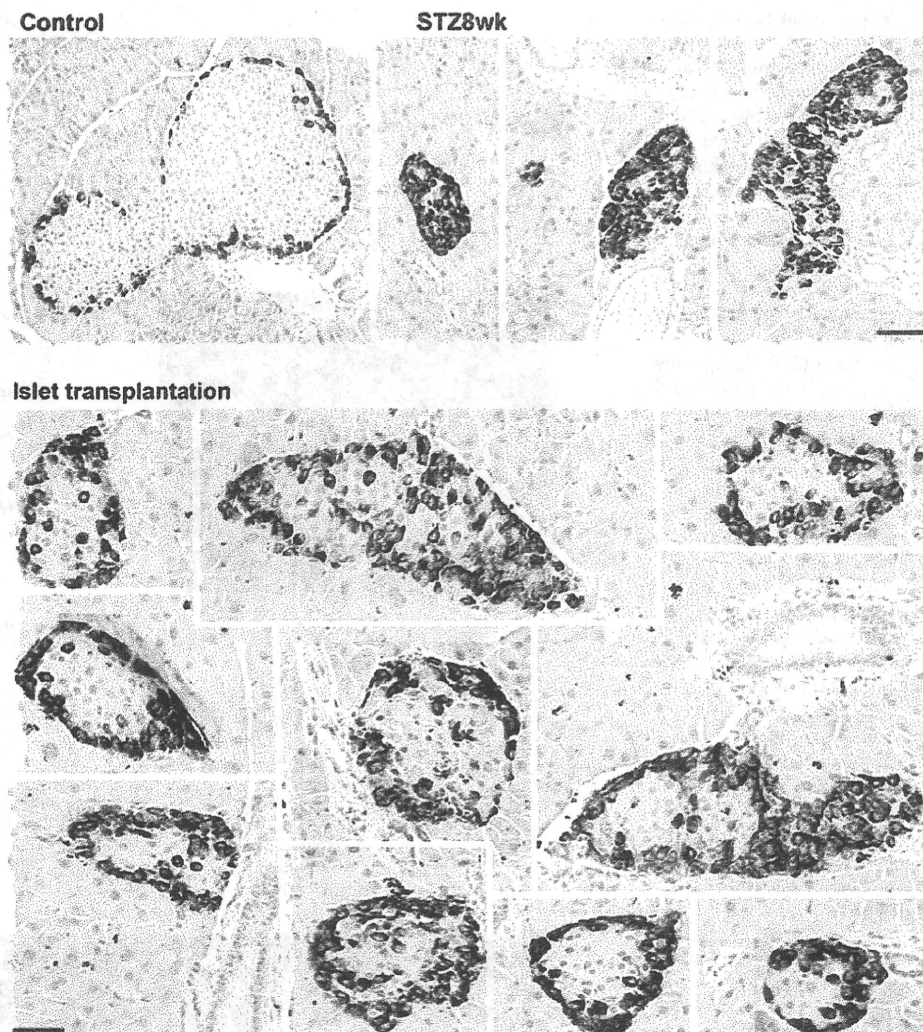**Fig. 3** The changes in the islet morphology. In STZ-diabetic mice, islets appear severely disorganized with a significantly increased proportion of glucagon-positive cells (*brown*). In contrast, islet morphology is greatly recovered and the typical islet morphology of the core of β cells is seen with a mantle of α cells in islet-transplanted mice. *Scale bar* 100 μm

that these changes are a direct result of the increased β cells due to normoglycemia.

Since the β cell increases through neogenesis and proliferation, these determinants of β-cell increase were examined. Because pre-existing β cells were destroyed almost completely with STZ as shown in Fig. 2c, g, newly born β cells and old β cells left in the islet with many α cells are morphologically distinguishable. Newly born β cells, as observed as scattered singlets-doublets of insulin-positive cells or clusters less than 6 β cells across, were frequently seen in transplanted mice (Fig. 4d). Dividing by β cell number (1–3 and 4–6 β cells), both small β-cell clusters were increased in transplanted mice (Fig. 4e), suggesting that neogenesis of β cells was enhanced in transplanted mice. Furthermore, using dual staining of insulin and Ki67, a marker for β-cell proliferation, we

found that Ki67-positive islets were increased in transplanted mice (Fig. 4f), suggesting that β-cell proliferation is enhanced in normoglycemia.

