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Normal relationship of beta and non-beta cells not needed for successful islet transplantation.

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Aileen J.F. King PhD [1,2,*], Justin R. Fernandes BA [1*], Jennifer Hollister-Lock BA [1], Cameron E. Nienaber BA [1], Susan Bonner-Weir PhD[1], Gordon C. Weir MD [1].

* These authors contributed equally.

Running title: Transplantation of purified beta cells.

1. Section on Islet Transplantation and Cell Biology, Research Division, Joslin Diabetes Center; and the Department of Medicine, Harvard Medical School, Boston, Massachusetts
2. Division of Reproduction and Endocrinology, Guy's Campus, King's College London, UK

Address for Correspondence:
Gordon C. Weir, M.D.,
Section on Islet Transplantation and Cell Biology
Joslin Diabetes Center,
One Joslin Place, Boston, MA 02215
E-mail: gordon.weir@joslin.harvard.edu.

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Abstract

Objective. Islets are comprised mostly of beta cells and therefore stem cell research has concentrated on generating purified beta cells, neglecting the other endocrine cell types in the islet. We investigated the presence of endocrine non-beta cells after islet transplantation. In addition, we studied whether the transplantation of pure beta cells, in volumes similar to that used in islet transplantation, would suffice to reverse hyperglycemia in diabetic mice.

Research Design and Methods. Rat islets were dispersed and beta cells were purified by FACS sorting according to their endogenous fluorescence. After reaggregation, 600 islet equivalents of the purified beta cell aggregates were implanted into diabetic SCID mice.

Results. In mice implanted with beta cell enriched aggregates, the hyperglycemia was reversed and good graft function over a 12 week period was observed with regard to glucose and insulin levels, glucose tolerance tests and graft insulin content. The endocrine cell composition of the beta cell enriched aggregates remained constant; prior to and 12 weeks after transplantation the beta cell enriched aggregates comprised of 95% beta cells and 5% endocrine non beta cells. However, islet grafts, despite originally having been comprised of 75% beta cells and 25% endocrine non-beta cells, comprised of just 5% endocrine non-beta cells after transplantation, indicating a loss of these cells.

Conclusion. Beta cell enriched aggregates can effectively reverse hyperglycemia in mice and transplanted intact islets are depleted in non-beta cells. It is therefore likely that islet non-beta cells are not essential for successful islet transplantation.

Islets of Langerhans consist of four main endocrine cell types; insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-producing delta cells and pancreatic polypeptide-producing PP cells. The most abundant cell is the beta cell, which makes up about 60-80% of islet cells in most mammalian species (1). The replacement of the beta cells by islet or pancreas transplantation is presently the only treatment of type 1 diabetes that can render the patient insulin independent (2, 3). However, the supply of donor pancreases is severely limited and the demand is far greater than the supply. To overcome this problem, much effort has been made in trying to derive beta cells from embryonic stem cells (4-7) and other putative adult stem cells, such as pancreatic duct cells (8) bone marrow cells (9) and acinar cells (10). This would potentially create an unlimited supply of beta cells for transplantation purposes. Moreover, it can be envisaged that the beta cells generated in vitro could be genetically manipulated to confer protection against damage after transplantation (11). It may be that stem cell technology will find a way to produce beta cells but not islet non-beta cells. Therefore, it is important to know whether a pure beta cell graft can effectively reverse hyperglycemia. The importance of the non-beta cells in the physiology of maintaining normoglycemia is well known (12). However, it is not clear that the non-beta cells of islets have a meaningful local influence on beta cell function. In fact, the microvasculature of the islet as well as functional studies indicate that non-beta cells are downstream from beta cell secretion suggesting that most beta cells see very little intraislet glucagon or somatostatin (13, 14). Also there are non-endocrine cell types present in the islets, including endothelial cells, which could potentially have an impact on islet transplantation outcome (15, 16). In order to obtain a purified population of beta cells, the islets had to be dispersed into single cells. Our initial study therefore investigated the effect of dispersion and re-aggregation of islet cells on the outcome of transplantation. We then studied the outcome of beta cell enriched transplants and investigated further non-beta cells in normal islet transplantation.

Research Design and Methods

Animals

Male Sprague Dawley rats were used as islet donors. Male ICR/SCID mice (Taconic, Germantown, NY) aged 6-10 weeks were used as recipients of grafts. Recipient mice were made diabetic with a single intraperitoneal injection of streptozocin (Sigma, St Louis, MO) 180 mg/kg body wt, freshly dissolved in citrate buffer (pH = 4.5). Only those mice with a blood glucose concentration greater than 350 mg/dl at 5 days were used as recipients. Blood glucose concentrations were determined using a glucose meter (Precision QID, Abbott Labs, Bedford, MA) with blood obtained from a snipped tail. All animal experiments were approved by the Joslin Animal Care Committee.

Islet Isolation and Culture

Rat islets were isolated by using a collagenase digestion followed by separation using a density gradient as previously described in detail (17). Briefly, under anaesthesia, a laparotomy was performed and the pancreas exposed. After ligation at the ampulla of Vater, 9ml of a collagenase solution (Liberase RI, Roche, Indianapolis, IN) was injected into the pancreas via the common bile duct. The pancreas was removed and incubated in a stationary water bath for approximately 25 min at 37 °C. The islets were separated by a density gradient (Histopaque-1077, Sigma) and centrifuged at 1750 g for 20 min. After washing, islets were handpicked and cultured overnight in RPMI 1640 + 10% foetal calf serum before islet transplantation or dispersion.

