

# Fewer Islets Survive from a First Transplant than a Second Transplant: Evaluation of Repeated Intraportal Islet Transplantation in Mice

Cell Transplantation  
2019, Vol. 28(11) 1455–1460  
© The Author(s) 2019  
Article reuse guidelines:  
sagepub.com/journals-permissions  
DOI: 10.1177/0963689719866685  
journals.sagepub.com/home/cll  


Hanna Liljebäck<sup>1,2</sup>, My Quach<sup>1</sup>, Per-Ola Carlsson<sup>1,2</sup> ,  
and Joey Lau<sup>1</sup>

## Abstract

Beta cell replacement is an exciting field where new beta cell sources and alternative sites are widely explored. The liver has been the implantation site of choice in the clinic since the advent of islet transplantation. However, in most cases, repeated islet transplantation is needed to achieve normoglycemia in diabetic recipients. This study aimed to investigate whether there are differences in islet survival and engraftment between a first and a second transplantation, performed 1 week apart, to the liver. C57BL/6 mice were accordingly transplanted twice with an initial infusion of syngeneic islets expressing green fluorescent protein (GFP). The second islet transplant was performed 1 week later and consisted of islets isolated from non-GFP C57BL/6-mice. Animals were sacrificed either 1 day or 1 month after the second transplantation. A control group received a saline infusion instead of GFP-expressing islets, 1 week later obtained a standard non-GFP islet transplant, and was subsequently sacrificed 1 month later. Islet engraftment in the liver was assessed by immunohistochemistry and serum was analyzed for angiogenic factors induced by the first islet transplantation. Almost 70% of islets found in the liver following repeated islet transplantation originated from the second transplantation. The vascular density in the transplanted non-GFP-expressing islets did not differ depending on whether their transplantation was preceded by a primary islet transplantation or saline administration only nor did angiogenic factors in serum prior to the transplantation of non-GFP islets differ between animals that had received a previous islet transplantation or a saline infusion. We conclude that first islet transplantation creates, by unknown mechanisms, favorable conditions for the survival of a second transplant to the liver.

## Keywords

islet transplantation, type 1 diabetes, engraftment, GFP

## Introduction

Islet transplantation is an available treatment for a selected group of type 1 diabetes patients with the most severe glycemic lability. The liver has been the site of choice from the start of clinical islet transplantation<sup>1,2</sup>. Since Shapiro et al. introduced the Edmonton protocol<sup>3</sup>, the outcome of islet transplantation has steadily improved owing to progress in the islet isolation techniques and refined immunosuppressive regimes<sup>2</sup>. Currently, insulin independent success rate in allogeneic islet transplantation is reported in up to 50% of patients after 5 years<sup>2,4</sup>. Although some patients revert to exogenous insulin therapy, a majority achieve freedom of severe hypoglycemic events 1 year after islet transplantation and also report improved quality of life<sup>5</sup>. However, repeated islet transplantation is often required to achieve insulin

independence in the clinical setting. Islet survival is influenced by several factors, and especially when transplanting to the liver, many islets are lost due to an instant blood-mediated inflammatory reaction when islets come into contact with the blood<sup>6,7</sup>. Moreover, transplanted islet mass is a well-known predictor for transplantation outcome<sup>8,9</sup>.

<sup>1</sup>Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden

<sup>2</sup>Department of Medical Sciences, Uppsala University, Uppsala, Sweden

Submitted: June 3, 2019. Accepted: June 28, 2019.

### Corresponding Author:

Hanna Liljebäck, Department of Medical Cell Biology, Uppsala University, Box 571, Uppsala SE 75123, Sweden.  
Email: hanna.liljeback@mcb.uu.se



At transplantation, the pancreatic islets are infused into the portal vein and embolize the liver where portal vein tributaries become too narrow to allow their passage. Transient transaminase increases, as well as acute innate inflammatory reactions, are commonly seen after islet transplantation, which may cause regenerative changes in the liver<sup>10</sup>. Lodging of islets from a primary transplantation may influence the potential distribution of islets in an additional transplantation. The present study tested the hypothesis that changes in the liver milieu may affect the engraftment of a second islet transplantation.