Acute damages in the liver and kidney

Since STZ induces cytotoxicity in multiple organs, including liver and kidney [31–36], we finally examined the recovery from these toxicities in the liver and kidney from both the Detemir injected group and islet transplanted group. After 8 weeks, STZ 8 weeks mice showed dyslipidemia (Table 1), but both treatments significantly reduced the elevated total cholesterol. Liver weight/body weight and AST/ALT levels were significantly higher in STZ 8 weeks and Detemir injected mice, but normalized in transplanted mice (Table 1). The liver section also showed hepatic steatosis

Fig. 4 Quantification of β cells. **a** All insulin-positive cells on the cross section were photographed and counted; 4–7 sections/animal at least 150 μm apart were evaluated. **b** Islets were divided by their size (6–20, 21–100, and over 100 β cells in the islet). In transplanted mice, not only small islets but also larger islets were found. **c** The changes of proportion of islets. All insulin-positive and glucagon-positive cells were counted as above. **d** Newly formed β cells. Scattered singlets-doublets of insulin-positive cells or clusters less than 6 β cells across were frequently seen in transplanted mice. Because pre-existing β cells were destroyed almost completely with STZ, newly born β cells and old β cells left in the islet with many α cells are morphologically distinguishable. **e** Quantification of newly born β cells (1–6 β-cell clusters cross section). Newly born β cells were divided by their size (1–3, 4–6 β cells). **f** Quantification of Ki67-positive islets/total islets. Pancreatic sections were double immunostained for insulin and Ki67, a marker for cell proliferation; 3–7 sections/animal 150 μm apart were evaluated. Results are expressed as mean ± SEM. STZ diabetic group (*black*), islet transplanted (IT) group (*gray*), control mice (*white*)
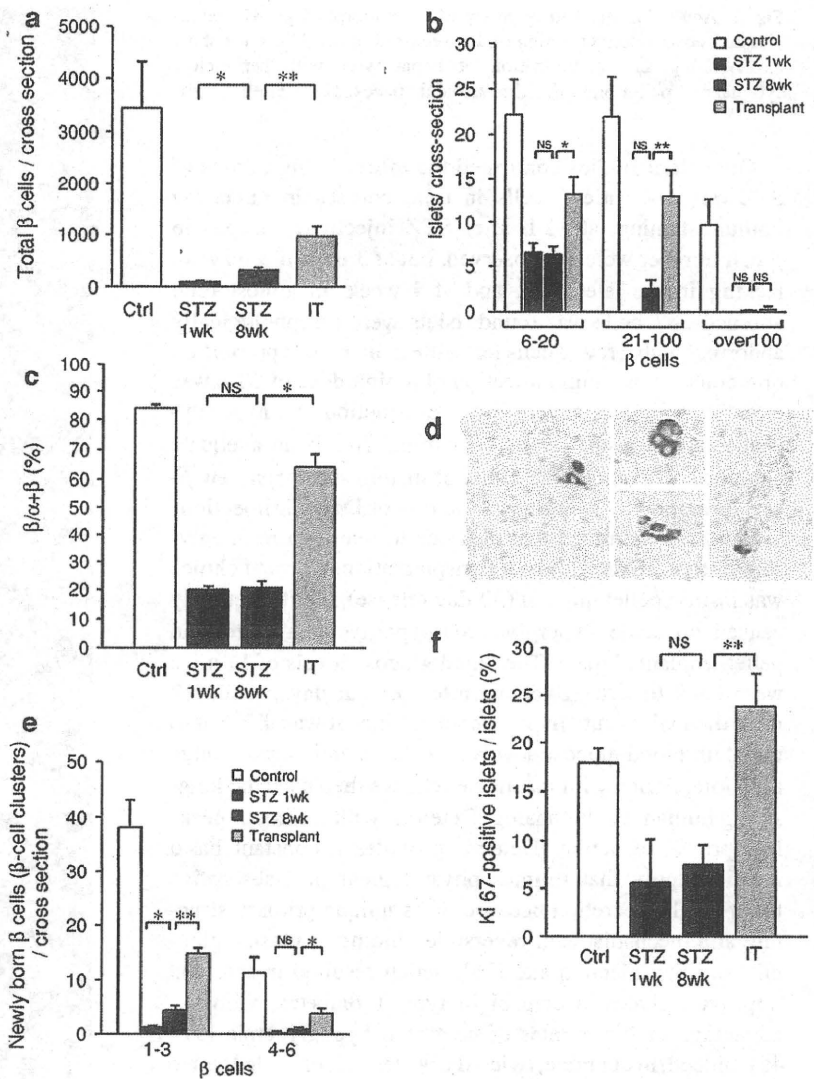
(Fig. 5). The severity of degenerative changes was lessened by islet transplantation in the islet-transplanted mice compared to the STZ mice (Fig. 5).