Dispersion

The rat islets were washed 2 times in PBS. As previously described (18), solution of 1mg/ml trypsin and 30 µg/ml DNAse was then added to the islets which were then incubated for 15 min in a 37 °C incubator. During the digestion, the islets were vortexed every 5 min for 10 seconds. Cold media with serum was then added to stop the digestion and the cells were washed two times. The cells were then counted and either sorted or aliquoted for either transplantation or re-aggregation.

Beta cell sorting

Prior to sorting, cells were filtered through a 35 µm mesh to exclude cell clumps. Cells were sorted using a DakoCytomation MoFlo Cytometer (Dako, Ft. Collins, CO), where cells were gated according to forward scatter (19, 20), and then sorted on the basis of endogenous fluorescence (21). Sorted cells were collected on ice and put into culture for re-aggregation as described below.

Re-aggregation

Cells were cultured in RPMI 1640 and 10% fetal calf serum for a period of 72h in Corning Ultra Low Attachment plates (Corning/Costar, Ithaca, NY). During this time the cells aggregated to islet-like clusters approximately 50-150 µm in size. These re-aggregates were counted and calculated as islet equivalents (IE) (22).

Transplantation

Cells were counted and aliquoted into groups of 1.2×10^6 or 2.5×10^6 cells. Islets or clusters were counted and aliquoted into groups of 600 islet equivalents. The cells, cell clusters or islets were then centrifuged in PE50 polyethylene tubing (Becton Dickenson, Sparks, MD) to form pellets. Male ICR/SCID mice were anesthetized using Avertin (Sigma) and placed on a heated plate for surgery. The left kidney was exposed through a lumbar incision and the kidney capsule was incised. Using a Hamilton syringe (Fisher, Pittsburg, PA) and the PE50 polyethylene tubing, rat islets, aggregates or dispersed cells were placed under the kidney capsule as previously described (23). The incision in the kidney capsule was then cauterized and the peritoneum was sutured. The skin was then closed using staples, and mice were allowed to recover under a warm lamp.

Graft function

The blood glucose and weights of the ICR/SCID recipient mice implanted with dispersed rat islet cells or reaggregated rat islet cells were monitored weekly for 12 weeks. Intraperitoneal glucose tolerance tests (IPGTTs) were carried out 12 weeks after transplantation in recipient mice and weight-matched controls to assess graft function. In the IPGTTs, 2g/kg glucose was injected into mice with a fasted blood glucose concentration of <200 mg/dl. Blood glucose concentrations were measured before the injection (time 0) and then at 15, 30, 60, 90 and 120 minutes. At the end of the experiment, a nephrectomy was then carried out to remove the graft.

In a second set of experiments, diabetic mice were implanted with either 600 IE of whole rat islets or 600 IE of rat beta cell enriched aggregates for 12 weeks. Blood glucose concentrations were measured, weights were monitored weekly and IPGTTs were carried out 4 and 12 weeks post transplantation in recipient mice and age-matched controls as described above. In addition, at 4 and 12 weeks post-transplantation, blood samples were collected at the 0, 30, 60 and 120 min time points during the IPGTT. Serum insulin was then quantified using ELISA. The grafts were removed by nephrectomy and some were used to measure graft insulin content while other grafts were processed for histology and their composition studied as described below. Furthermore, additional diabetic mice were implanted with 600 IE intact rat islets for a period of one or two weeks to study graft composition.

Quantification of beta cells versus non-beta cells

Cell aggregates and graft-bearing kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained for insulin and other islet endocrine cells (glucagon, somatostatin, pancreatic polypeptide). Briefly, sections were hydrated and donkey serum (1:50) was added for 30 min. An antibody cocktail was added which contained the following antibodies: rabbit anti glucagon (1:3000), rabbit anti somatostatin (1:200) and rabbit anti pancreatic polypeptide (1:200). Alternatively, some slides were incubated with the rabbit anti glucagon antibody alone (1:3000). The slides were stored at 4 ^{o}C in a humidity chamber overnight. After washing, donkey biotin anti-rabbit (1:400) was added for one hour before washing and the addition of Strept-avidin Alexofluor (1:400) for an additional hour. The slides were then washed and guinea-pig anti bovine insulin (1:200) was added to the sections for one hour before washing and a Texas red conjugated anti guinea-pig antibody (1:200) was added for one hour. Slides were mounted using an antifade mounting media containing DAPI (1µg/ml). Complete grafts were photographed with the confocal mode of a LSM410 microscope. The number of insulin positive cells (red) and non-beta endocrine cells (green) was quantified on each image of each graft by independent counting by two individuals who were blinded to the origin of the slide. On average 1200 cells were counted in each preparation. Eight of each (islet and beta cell-enriched) grafts were analyzed. In addition, the percentage

of insulin positive cells that were further than 50 μm from any non-beta endocrine cells was calculated. This was done to calculate how many beta cells were so far from any endocrine non-beta cell that paracrine interactions were unlikely. To do this, circles with a radius of 50 μm were drawn around each non-beta endocrine cell (green) and the number of insulin positive cells (red) not falling within any circle was counted and expressed as a percentage of total insulin positive cells.

Statistics

The Student's t-test was used to compare two groups. When the data was not normally distributed, or did not have equal variance, Mann Whitney rank sum test was used. When more than two groups were compared, an analysis of variance (ANOVA) was carried out, with a Bonferroni post hoc test. In cases where the data was not normally distributed or did not have equal variance, an ANOVA on ranks was carried out. The statistical test used is specified for each data set. All analysis was carried out using Sigmastat (Systat Software, Point Richmond, CA).