## Materials and Methods

### Animals

C57BL/6 male mice (Taconic M&B, Ejby, Denmark) were used as donors and recipients for islet transplantation. Some of the C57BL/6 mice used for islet isolation expressed green fluorescent protein (GFP) under the control of the mouse insulin I gene promoter. All experimental procedures were approved by the local animal ethics committee of Uppsala University.

### Islet Isolation and Culture

Islet isolation was performed as previously described<sup>11</sup>. Briefly, mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (200 mg/kg; Apoteket, Stockholm, Sweden). Collagenase A from *Clostridium histolyticum* (2.5 mg/mL; Roche Diagnostics, Mannheim, Germany) was injected via the common bile duct. The pancreas was inflated and surgically removed and islet separated from exocrine tissue by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). Islets were cultured 1–2 days before transplantation. Culture medium consisted of RPMI 1640 supplemented with L-glutamine (2 mmol/L; Sigma-Aldrich), FCS (10% vol/vol; Sigma-Aldrich) and penicillin streptomycin (100 U/mL and 0.1 mg/mL, respectively; Roche, Sigma-Aldrich).

### Islet Transplantations

Most animals received two islet transplantations 1 week apart, while mice in the control group received a sham transplantation with saline infusion followed by a single transplantation 1 week later. The first islet transplant consisted of 125 syngeneic GFP-expressing islets, while the second transplant consisted of 125 syngeneic non-GFP islets of similar size (50–100  $\mu\text{m}$ ). Animals were anesthetized by an intraperitoneal injection of Avertin (0.02 mL/g body weight; Kemila, Stockholm, Sweden) or anesthetized under spontaneous inhalation of isoflurane (Baxter Medical, Kista, Sweden). A mid-line incision in the skin and the abdominal muscle was made and the portal or appendicular vein used for islet injection through a butterfly needle (25G). Different parts of the portal system were used to avoid scar tissue. The total volume of

infusion was  $\leq 150 \mu\text{L}$  at each time point. The animals were sacrificed 1 day ( $n = 4$ ) or 1 month ( $n = 6$ ) after the second transplantation. Animals ( $n = 7$ ) in the control group were sacrificed 1 month after the transplantation of islets.

### Tissue Sampling and Sectioning

Graft-bearing livers were initially fixated in 4% vol/vol paraformaldehyde overnight at 4°C and subsequently incubated in 15% wt/vol sucrose PBS (2 h) and 30% wt/vol sucrose PBS overnight at 4°C to preserve GFP expression. The livers were frozen and sectioned (10  $\mu\text{m}$ ) until a depth of 4.0 mm. Islet grafts were identified in sections stained with hematoxylin, while consecutive sections were collected for immunostainings. Only islets with an area exceeding 50  $\mu\text{m}^2$  were evaluated in order to avoid misestimations based on single-cell residues.

