Introduction

Insulin-secreting pancreatic beta-cells proliferate in response to increasing demand for insulin and also after physiological injury [1,2,3,4,5,6,7,8]. It is generally accepted that beta-cells have a finite life span and that dying beta-cells are continuously replaced [3,9,10,11,12]. This notion raises the possibility of enhancing baseline replication of beta-cells as a therapeutic approach for the treatment of diabetes patients with type 1 or type 2 diabetes. Indeed, there are clinical case-reports of beta-cell regeneration allowing the complete recovery from type 1 diabetes [13]. However, in the majority of patients, the reported level of glycemic control with better control being associated with improved recovery. Finally, real-time bioluminescent imaging can be used to monitor beta-cell recovery in living MIP-luc transgenic mice.

Materials and Methods

Mice

C57BL/6 mice obtained NCI (Frederick, MD) or Jackson Laboratory (Bar Harbor, ME) were used as donors and recipients of kidney subcapsular islet transplants. CD1 transgenic mice expressing firefly (Photinus pyralis) luciferase (luc) under the control of the mouse insulin 1 promoter (MIP- luc) [15] were used as recipients of kidney subcapsular islet transplantation. 9–12 week
old female mice were made diabetic by a single intraperitoneal (IP) injection of STZ (150 mg/kg, Sigma Chemical, St. Louis, MO). Diabetic mice with non-fasted blood glucose values >400 mg/dl for more than 2 consecutive days (SureStep; Lifescan, Milpitas, CA) were used as recipients of islet grafts or insulin implants. All studies were performed in accordance to protocols approved by the University of Chicago Institutional Animal Care and Use Committee.

**Islet Isolation and Transplantation**

Syngeneic islets from C57BL/6 mice were isolated following intraductal collagenase digestion (Collagenase P, 0.3 mg/ml; Roche, Indianapolis, IN) and purification by Ficoll gradient centrifugation (Sigma, St. Louis, MO) as previously described [14,16]. Approximately 200 islets were transplanted under the kidney capsule.

**Exogeneous Insulin Implants**

Insulin was administered via subcutaneous ‘LinBit Implants for Mice’ (LinShin, Toronto, ON, Canada). The implants were titrated for blood glucose levels of <250 mg/dl.

**Blood Glucose Monitoring**

Random non-fasted blood glucose levels were determined three times weekly from the tail vein using a SureStep glucometer.

**Bioluminescent Imaging**

Bioluminescent optical imaging was performed using a Xenogen IVIS 200 imaging system (Xenogen, Alameda, CA) as previously described [15]. Briefly, MIP-luc mice were fasted for 4 h, shaved, then anesthetized with isofluorane (using the Xenogen system). Mice were placed on their sides on the imaging stage and an overlay image was initially taken. Mice were then injected IP with 15 mg/ml D-luciferin in sterile PBS (150 mg/kg) and exactly 14 min after injection of D-luciferin, a bioluminescent image was captured utilizing an exposure time of 1 minute. Subsequent image processing including quantification of bioluminescence was conducted using Living Image Software v. 2.05 (Xenogen).

**Histological Studies**

Beta-mass was determined as described previously [14]. Briefly, the pancreas was removed, weighed, fixed, embedded and then serially step-sectioned. Every 10th section was stained with anti-insulin polyclonal antibody (Zymed, South San Francisco, CA; ~10 sections per mouse) and images of each section were captured on a Zeiss Axiolicht 200 M microscope. The insulin positive and total pancreas area were quantified with Image J (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/), and the relative ratio of insulin positive areas to the entire pancreas area was determined. The beta-cell mass was calculated by multiplying the relative ratio by the total weight of the pancreas.

**Intraperitoneal Glucose Tolerance Test**

An intraperitoneal glucose tolerance test (IPGTT) was performed as previously described [14]. Briefly, after four hours of fasting, the mice received an intraperitoneal injection of dextrose (2 g/kg), and blood glucose levels determined from the tail vein at 30-minute intervals.

**Statistics**

Data are presented as means±SEM and evaluated for statistical significance by ANOVA (SuperANOVA v. 1.11; Abacus Concepts, Berkeley, CA). A P value of <0.05 was considered to be significant.