Results

Transplantation of non-sorted dispersed and reaggregated islet cells

Transplantation of reaggregated non-purified islet cells (600 islet equivalents, which contain 0.9-1.2x10^6cells) produced better outcomes than 1.2 x 10^6 dispersed islet cells, showing the benefit of reaggregation (Figure 1a). However, a larger number of transplanted non-purified dispersed islet cells, 2.5x10^6 had the same success as the aggregates, which had half the number of cells. In terms of how many animals in each group had cured, at 84 days 100 % of mice transplanted with 2.5 x 10^6 cells (n=8) or 600 islet equivalent reaggregated clusters (n=5) had cured, compared to just 33% of mice transplanted with 1.2 x 10^6 cells (n=6). Twelve weeks after transplantation, an intraperitoneal glucose tolerance was carried out, using only the animals that had non-fasted blood glucose levels of less than 200mg/dl (Figure 1b). Due to the small number of animals that had a non-fasting blood glucose of less than 200mg/dl in the group transplanted with 1.2 x 10^6 cells (n=2), this group was excluded from the statistical analysis. At time point 0 (i.e. before glucose injection), mice transplanted with 2.5 x 10^6 cells had significantly lower blood glucose than weight matched non-diabetic non-transplanted control mice (ANOVA with Bonferroni post hoc test, $p<0.05$, n=4-5). In addition, at 60 min after glucose injection, the mice transplanted with 2.5 x 10^6 cells had lower blood glucose levels than mice transplanted with islet cell aggregates (ANOVA with Bonferroni post hoc test, $p<0.05$, n=5). Of the two cured mice which had received 1.2 x 10^6 cells, one was glucose intolerant (blood glucose 60 min after glucose injection: 296 mg/dl) and the other had quite low blood glucose levels 60 min after injection (67 mg/dl), which rose to 76 mg/dl at the two hour time-point.

Transplantation of beta cell enriched aggregates

No difference was seen in the non-fasted blood glucose of recipients of intact islets (600 islet equivalents) and recipients of beta cell enriched aggregates (600 islet equivalents) (Figure 2). During an intraperitoneal glucose tolerance test four weeks after transplantation, blood glucose levels were lower at 60 and 90 minutes after injection in mice transplanted with beta cell enriched aggregates than in mice transplanted with islets (Figure 3a, $p<0.05$, ANOVA with Bonferroni post hoc test, n=13-14). Age-matched non-transplanted controls had higher blood glucose levels 90 and 120 minutes after glucose injection compared with islet recipients. Moreover, age-matched controls had higher fasting blood glucose levels (87.0±5.3 mg/dl) than mice that had received an islet transplant ($p<0.05$, ANOVA with Bonferroni post hoc test, n=6). There was no difference in fasting blood glucose levels in mice that had received an islet transplant versus an enriched beta cell aggregate transplant (68.7±3.7 mg/dl vs 64.5±4.9 mg/dl respectively). At the 4-week IPGTT, the weights of the mice which had a received an enriched beta cell aggregates transplant were similar to those which had received islets (29.9±0.83 g vs 30.2±0.85 g). However the age-matched controls weighed on average 35.4± 1.6 g. When glucose tolerance was tested twelve weeks after transplantation, there were no differences between mice implanted with islets or beta cell enriched cell aggregates (Figure 3b). Age-matched controls had higher blood glucose levels than islet transplant recipients 60, 90 and 120 minutes after transplantation ($p<0.05$, ANOVA with Bonferroni post hoc test, n=8-14). The weight of the age-matched controls at this time-point were 39.9±1.8 g compared with 30.3±0.9 g and 32.8±1.2 g in mice which had received enriched beta cell aggregate grafts and islet grafts, respectively.

Four weeks after transplantation, there were no significant differences in serum insulin levels in mice implanted with islets or beta cell enriched aggregates prior to and after a glucose tolerance test. However, sixty minutes after glucose injection, age-matched control mice had higher serum insulin levels than in the mice transplanted with islets (Table 1. $p<0.05$, ANOVA with Bonferroni post hoc test, n=6). Twelve weeks after transplantation, serum insulin levels after an intraperitoneal glucose tolerance test did not differ among any of the groups ($p>0.05$, ANOVA, n=6).

Twelve weeks after transplantation the mice were nephrectomized, after which all animals reverted to hyperglycemia. In some mice, graft composition was quantified (see below). In others, the insulin content of the graft was measured. No differences could be detected in graft insulin content when comparing islet grafts with beta cell enriched aggregate grafts (5.7±1.7 μg in islet grafts vs 3.4±1.0 μg in beta cell aggregates grafts. p=0.28, t-test; n=5).

Graft composition

As shown in Table 1, the beta cell enriched aggregates contained a significantly higher percentage of beta cells than islets prior to transplantation

(Table 2, Figure 4 a,b). However, twelve weeks after transplantation, the islets contained a similar percentage of beta cells as the islet grafts (Table 2, Figure 4 c,d), with ranges between 92.7%-97.4% beta cells in the islet grafts and 93.2%-98.5% beta cells in the beta cell enriched aggregate grafts. The decrease in percent of non-beta cells in islet grafts compared with islets prior to transplantation was highly significant (t-test, $p < 0.001$). Similar results were seen when a glucagon antibody alone was used rather than the cocktail of glucagon, somatostatin and PP antibodies, with the percentage of glucagon positive cells falling from 26.1% to 4.4% after transplantation. In addition, in the grafts, the majority of beta cells were further than 50 μm from any non-beta cell (Table 2, Figure 5). In one and two week-old grafts, the percentage of endocrine cells that were beta cells were 93.7±0.7% and 98.0±0.6%, respectively (average cells counted per graft: 931±102, n=4 in each group).