### Immunohistochemistry

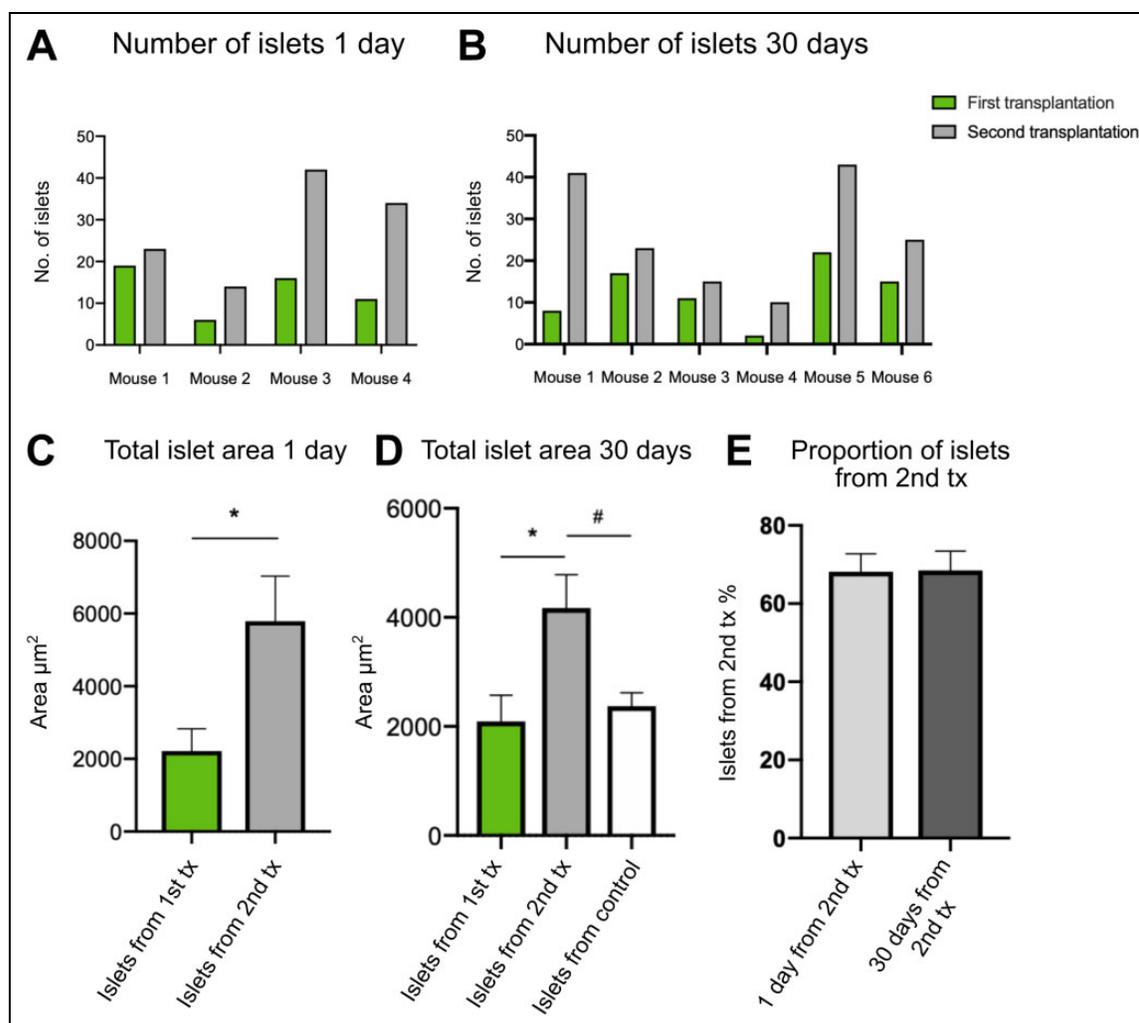
The primary antibodies used were purified rat anti-mouse CD31 (1:100; BD Biosciences, San Jose, CA, USA), guinea pig anti-insulin (1:400; Kem-En-Tec Nordic, Tåstrup, Denmark), and rabbit anti-Ki67 (1:250; Abcam, Cambridge, UK). All secondary antibodies used were diluted 1:300 in PBS containing 3% donkey serum, and purchased from Jackson ImmunoResearch Laboratories, West Grove, PA, USA: Alexa Flour 647 (donkey anti-rat), Alexa Flour 488 (donkey anti-guinea pig) and Alexa Flour 488 (donkey anti-rabbit). Slides were counterstained with Hoechst (1:10 000; Life Technologies, Carlsbad, CA, USA). For image analysis of islet area and vascular density, a macro was built in Fiji software with the assistance of staff at the BioVis facility platform of Uppsala University.

### Angiogenic Factors

Approximately 200  $\mu\text{L}$  of blood was collected from the tail vein of the animals 1 day prior to the transplantation of non-GFP islets. A mouse angiogenesis array kit (R&D Systems, Minneapolis, MN, USA) was performed according to the manufacturer's instructions in order to detect the relative expression level of 53 angiogenesis related proteins in 30  $\mu\text{L}$  serum from each animal. Readings of the array membrane were captured in Bio-Rad Universal Hood II (Bio-Rad Laboratories, Hercules CA, USA) and data analysis was performed using the Image Lab software (Bio-Rad Laboratories).

