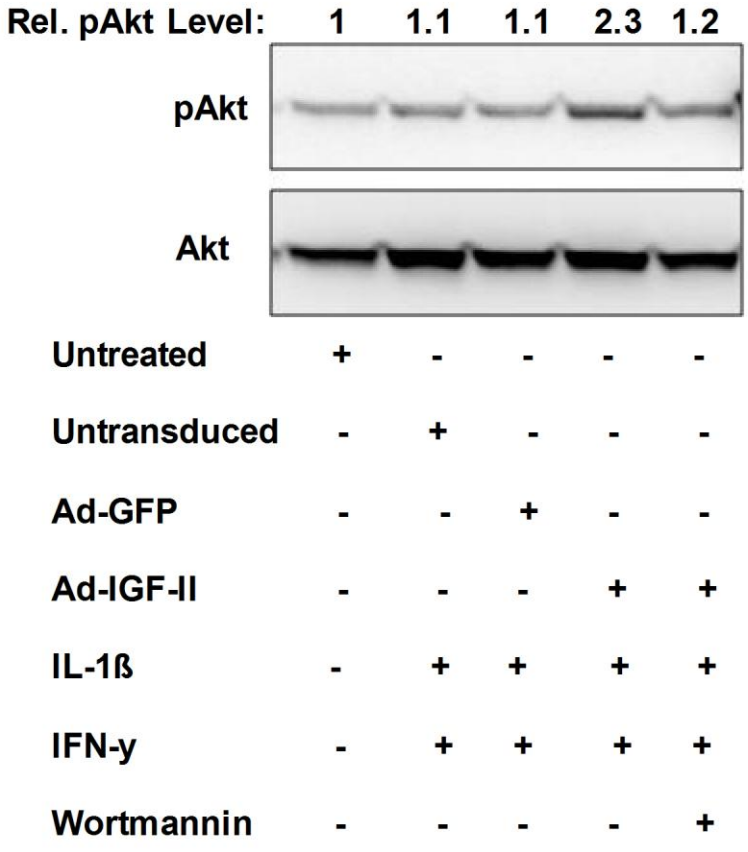
500 ng/ml rIGF-II
Ad-IGF-II MOI 100

Fig. 4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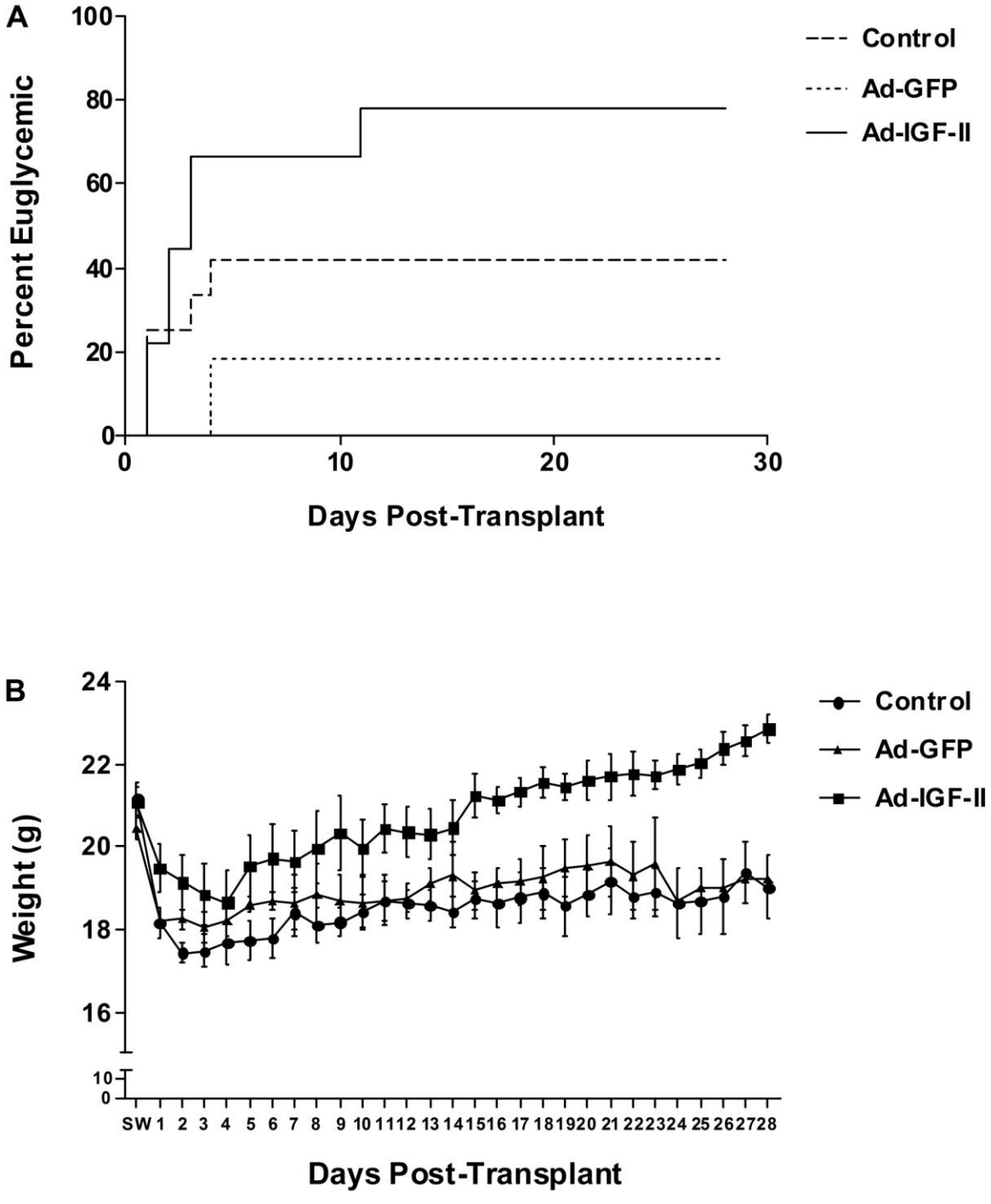
IL-1ß - + + + +
IFN-y - + + + +
Wortmannin - - - - +

Fig. 5


A
Percent Euglycemic
100
80
60
40
20
0
0
10
20
30
Days Post-Transplant
Control
Ad-GFP
Ad-IGF-II
B
Weight (g)
24
22
20
18
16
10
0
SW 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Days Post-Transplant
Control
Ad-GFP
Ad-IGF-II