Discussion

This study provides evidence that beta cells can function well in a graft site without nearby non-beta cells. To study beta cell enriched grafts, islets had to be dispersed into single cells to allow fluorescence activated cell sorting. Our initial studies therefore investigated the effect of islet dispersion and reaggregation on transplantation outcome. When the dispersed cells were reaggregated prior to transplantation, fewer cells were needed to reverse hyperglycemia. After intraperitoneal glucose tolerance tests, reaggregated islets had a similar glucose profile to weight-matched non-diabetic, non-transplanted control mice. However, mice transplanted with 2.5 million dispersed islet cells had even lower blood glucose levels after the glucose tolerance test. This finding indicates that transplantation of dispersed islet cells can be efficient, providing sufficient numbers are transplanted. However, if islet cells are reaggregated prior to transplantation, half the number of cells can restore normoglycemia. These results are in line with several previous studies that have shown that insulin secretory responses are decreased in dispersed islet cells and are improved by reaggregation of the cells (24-27). This superior result may be due a positive influence of cell communication upon insulin secretion and raises questions about whether aggregated cells are more resistant to apoptosis. It is likely that the dispersed cells formed clusters in vivo after transplantation but this may occur after many cells are lost in the first days after transplantation. In summary, it is evident that larger numbers of dispersed cells are initially required to reverse hyperglycemia.

In our next study, dispersed islet cells were enriched for beta cells by use of fluorescent activated cell sorting. Beta cells were sorted according to their endogenous fluorescence and forward scatter (size), as has been previously described (19-21). Using this technique, we were able to obtain cell populations of about 95% beta cells. These beta cell enriched fractions were then reaggregated prior to transplantation. Mice that had been transplanted with beta cell enriched

aggregates, had a rapid reversal of hyperglycemia in a similar manner to mice transplanted with a similar volume of whole islets. It should be noted that the number of beta cells would have been lower in the islet transplants compared with the beta cell transplants. It is likely that the beta cell transplants, with 95% beta cell purity contained approximately 20% more beta cells than in the islets. However, the aim of the study was to establish whether purified beta cells, transplanted in volumes similar to those used in islet transplantation, could reverse diabetes, to establish whether beta cells derived from stem cells could ever be useful for transplantation of diabetes patients. In glucose tolerance tests 4 weeks after transplantation, mice transplanted with beta cell-enriched aggregates had slightly lower blood glucose levels and tended to have slightly higher insulin levels than those transplanted with islets. However, by 12 weeks there was no difference between these groups with regard to glucose tolerance, insulin levels or graft insulin content. In this experimental series, the non-diabetic, non-transplanted control mice were age-matched rather than weight matched. Whereas the weights of the transplanted mice had remained stable or dropped during their period of diabetes, the control mice continued to gain weight throughout the study. Their poor glucose tolerance and higher insulin levels suggest these control mice had become insulin resistant. In addition, the lower glucose levels of the transplanted mice may be explained by the fact that rat islets were implanted, as rats typically have lower glucose levels than mice and may have a lower set-point for glucose-stimulated insulin secretion.

These results show that transplantation outcome of beta cell enriched aggregates is similar to that of islets. Transplantation of a purified population of beta cells has been previously shown by Keymeulen *et al* to be successful (28), although it was suggested in this study that mixing non-beta endocrine cells with beta cells prior to transplantation improved graft function. In the Keymeulen study, the grafts were studied for 64 weeks rather than 12 weeks in this study. Their rat beta cell grafts and mixed grafts of rat beta cells + rat endocrine non-beta cells functioned well in rats until about 20 weeks when some of the grafts started to fail. By 64 weeks, all grafts, regardless of whether they contained purified beta cells or beta cells mixed with other endocrine non-beta cells, had partially or fully failed. However, they implanted about 1.2-1.7 million beta cells into rats, which could be regarded as a minimal mass model. This thorough study also showed that beta cells from older rat donors do not function as well as beta cells from younger rat donors. Thus, the Keymeulen study complements the findings of the present study in that both show impressive function of grafts consisting of purified beta cells. Another cell type within islets that could potentially contribute to the outcome of transplantation is the endothelial cell, which has been reported to contribute to the revascularization of transplanted islets (15, 16). However, it is not clear there are enough of these cells to make a meaningful contribution. Nonetheless, it is evident from the current studies that although endocrine non-beta cells

and donor endothelium may be beneficial to the function of transplanted islet grafts, they do not seem to be essential for successful transplantation. This finding indicates that beta cells derived in vitro from stem cells or some other source could indeed be very useful for transplantation purposes.

When graft composition was studied 12 weeks after transplantation, the beta cell-enriched aggregates had virtually the same 5% of non-beta cells in the grafts as was in the initial preparations. While this was not surprising, it was remarkable to find that islets that initially had 25% non-beta cells, had only 5% within the grafts. The vast majority of these cells are glucagon containing alpha cells. If the beta cell enriched grafts had lost non-beta cells to the same degree as the islet grafts, one would have expected them to have only one percent of non-beta cells after transplantation. It is also intriguing that the loss of non-beta cells in the islet grafts seemed to stop at 5%, which was the same percentage of non-beta cells found in the beta cell enriched grafts. In agreement with these findings, we also found that when immature neonatal porcine pancreatic cell clusters were transplanted into nude mice, the grafts many months later were about 95% beta cells (29). This phenomenon of the present experiments with rat islets might be explained by preferential death of the non-beta cells as described before (30-33). These studies and our own conclude that the decrease is mainly accounted for by glucagon producing cells (30-33). We do not know whether delta and PP cells also disappear. The peripheral localization of alpha cells in rodent islets could potentially make them more vulnerable to damage during the isolation. Although we have not studied this in detail in the present study, previous studies have indicated that this is not the major reason for alpha cell loss after transplantation. In a study by Lau et al, islets implanted in the liver had lost alpha cells at 4 weeks after transplantation whereas islets implanted in kidney from the same isolation had not, indicating that isolation related events had not caused the loss of these peripheral cells (33). In that study, islets implanted intraportally showed loss of alpha cells whereas islets implanted under the kidney capsule showed no loss of alpha cells. In the present study, we saw loss of non-beta cells in islets under the kidney capsule one week after implantation. One difference between their study and this study is that they implanted islets into normoglycemic rats whereas in the present study the recipients were hyperglycemic mice. It is possible that differences in metabolic load on the islet graft may affect the alpha cell survival. It cannot be ruled out that alpha cell loss is more rapid in the liver site, as Lau et al reported. Indeed, Gunther et al reported the loss of alpha cells as early as 2 days after implantation into the liver of diabetic rats (31). Since normal levels of glucagon and other non-beta cell derived peptides are still produced by the endogenous pancreas, it is possible that the non-beta cells in the grafts disappear due to involution to prevent hyperglucagonemia.