Renal hypertrophy, which is expressed as the ratio of the combined weight of the two kidneys to body weight, was prominent in STZ mice (Table 2). Histologically, glomerular hypertrophy, the characteristic phenomenon of diabetes, was prominent, and glomerular size in the superficial cortex is small in STZ mice (data not shown). BUN was also significantly high in STZ mice. These acute damages of renal function were restored in transplanted mice, but not in Detemir-injected mice (Table 2). There was no significant difference in serum creatinine and serum albumin among the three groups, STZ, transplanted, and Detemir-injected mice.

Table 1 The liver toxicity by STZ

Group	Liver weight/body weight ($\times 10^2$)	AST/ALT	Total cholesterol (mg/dl)
STZ	7.0 ± 0.39*	4.7 ± 1.1*	152.8 ± 11.2*
Insulin (Detemir)	6.4 ± 0.10*	3.8 ± 0.3*	108.0 ± 1.7
Islet transplant	4.3 ± 0.04	1.6 ± 0.01	106.9 ± 8.2
Control	4.5 ± 0.02	1.9 ± 0.2	108.8 ± 4.3

* $P < 0.05$ versus control

Discussion

In this study, we examined whether new β-cell formation occurs when β cells face being severely destroyed and hyperglycemia was restored with two different methods.

Fig. 5 Acute damage and recovery of liver morphology. Hematoxylin and eosin (H&E) staining of 16-week-old mice. STZ mice show the swelling and vacuolization of hepatocytes with perinuclear cytoplasmic pallor and dilated sinusoidal spaces. *Scale bar* 100 μm

Our initial studies confirmed the effect of high doses of STZ on pancreatic β cells in time courses in mice. By immunostaining, at 12 h after STZ injection, changes in β-cell number were not observed, but at 3 days β cells were lacking in the islet core, and at 1 week an almost total absence of β cells was found. Islets were morphologically abnormal with a few β cells left with an increased proportion of α cells. Thus, single injection of a high dose of STZ was effective to induce severe β-cell destruction and hyperglycemia due to insufficiency of insulin. This is an adequate condition to examine the effect of insulin supply on new β-cell formation by islet transplantation or Detemir injection.

We then examined two methods to maintain normoglycemia to compare with islet transplantation. Our first choice was insulin pellet implant (90 day release), but it frequently caused an acute hyperglycemia-hypoglycemia pattern in pellet-implanted mice. The blood glucose levels of 11 mice were back to hyperglycemia after several days, while 17 mice died with acute hypoglycemia. Since it was difficult to maintain blood glucose levels in a stable and normal range for a long period with insulin pellets, we then used the long-acting human insulin analog, Detemir, with a smooth peakless profile of action. Detemir provides a constant basal insulin supply that mimics physiological post-absorptive basal insulin secretion because of its unique primary structure and mechanisms of reversible binding to plasma albumin and the injection site [26], which resulted in safe and improved glycemic control in type 1 diabetes, with the advantage of lower rates of nocturnal hypoglycemia [37–45]. Indeed, in our mice, twice-daily Detemir provided stable glycemic control, which is comparable to islet transplantation. Because of the long duration of action and carry-over effect of Detemir, some mice needed to have lower doses at the second injection of the day for good glucose curves.