### Resistance to Cellular Stress in Vitro

GFP or non-GFP-expressing islets from C57BL/6 male mice were isolated and incubated with cytokines for 24 h (50 U/mL; IL-1 $\beta$ , 1,000 U/mL; murine IFN- $\gamma$ , PeproTech, London, UK). Evaluation of cell death was performed by staining with Live-or-Dye NucFix™ Red Staining Kit (Biotium, Fremont, CA, USA) and bisbenzimidazole (20  $\mu\text{g}/\text{mL}$ ; Hoechst 33324, Sigma-Aldrich). A macro using Fiji software performed



**Figure 1.** Islet characteristics. Repeated islet transplantation was performed using green fluorescent protein (GFP) and non-GFP-expressing islets and resulted in an increased islet number (A, B) and a greater islet area (C, D) found from the second islet transplantation. The same result was seen regardless of whether evaluated 1 day (A and C) or 30 days (B and D) after the second islet transplantation. In the control group, a lower islet area was found compared with the non-GFP islets which had been preceded with a GFP-expressing islet transplantation (D). The proportion of islets derived from the second transplantation was the same (68%) for the 1-day and 30-day group (E). All values are given as means  $\pm$  SEM for 4-7 experiments. \*denotes  $p < 0.05$  when comparing islets from first and second transplantation. #denotes  $p < 0.05$  when comparing islet area from second transplantation with that of control.

threshold-based calculations of the ratio of dead to living cells, taken as a relative measure of islet resistance to stress.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). All values are given as means standard error of the mean (SEM). Comparisons between two groups were performed by a paired or unpaired two-tailed Student's *t*-test. Comparisons between three groups were performed using analysis of variance (ANOVA) and Tukey's multiple comparisons test. Multiple comparisons of the angiogenic factors were performed using a two-way ANOVA with Sidak's multiple comparisons test.

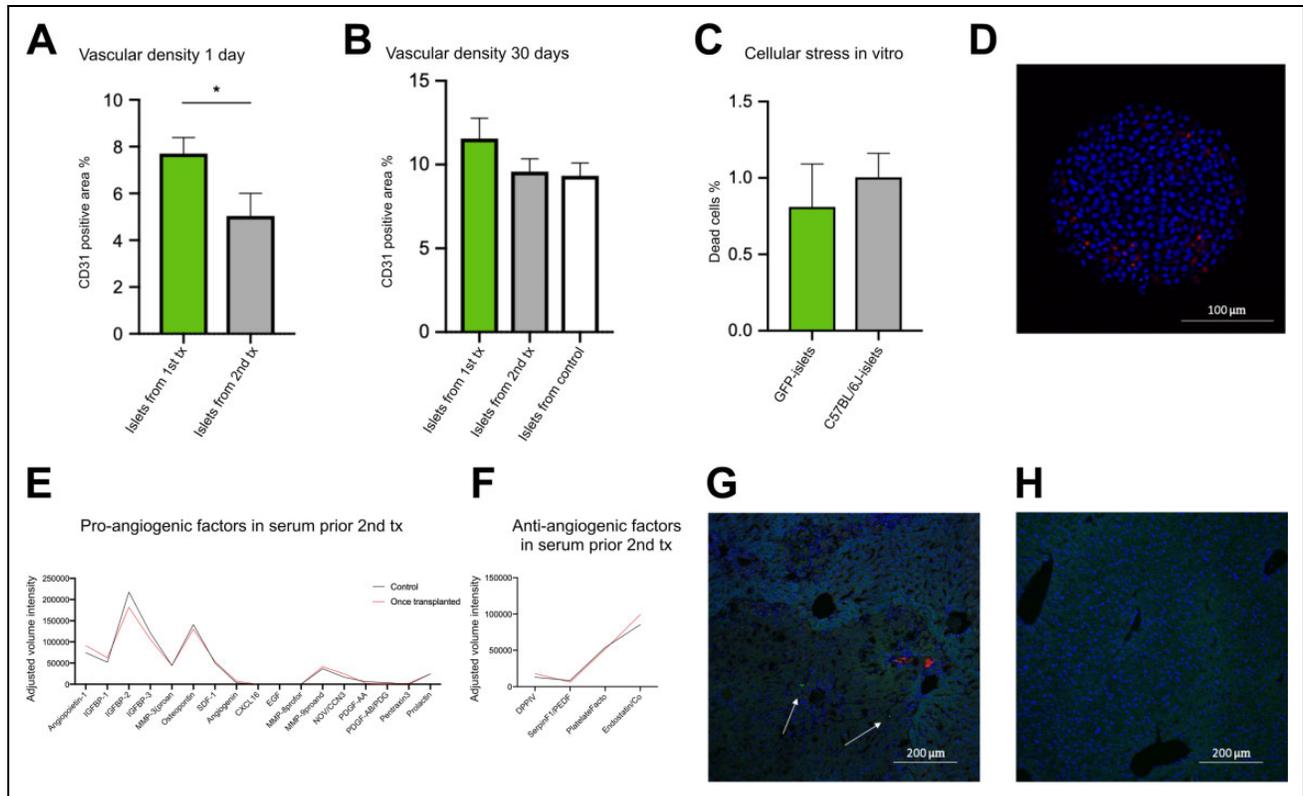
For all comparisons,  $p$ -values  $< 0.05$  were considered statistically significant.