The hypothetical question, which may one day be of practical importance, is whether a graft of pure

beta cells would provide an adequate transplant result. In terms of whole body metabolic effects of glucagon secretion from grafts, it appears that secretion from grafts provides minimal protection against acute hypoglycemia (34), yet human islet transplants can maintain near normal glucose levels. The persistent glucagon secretion from the pancreas may make some contribution to glucose homeostasis, but, again, little protection against acute hypoglycemia. These deficient glucagon responses could make recipients more prone the hypoglycemia during exercise (35, 36), but this is unlikely to outweigh the benefits. The next question is whether there is some important local influence of islet non-beta cells on beta cell function. Considering the islet microvascular anatomy and the physiological experiments showing that non-beta cells are downstream of beta cells (13, 14), it seems likely that most beta cells are not influenced by non-beta cells. Some insight may be provided by our measurements that determined how far beta cells were away from non-beta cells. Indeed, when we quantified the number of beta cells that were in close proximity to a non-beta cell, it was found that 65-75% of beta cells were 50 μm from a non-beta cell. It seems unlikely that local secretion from non-beta cells could have much paracrine influence at that distance. Moreover, from studies of others (37, 38), we know that the microvasculature of transplanted islets is very different from normal. Thus, the anatomy of islet grafts is very different from islets in the pancreas, yet these grafts are still efficacious in reversing hyperglycemia in diabetic recipients.

In conclusion, we found that beta cell enriched aggregates can effectively reverse hyperglycemia in mice and that transplanted intact islets are depleted in non-beta cells. It is therefore likely that islet non-beta cells are not essential for successful islet transplantation.

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Legends to the Tables

Table 1. Insulin serum levels (ng/ml) in ICR/SCID mice after an IPGTT (2g/kg) 4 and 12 weeks after transplantation of rat islets or beta-cell enriched aggregates. *=$p<0.05$ vs age-matched control mice (ANOVA with Bonferonni post hoc test, $n=6$).

Table 2. Percent of beta cells in rat islet and beta-cell enriched grafts prior to and 12 weeks after transplantation. The percentage of beta cells further than 50 μm from any endocrine non-beta cell is also shown.

Table 1. Serum insulin levels (ng/ml) in mice after intraperitoneal injection of 2g/kg glucose.

Time after glucose injection (minutes)	**0**		**30**		**60**	
	4 weeks	12 weeks	4 weeks	12 weeks	4 weeks	12 weeks
Non-transplanted, non-diabetic control mice	0.69±0.19	0.61±0.11	0.65±0.11	0.51±0.07	0.96±0.11	0.72±0.10
Islet recipients	0.39±0.06	0.35±0.11	0.38±0.09*	0.40±0.08	0.53±0.11	0.48±0.10
Beta cell aggregates recipients	0.48±0.06	0.34±0.03	0.63±0.07	0.42±0.07	0.72±0.06	0.42±0.07

Table 2. Composition of islets and beta cell aggregates pre and post transplantation.

Graft characteristics	Islets Pre-transplantation (n=7)	Islets Post-transplantation 12 weeks (n=8)	Beta cell aggregates Pre-transplantation (n=4)	Beta cell aggregates Post-transplantation 12 weeks (n=8)
Percent beta cells	**75.3±1.5**	**95.1±0.6**	**96.7±1.0**	**94.3±0.8**
Percent beta cells > 50 μm from an endocrine non-beta cell	**23.4±6.2**	**70.3±1.8**	***63.8±2.1***	**67.2±5.1**

Legends to the Figures

Figure 1. Blood glucose after transplantation 1.2 x10^6 dispersed rat islet cells (filled circles) or 2.5 x10^6 dispersed rat islet cells (open circles) or 600 re-aggregated rat islet equivalents (approximately 0.9-1.2x10^6 cells (filled triangles) (Figure 1a). Intraperitoneal glucose tolerance test (2g/kg bw) 12 weeks after transplantation in cured mice transplanted with 2.5 x10^6 dispersed rat islet cells (open circles) or 600 re-aggregated rat islet equivalents (filled triangles). Mice transplanted with 1.2 x10^6 dispersed rat islet cells are excluded from the graph due to too few mice curing. Controls are weight matched non-transplanted, non-diabetic control mice (filled circles, broken line) (Figure 1b) *=$p<0.05$ vs non diabetic non-transplanted controls at time 0 and 600 re-aggregated islet equivalents at time 60 minutes (ANOVA with Bonferroni post hoc test, n=4-5).