Although Detemir injection could effectively reverse hyperglycemia and glycemic control was successful during experiments, there was no β-cell increase, new formation, or recovery of islet morphology in Detemir-treated mice. Here we demonstrate for the first time that the effects of Detemir on the β cell in the pancreas were very different from those of islet transplantation. One possibility could be the low binding affinity to receptors and weak signal transduction of Detemir. It has been reported that Detemir has only 16–18% binding affinity of human insulin (100%) to insulin receptor and IGF-I receptor [46]. Moreover, other study has demonstrated that Detemir has remarkably lower induction of phosphorylation of the insulin receptor, IRS-1, Akt, and
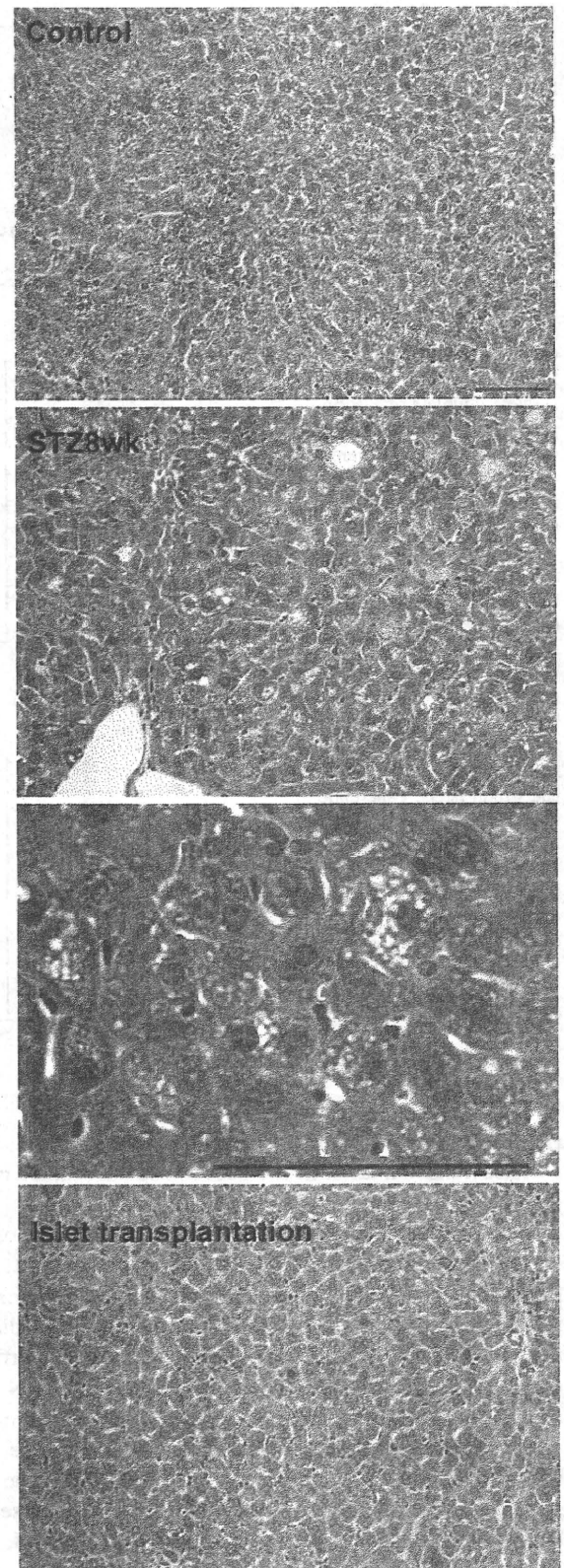
Table 2 The acute damage of renal function by STZ

Group	Kidney weight/body weight ($\times 10^2$)	BUN (mg/dl)	Serum creatinine (g/dl)	Serum albumin (g/dl)	Urine volume (ml)	Urine glucose (mg/dl)
STZ	1.7 ± 0.11*	32.7 ± 1.4*	0.16 ± 0.01*	3.0 ± 0.2*	23.5 ± 0.6*	++++
Insulin (Detemir)	1.5 ± 0.03	31.5 ± 1.8*	0.17 ± 0.01*	2.7 ± 0.1*	6.0 ± 0.9*	+++
Islet transplant	1.2 ± 0.09	27.1 ± 0.3	0.17 ± 0.01*	3.0 ± 0.1*	ND	±
Control	1.4 ± 0.03	25.9 ± 0.3	0.13 ± 0.01	3.7 ± 0.04	1.1 ± 0.2	−

ND not determined

* $P < 0.05$ versus control

GSK3, key signalling molecules involved in cell growth and cell differentiation, than human insulin in myocytes, hepatocytes, and vascular smooth muscle cells [47]. As a result of the low potential to activate insulin receptors and weak signal transduction, the mitogenic potency of Detemir was only 11–15% of human insulin (100%). Therefore, Detemir may not have the potential to increase β cells. Since only the effect of Detemir was examined in this study, further studies of the effects of other insulin analogs such as Glargine, which has a high binding affinity to the IGF-I receptor [46], will be required to reveal mechanisms that promote β-cell increase and survival.