## Results

### Islet Numbers and Graft Area

The number of GFP islets found in the livers 1 day after the second transplantation was  $13 \pm 3$  islets, while the number of non-GFP islets, derived from the second transplantation, was  $28 \pm 6$  (Fig. 1A). In the 30-day group, the corresponding numbers were  $13 \pm 3$  and  $26 \pm 6$ , respectively (Fig. 1B).

Total islet area, taken as a relative measurement of preserved islet mass, was consistently larger in the second islet



**Figure 2.** Vascular density, angiogenic factors and proliferation. Islet vascular density was evaluated by a CD31 staining and the percentage of CD31-positive area of islet area was calculated. One day after second transplantation, the green fluorescent protein (GFP)-expressing islets showed an increased vascular density compared with the 1-day-old islet transplants (A). One month after the second transplantation, no difference was found between islets from the double-transplanted group or the single-transplanted control group (B). The ratio of live and dead cells was evaluated after incubation of isolated GFP-expressing islets and non-GFP islets with IL-1 $\beta$  and IFN- $\gamma$  for 24 h. No difference was found in susceptibility to cellular stress between the two groups (C). The Live-or-Dye kit used permits a dye to enter and label the nuclei of dead cells that have compromised membrane integrity and images were capture with the Zeiss LSM 780 confocal. Dead cells were labeled in red and all nuclei were stained blue by bisbenzimidazole (D). Angiogenic factors in serum were assessed the day prior to the second transplantation for animals that already had received a GFP islet transplantation compared with a control group that had received a saline infusion. No difference was found between the two groups for any of the investigated pro-angiogenic factors (E) or anti-angiogenic factors (F) at this time point. Areas surrounding islets 1 day after transplantation often displayed regions of infarction and scattered Ki67-positive cells (arrows) were found in close proximity of 13% of the islets (G, insulin stained in red). Liver from control animal, not subjected to islet transplantation, with no proliferative cells (H). All values are given as means  $\pm$  SEM for  $n = 4-7$  animals in each group for the vascular density, and  $n = 38-78$  experiments for the live-or-dead evaluation. \*denotes  $p < 0.05$ .

transplants when compared with the first, both when evaluated 1 day and 1 month after the second transplantation (Fig. 1C, D). Interestingly, the total islet area found in the control group after 1 month was on par with the first transplant, that is, only 60% compared with the non-GFP islet area when preceded by a first transplantation (Fig. 1D). The proportion of islets derived from the second transplantation was similar in both animals investigated 1 day or 1 month after the second transplantation (Fig. 1E). There was no immune infiltration in any of the investigated islet grafts.

### Vascular Density

Islet vascular density was assessed by measuring the percentage of CD31-positive area in the islets. One day after

the transplantation of non-GFP islets, the GFP-expressing islets transplanted 1 week before demonstrated higher vascular density (Fig. 2A). One month later, there was no difference in vascularization between non-GFP-expressing islets and GFP-expressing islets (Fig. 2B). There was no difference in vascular density between the non-GFP islets of controls and grafts of non-GFP islets preceded by transplantation of GFP-expressing islets (Fig. 2B).