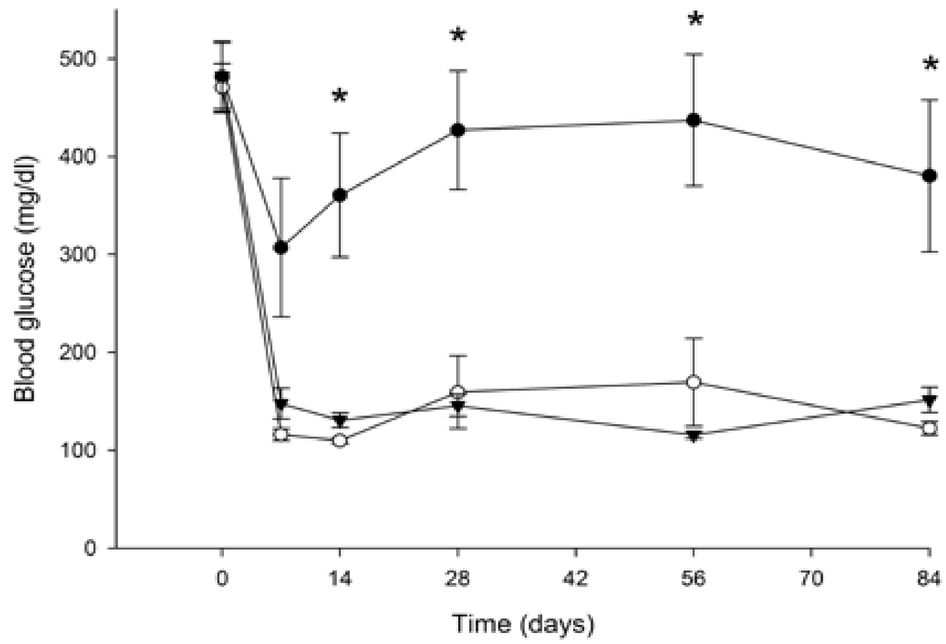
Figure 2. Blood glucose diabetic ICR/SCID mice after transplantation of 600 intact rat islet equivalents (filled circle, n=14) or 600 rat beta cell enriched reaggregates (open circle, n=13).

Figure 3. Blood glucose after an intraperitoneal glucose tolerance test (2g/kg bw) in mouse recipients of 600 intact rat beta cell enriched reaggregates (open circle), 600 rat islet equivalents (filled circle) 4 weeks (Figure 3a) or 12 weeks (Figure 3b) after transplantation or age matched controls (broken line, filled triangle). *=$p<0.05$ vs mice receiving islets. ANOVA with Bonferroni post hoc test.

Figure 4. Representative section of rat islets (panel a) and beta cell-enriched aggregates (panel b) prior to transplantation and twelve weeks after transplantation (panel c and d, respectively). Insulin is stained red and glucagon, somatostatin and PP are stained green.

Figure 5. To calculate how many beta cells were further than 50 μm from any endocrine non-beta cell, circles with a radius of 50 μm were placed around each endocrine non-beta cell (stained green for glucagon, somatostatin and PP). The number of insulin positive cells (stained red) which were outwith any circle was then expressed as a percentage of total beta cells.