**Results**

**Effect of Insulin Treatment on Beta-Cell Function in STZ-Diabetic Mice**

The experimental design to study beta-cell regeneration was divided into two phases: treatment and monitoring.

**Treatment Phase.** STZ-induced C57BL/6 diabetic female mice, with non-fasted blood glucose values >400 mg/dl for more than 2 consecutive days, were randomly assigned to the islet transplant or insulin implant group. Mice (N=10 per group) were then monitored for either 60 or 120 days with three times weekly blood glucose measurements.

**Monitoring Phase.** After either 60 or 120 days, the islet transplant was removed by nephrectomy and the insulin implant removed. Blood glucose monitoring was continued for an additional 30 days to allow for the assessment of endogenous beta-cell function. At the end of this 30 day monitoring phase (i.e. 90 or 150 days after induction of diabetes with STZ), the mice were sacrificed and histology performed to quantify beta-cell mass.

Both islet transplantation and insulin treatment were effective at preventing hyperglycemia and recurrence of diabetes (Table 1 and Fig. 1A), with blood glucose control by islet transplantation superior to insulin implants (139.4±7.5 and 181.6±8.3 mg/dl respectively, P<0.05). Following the removal of the transplanted islets or discontinuation of insulin therapy, all treatment groups showed a significant (P<0.05) increase in blood glucose levels. Both the 60-day islet transplant and implant groups redeveloped diabetic glucose levels during the Monitoring Phase (253.2±25. and 280.9±35.8 mg/dl, respectively). In contrast, the 120-day islet transplant and implant insulin implants maintained stable non-diabetic blood glucose levels (188.6±31.7 and 194.2±27.3 mg/dl, respectively). At the end of the 30-day monitoring phase, mice from both 120-day groups underwent an IPGTT (Fig. 1B). Both groups showed an abnormal glucose profile compared to non-diabetic controls.

**Effect of Insulin Treatment on Beta-Cell Mass in STZ-Diabetic Mice**

There was no significant increase in beta-cell mass in the 60-day-insulin-treated groups compared to STZ-treated controls (untreated STZ-control group, 0.09±0.01 mg; islet transplant, 0.14±0.09 mg; and insulin implant, 0.18±0.13 mg) (Table 2). In contrast, there was a significant increase in beta-cell mass in both the 120-day-insulin-treated groups compared to untreated STZ-induced diabetic mice (islet transplant, 0.91±0.23 mg; insulin implant, 0.69±0.22 mg). The maximum beta-cell regeneration occurred in the 120-day islet transplant group achieving a 60% recovery of beta-cell mass that was statistically greater than the 45% recovery in 120-day insulin implant group (P<0.05). The islets from the 120-day islet transplant group were large and densely populated with insulin-producing beta-cells as compared to the other treatment groups (Fig. 2).

**Real-Time Quantification of Beta-Cell Recovery**

Traditional measures of beta-cell mass preclude real-time quantification and thus the kinetics of beta-cell regeneration cannot be accurately ascertained. Our data suggest that significant beta-cell regeneration occurred in the 60–120 day post-STZ treatment period, but we were not able to define the kinetics of
regeneration within this 60 day period. We therefore tested whether biweekly bioluminescence imaging could successfully be used to quantify beta-cell mass following destruction and regeneration. Because bioluminescence emission is a dynamical chemical reaction dependent on luciferin availability, numerous optimization experiments were initially performed to determine the time point (14 minutes post luciferin injection) at which maximal bioluminescence emission could be captured. All bioluminescence measurements were then performed in precisely the same manner to allow for results at different time points to be compared.