### Islet Susceptibility to Cellular Stress in Vitro

There was no difference in cell death rates in response to cytokine-induced cellular stress between C57BL/6 islets with or without GFP expression (Fig. 2C, D).

## Angiogenic Factors in Serum

A proteome profile assay was performed on serum collected 1 day prior to the second transplantation and compared with control animals infused with saline instead of first transplantation. No difference was found in either the pro- or anti-angiogenic expression among the animals transplanted with islets or those having received a saline infusion (Fig. 2E, F).

## Liver Morphology

One day after the second islet transplantation, liver parenchyma surrounding the islets showed regions of infarction. Sections stained for the cell proliferation marker Ki67 showed single proliferating cells in control livers of C57BL/6 mice, while proliferative events occurred in nearby liver tissue of 13% of the islets 1 day posttransplantation (Fig. 2G–H).

## Discussion

In the clinic, repeated islet transplantation to the liver is common in order to attain and sustain insulin independence of transplant recipients. However, while the engraftment of islets transplanted to the liver has repeatedly been characterized, there have been no studies on whether the conditions change when repeating the procedure. The present study investigated the possibility that changes in the liver induced by a first transplantation may affect the engraftment of a second transplantation. Interestingly, we discovered a consistent finding that, regardless whether evaluated as number of surviving islets or as surviving islet area, the secondary transplantation was more successful than the first. A tentative explanation that we tested was that the first transplantation induced an angiogenic niche through tissue expression of hypoxia, inflammatory processes, and liver microinfarctions. However, there was neither any increase in angiogenic factors in serum immediately preceding the second transplants when compared with a control group, nor could any increased vascular density be recorded in the second islet transplant when compared with the control group. The reasons for the obtained findings are therefore obscure, but it is likely that the lodging of the second transplant in the liver differs from the first transplant due to the previous obstruction of some of the portal vein tributaries. Moreover, it is possible that gene expression of the liver parenchyma is changed by a first transplantation, inducing hypoxia and survival genes not necessary reflected in circulating concentrations of angiogenic factors. Indeed, in line with previous studies, we found that islets transplanted intraportally into the liver sometimes cause infarction in surrounding liver parenchyma<sup>12</sup>. Staining for proliferative events in the liver parenchyma showed some scarce Ki67-positive hepatocytes in close proximity of 13% of the islets 1 day after the second transplantation. Hepatocytes and liver sinusoidal endothelial cells have also previously been reported to express Ki67 in

conjunction with tissue damage and liver regeneration<sup>13</sup>. It is interesting to note that disruption of islets or compensatory growth (after unilateral nephrectomy or partial hepatectomy) in recipient tissue have previously been reported to have a positive impact on islet implantation<sup>11,14</sup>.

We do not expect there to have been any difference in graft rejection between the primary and second transplantation, since no immune infiltration of the islets could be observed. The GFP-expressing islets were on C57BL/6 background and with no reported immunogenicity of GFP per se<sup>15,16</sup>. All islets were identified by their morphological appearance in bright field microscopy, and therefore the GFP expression itself did not affect the identification of islets. The investigated tissue depth was consistent for all sectioned livers (4.0 mm) to enable comparisons between groups, an otherwise challenging task when using the liver as implantation site. Moreover, when only performing a single transplantation with non-GFP islets (control group), the surviving islet area was lower than the corresponding secondary islet transplant, indicating that neither the site of islets infusion to the liver (portal or appendicular vein), nor the use of GFP-expressing islets for first transplantation affected our findings. Moreover, we did not record any difference in susceptibility to cellular death between GFP and non-GFP-expressing islets when exposed to cytokines. All in all, our findings suggest that a first transplantation paves the way for enhanced engraftment of later transplanted islets.

## Ethical Approval

Ethical approval was obtained for all experimental procedures by the local animal ethics committee of Uppsala University, Sweden (C 248/12).

## Statement of Human and Animal Rights

This article does not contain any studies with human subjects. All procedures with animals in this study were conducted in accordance with the local animal ethics committee of Uppsala University, Sweden.

## Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Funding for this study was obtained from the Swedish Research Council (2017-01343), the Swedish Child Diabetes Fund, the Swedish Diabetes Foundation, EXODIAB, The Erling-Persson Family Foundation and Diabetes Wellness Sverige.