Figure 1



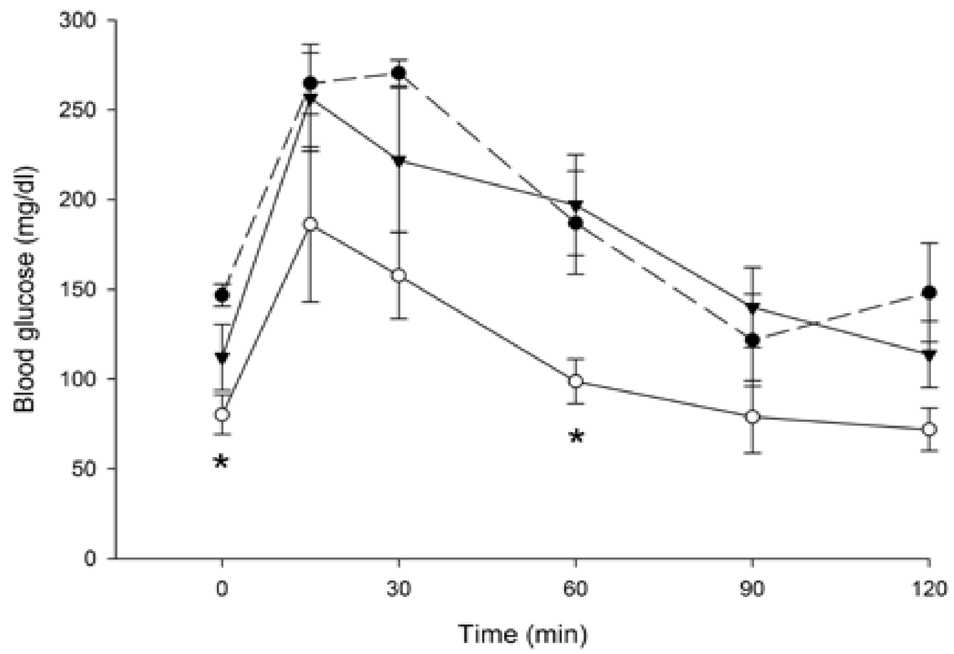


Figure 2

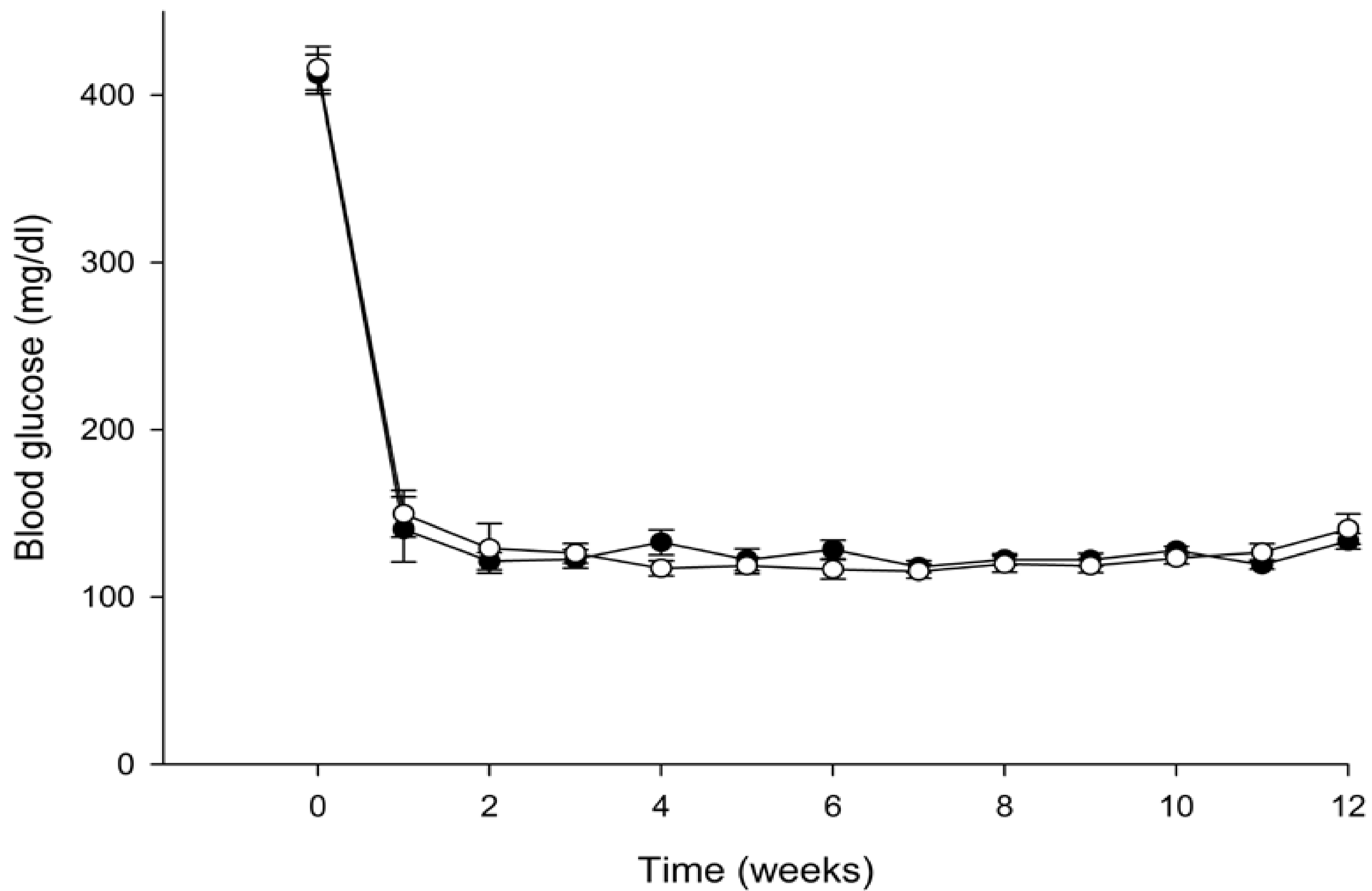
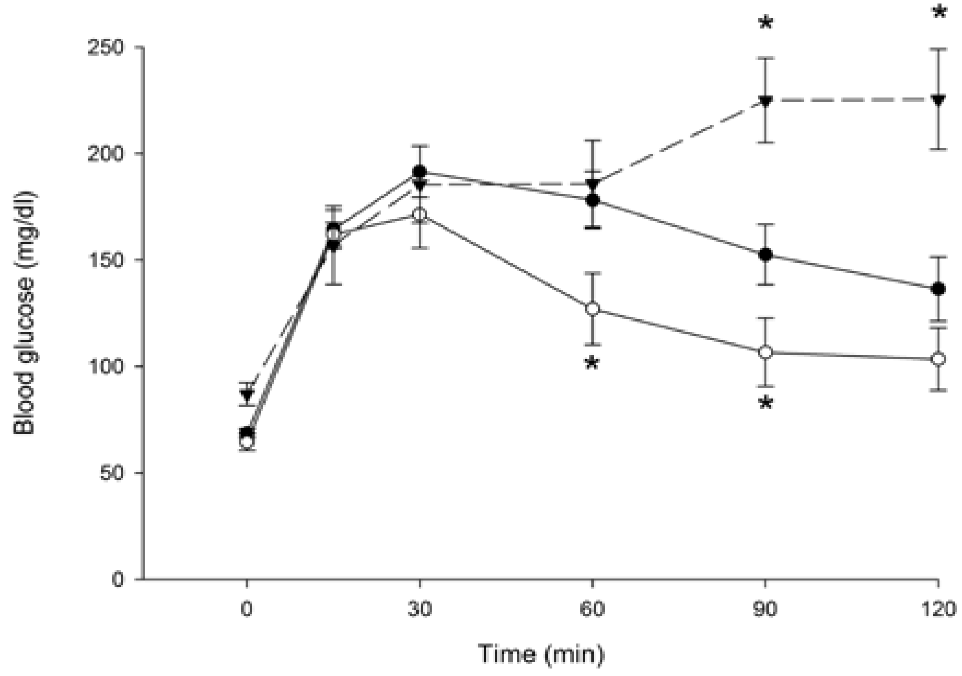