In the present study, continuous hyperglycemia (8 weeks) did not restore β-cell regeneration. Our results agree with a previous study that confirmed that prolonged exposure to elevated glucose levels (3–4 weeks) inhibits the proliferative capacity of β cells and increases DNA fragmentation in cultured islets [48]. On the other hand, β-cell regeneration was restored when hyperglycemia was reversed by islet transplantation. It was striking that the number of β cells and islets was increased and islet structure was greatly recovered. This recovery involved both increased neogenesis and replication. Thus, islet transplantation was effective and provided not only stable glycemic control for a long period, but also a trigger for the induction of new formation. The source of new β cells after birth has been debated, and pancreatic duct cells, bone marrow cells, and acinar cells have been reported as potential progenitor cells [16, 49, 50]. Since the embryonic islets are polyclonal [51], similar polyclonality in the newly formed islets in adults would be expected.

Recently, it has been reported that glycemic control by islet transplantation and insulin pellet implants could increase β-cell mass [52]. In that study, 200 islets or insulin pellets were implanted into STZ-induced diabetic female mice, and this showed that there was a significant increase in β-cell mass in both treatment groups with a long treatment period (120 days). However, it is unclear whether the increased β cells were from neogenesis or replication of the remaining cells, as the issue of new formation is not addressed. In the present study, we saw the number of β cells and islets was increased and the islet structure was greatly recovered in the transplanted group with a shorter

treatment period (10 weeks), but not in the Detemir-treated group. Differences between their study and this study are that they used only 200 islets or insulin pellets for female mice with less-restricted blood glucose control (<250 mg/dl), whereas in our study, we used 500 islets or Detemir injection for male mice with restricted blood glucose control (<160 mg/dl). It is possible that mild transient hyperglycemia might have enhanced β-cell replication and cell size. In addition, it has been reported that there are gender differences in β-cell death to STZ toxicity [53]. Females were protected and retained a normal islet architecture after STZ injection (day 8), whereas males were vulnerable to STZ. Thus, it is possible that in females the β-cells could increase with small amounts of insulin or insulin pellet.

The effectiveness of islet transplantation might be explained by the properties and metabolic potencies of insulin and the graft composition. Compared to other insulin analogs at equivalent concentrations, insulin supplied from the graft islet cells has a high potential for binding affinity to insulin receptors and IGF-I receptors [46], which induces phosphorylation of insulin receptor [47]. Insulin receptor phosphorylation (activation) is directly related to signal transduction strength [47], and continuous activation of the insulin receptor is required for mitogenic activity [54]. The importance of the relationship between insulin and β-cell-specific insulin receptors in β-cell mass is also demonstrated by knockout mice study [55]. Thus, insulin from the graft could stimulate β-cell proliferation and increase β-cell mass through insulin receptor and downstream processes. The second advantage is that insulin from the graft can respond to the changes in blood glucose levels. The third possibility could be the graft composition. Islets contain both endocrine and non-endocrine cell types, including endothelial cells, which could contribute to revascularization of islet grafts [56, 57]. On the other hand, recent study has shown a pure β-cell graft could effectively reverse hyperglycemia and non-β-cells are not essential [58]. However, it is not clear that the residual islets in the pancreas can recover with a pure β-cell transplantation. Therefore, the question still remains whether other hormones secreted from non-β cells can also

play a role in proliferation or new formation of pancreatic β cells. Taken together, islet transplantation, pure β-cell transplantation, or Detemir injection can effectively reverse hyperglycemia, but only islet transplantation could contribute to β-cell proliferation or new formation in the pancreas.

We further demonstrated that only islet transplantation could reverse the damaged liver and renal function and the changed structures.

In conclusion, our data showed β-cell's capability for new formation and replication when β cells were severely destroyed and hyperglycemia was reversed.

Our goal is to develop a regenerative therapy in which enough β cells are served by proliferation or new formation in the pancreas. What stimulates β cells to proliferate and how β cells newly form (the mechanisms of neogenesis) remain to be investigated.

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