## ORCID iD

Per-Ola Carlsson  <https://orcid.org/0000-0003-4804-5091>

## References

1. Kemp CB, Knight MJ, Scharp DW, Ballinger WF, Lacy PE. Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. *Diabetologia*. 1973;9(6):486–491.
2. Barton FB, Rickels MR, Alejandro R, Hering BJ, Wease S, Naziruddin B, Oberholzer J, Odorico JS, Garfinkel MR, Levy M, Pattou F, et al. Improvement in outcomes of clinical islet transplantation: 1999–2010. *Diabetes Care*. 2012;35(7):1436–1445.
3. Shapiro AM, Lakey JR, Ryan EA, Korbitt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*. 2000;343(4):230–238.
4. Bellin MD, Barton FB, Heitman A, Harmon JV, Kandaswamy R, Balamurugan AN, Sutherland DE, Alejandro R, Hering BJ. Potent induction immunotherapy promotes long-term insulin independence after islet transplantation in type 1 diabetes. *Am J Transplant*. 2012;12(6):1576–1583.
5. Foster ED, Bridges ND, Feurer ID, Eggerman TL, Hunsicker LG, Alejandro R. Improved health-related quality of life in a phase 3 islet transplantation trial in type 1 diabetes complicated by severe hypoglycemia. *Diabetes Care*. 2018;41(5):1001–1008.
6. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, Ostraat O, Salmela K, Tibell A, Tufveson G, Elgue G, et al. Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet*. 2002;360(9350):2039–2045.
7. Eich T, Eriksson O, Lundgren T. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. *N Engl J Med*. 2007;356(26):2754–2755.
8. Suszynski TM, Wilhelm JJ, Radosevich DM, Balamurugan AN, Sutherland DE, Beilman GJ, Dunn TB, Chinnakotla S, Pruett TL, Vickers SM, Hering BJ, et al. Islet size index as a predictor of outcomes in clinical islet autotransplantation. *Transplantation*. 2014;97(12):1286–1291.
9. Ryan EA, Lakey JR, Paty BW, Imes S, Korbitt GS, Kneteman NM, Bigam D, Rajotte RV, Shapiro AM. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. *Diabetes*. 2002;51(7):2148–2157.
10. Rafael E, Ryan EA, Paty BW, Oberholzer J, Imes S, Senior P, McDonald C, Lakey JR, Shapiro AM. Changes in liver enzymes after clinical islet transplantation. *Transplantation*. 2003;76(9):1280–1284.
11. Henriksnas J, Lau J, Zang G, Berggren PO, Kohler M, Carlsson PO. Markedly decreased blood perfusion of pancreatic islets transplanted intraportally into the liver: Disruption of islet integrity necessary for islet revascularization. *Diabetes*. 2012;61(3):665–673.
12. Liljebäck H, Grapensparr L, Olerud J, Carlsson PO. Extensive loss of islet mass beyond the first day after intraportal human islet transplantation in a mouse model. *Cell Transplant*. 2016;25(3):481–489.
13. Wabitsch S, Benzing C, Krenzien F, Splith K, Haber PK, Arnold A, Nosser M, Kamali C, Hermann F, Gunther C, Hirsch D, et al. Human stem cells promote liver regeneration after partial hepatectomy in balb/c nude mice. *J Surg Res*. 2019;239:191–200.
14. Dunger A, Korsgren O, Andersson A. DNA replication in mouse pancreatic islets transplanted subcapsularly into the kidney or intraportally into the liver. Influence of unilateral nephrectomy or partial hepatectomy. *Transplantation*. 1990;49(4):686–689.
15. Tanaka T, Kojima D, Mera T, Matsumoto M, Yasunami Y, Yanase T. Expansion of transplanted islets in mice by cotransplantation with adipose tissue-derived mesenchymal stem cells. *Heliyon*. 2018;4(5):e00632.
16. Hamamoto Y, Akashi T, Inada A, Bonner-Weir S, Weir GC. Lack of evidence for recipient precursor cells replenishing beta-cells in transplanted islets. *Cell Transplant*. 2010;19(12):1563–1572.