Figure 3



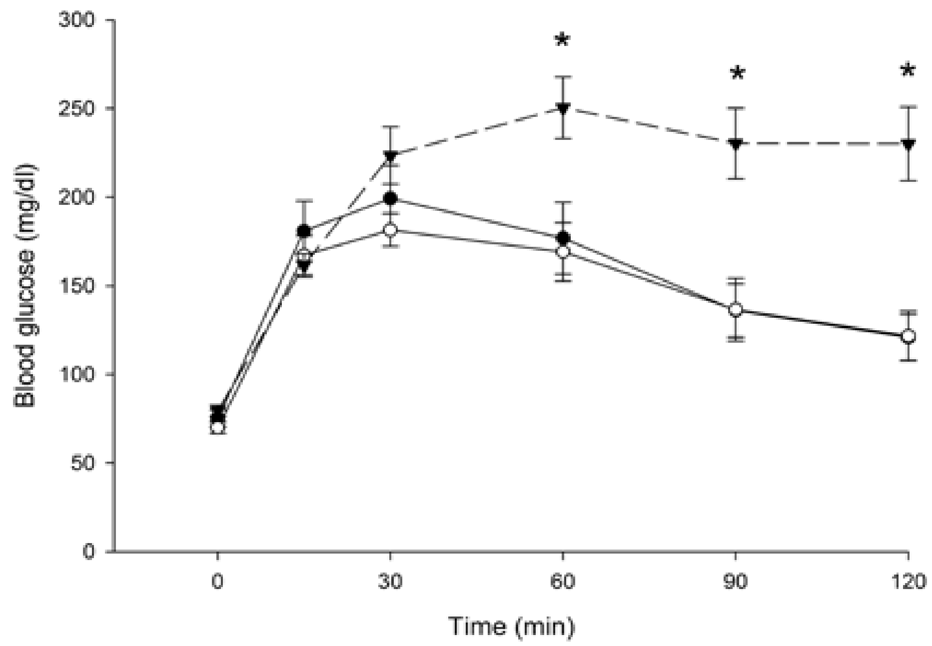


Figure 4

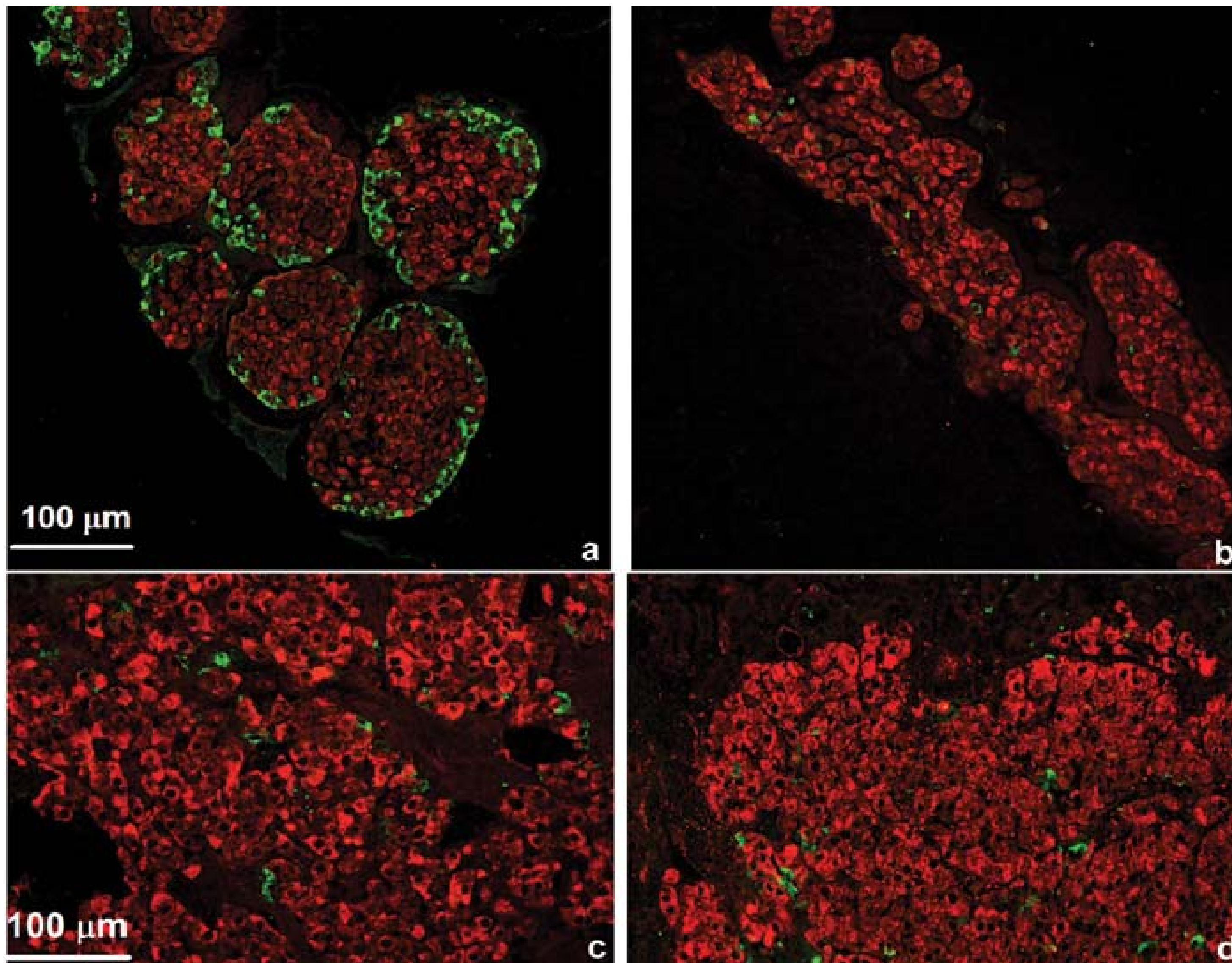


Figure 5