STZ-induced diabetic MIP-luc female mice (C57BL/6 background) were treated with 200 syngeneic wild-type C57BL/6 islets placed under the kidney capsule. The transplanted islets do not express the MIP-luc transgene, and consequently, the bioluminescent signal reflects the endogenous beta-cell mass. The transplanted islets were removed at 120 days via nephrectomy beta-cell function monitored for an additional 30 days. The baseline bioluminescent signal was similar for all five MIP-luc mice. After STZ-induced diabetes, bioluminescence decreased continued to do so, reaching a nadir approximately 60 days after the induction of diabetes in all five mice (Fig. 3). After 60 days post-STZ treatment phase, four of the five mice (M1, M3, M9 and M12) showed a persistent increase in bioluminescent signal. In contrast, M2 demonstrated a transient increase in bioluminescent that eventually was lost. At 120 days post-STZ treatment, the bioluminescent signal of M1, M3, M9, and M12 showed a 5.2±1.5 fold increase compared to their nadir while M2, did not.

All five MIP-luc mice maintained normal random blood glucose levels (148.3±26 mg/dl) prior to nephrectomy, indicating appropriate function of the transplanted islets. However, following nephrectomy, M1, M3, M9 and M12 were able to maintain glycemic control (180±47, 136±30, 166±20, and 152±37 mg/dl).
islet transplant (txp) under the kidney capsule for 60 or 120 days. Untreated STZ-diabetic mice treated with insulin implants or islet transplant under the kidney capsule.

Table 2. Beta-cell mass (mg) in STZ-diabetic mice treated with insulin by insulin implant or islet transplant under the kidney capsule.

| Non-diabetic control | Untreated STZ-diabetic mice | 60-day insulin implant | 60-day islet transplant | 120-day insulin implant | 120-day islet transplant |
|----------------------|-----------------------------|------------------------|-------------------------|------------------------|-------------------------|
| Beta-cell mass (mg)  | 1.52±0.25                   | 0.09±0.01              | 0.18±0.13               | 0.14±0.09              | 0.69±0.22*               | 0.91±0.23*               |

Untreated STZ-diabetic B6 mice were examined 4–8 days after STZ-induced diabetes, which when untreated results in severe diabetes and death. *P*,<0.05 compared to untreated STZ-diabetic mice.
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Table 2.

Discussion

Recent reports of functional pancreatic beta-cell regeneration in murine models [17,18] has generated much excitement and controversy [3,19]. Despite the increasingly accepted notion that pancreatic beta-cells have the ability to regenerate, either from remaining beta-cells or existing beta-cell precursors, and to restore euglycemia after the induction of diabetes in laboratory rodent models, the understanding and clinical relevance of beta-cell regeneration is still incomplete. In particular, observations that beta-cell regeneration derives primarily from existing beta-cells and that insulin secreting beta-cells remain functional in patients with established type 1 diabetes, raise the possibility that beta-cell regeneration may contribute to the recovery in patients with autoimmune diabetes [20,21,22,23]. The results of the studies described here suggest that insulin treatment by implants or islet transplantation promotes beta-cell regeneration in the STZ-diabetic mouse model of beta-cell regeneration, and does so in a time dependent manner with longer treatment periods associated with greater recovery. They also suggest that the overall degree of glycemic control affects regeneration with better control accelerating recovery of beta-cell mass and function. Further studies are needed to determine the optimal length of time of insulin treatment for complete recovery of beta-cell mass and function in this model.

Figure 2. Representative pancreatic islets showing insulin staining (brown) in untreated STZ-diabetic mice, non-diabetic control and STZ-diabetic mice treated with insulin implants or islet transplant (txp) under the kidney capsule for 60 or 120 days (40x).
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In conclusion, our studies of beta-cell regeneration in the STZ-diabetic mouse may have implications for patients with type 1 diabetes, indicating an increase in beta-cell mass and function. These findings suggest that beta-cell regeneration is not confined to the early stages of diabetes, but can occur throughout the disease process, potentially contributing to glycemic control in patients with type 1 diabetes.
diabetes and suggest that if the ongoing immunological destruction of beta-cells can be prevented, proliferation of the remaining beta-cells in the presence of insulin (and possibly other agents) may lead to a restoration of normal glucose tolerance.

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Author Contributions
Conceived and designed the experiments: EG DDL AC. Performed the experiments: EG DDL JT RAW. Analyzed the data: EG AC. Contributed reagents/materials/analysis tools: SYP GB. Wrote the paper: EG GB.
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