Blood glucose was measured after a 10 h fast and assayed using Antsense II (Horiba Industry, Kyoto, Japan). Plasma insulin was determined with an ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan). Insulin content was measured as described previously (23).

Bone marrow transplantation (BMT)

BM cells were flushed in bulk from the medullary cavities of femurs and tibias. BM donors were young (6 week old) sex-matched GFP transgenic mice and *Nos3*$^{-/-}$ or *Nos3*$^{+/+}$ mice. Recipient mice were lethally irradiated (10 Gy) and reconstituted a single intravenous infusion of 2 x 10^6 BM cells, from donor mice, through the tail vein. Tissues were analyzed 30-40 days after BMT. The percentage of GFP-positive cells among recipient BM cells was determined by fluorescence-activated cell sorting (FACS), using a FACS Caliber with CellQuest software (BD Pharmingen, Franklin Lakes, NJ).

BrdU in-situ detection

To identify proliferating cells in the pancreas, bromo-deoxyuridine (BrdU) was injected according to the BrdU In-Situ Detection Kit protocol (BD Bioscience, San Jose, CA). Mice were injected intraperitoneally with 1mg of BrdU, 24 h before pancreas extraction at 0, 3, 7, 10, 15 or 25 days after BMT. The labeled cells were immunostained with anti-BrdU antibody. To calculate numbers of islets and cells per islet and the percentage of BrdU-positive cells among islet cells, we microscopically examined the whole

pancreas in 30 μm sections, and counted the numbers of islets, islet cells and BrdU-positive nuclei in islets.

Immunohistochemistry

Mouse pancreases were excised and fixed overnight in 10% paraformaldehyde. Fixed tissues were processed for paraffin embedding and 3 μm sections were prepared. The streptavidin–biotin (SAB) method was performed with a Histofine SAB-PO kit (Nichirei, Tokyo, Japan) for immunostaining using antibody against insulin (Sigma-Aldrich) or GFP (Santa Cruz Biotechnology, Santa Cruz, CA). Slides were deparaffinized and immediately exposed to the blocking solution. Sections were incubated for 18h at 4℃ with antibody against human insulin or GFP diluted 1:1000 in phosphate buffered saline (PBS). Slides were incubated with the biotinylated IgG for 1h and then with peroxidase-conjugated streptavidin for 30 min at room temperature. Finally, immunoreactivity was visualized by incubation with a substrate solution containing 3,3′-diaminobenzidine tetrahydrochloride. For double staining of insulin and BrdU, the streptavidin-peroxidase method was applied, followed by incubation with Simple stain AEC (3-amino-9-ethyl carboxazole) solution (Nichirei,Tokyo, Japan).

Fluorescent immunohistochemistry

For double staining of insulin with glucagon, keratin/cytokeratin or CD45, the 3 μm sections of paraffin-embedded pancreases were incubated overnight with the respective antibodies at 4℃. Antibodies against insulin, glucagon (DAKO Corporation, Carpinteria,

CA), keratin/cytokeratin (Nichirei, Tokyo, Japan) and CD45 (Santa Cruz Biotechnology) were diluted 1:1000 in PBS. For PECAM-1 staining, sections were immunostained with rat anti-CD31 (1:10; BD Biosciences). Labeled cells were visualized with a biotin-conjugated secondary antibody with Streptavidin, Texas red conjugate (Vector Laboratories). For double staining of insulin with glucagon or keratin/cytokeratin, the sections were incubated for 1h at room temperature in a mixture of Alexa Fluor 488 goat chicken anti-mouse IgG (Molecular Probes, Eugene, OR) diluted 1:100 and Alexa Fluor 594 donkey anti-rabbit diluted 1:50 in PBS. For double staining of insulin and CD45, the sections were incubated in a mixture of Alexa Fluor 488 chicken anti-mouse IgG and Alexa Fluor 546 goat anti-rabbit IgG diluted 1:1000 in PBS. Sections were observed under a fluorescence microscope, LSM 5 PASCAL (Carl Zeiss) and the image was analyzed using the PASCAL system.

Statistical analysis

Data are expressed as means ± SE. Differences between experimental groups were evaluated using the unpaired Student's *t* test for several independent observations. A p value < 0.05 was considered significant.

RESULTS

Recipient BM was replaced with donor cells after irradiation followed by BM cell infusion, but not after simple BM cell infusion without pre-irradiation.

Six-week-old C57BL/6J mice were given STZ daily for 8 days, followed by lethal irradiation and subsequent infusion of BM cells (STZ+BMT mice). In these experiments, BM cells were obtained from GFP transgenic mice (Fig. 1A). A group of STZ-treated mice was simply infused with the same number (2×10^6) of BM cells without pre-irradiation (STZ+BM-infused mice). First, we confirmed replacement of recipient BM with that of donor mice using fluorescence microscopy and FACS analysis. As shown in Fig. 1B, there were no GFP-positive cells in the BM of C57BL/6J mice, while nearly all BM cells from GFP mice were GFP-positive. BM cells of STZ+BMT mice showed high donor chimerism, indicating the recipient BM to have essentially been replaced with donor BM cells. In contrast, STZ+BM-infused mice had no donor-derived GFP cells in their BM, suggesting that pre-irradiation is necessary for BM replacement.

BMT, but not simple BM cell infusion without pre-irradiation, improved hyperglycemia in STZ-treated mice.

As shown in Fig. 1C, STZ-treated mice receiving neither irradiation nor BM cell infusion (hyperglycemic controls) showed markedly higher fasting blood glucose than mice without STZ treatment (normoglycemic controls). Notably, blood glucose levels of STZ+BMT mice were significantly lower than those of hyperglycemic controls. Forty

days after the first STZ administration, blood glucose levels of STZ+BMT mice were similar to those of normoglycemic controls. However, blood glucose levels of STZ+BM-infused mice did not decrease, instead remaining similar to those of hyperglycemic controls for 50 days after STZ administration. We additionally examined the effects of BMT, performed 30 days after STZ treatment. This late BMT did not significantly decrease blood glucose levels (Supplemental Figure). Together, these findings suggest that BMT improves hyperglycemia after acute injury of pancreatic β cells with STZ treatment.

Next, we measured fasting plasma insulin levels on day 40 (Fig. 1D) in STZ+BMT mice. STZ administration markedly decreased plasma insulin levels, while BMT partially but significantly restored these levels by day 40.

STZ administration followed by BMT increased pancreatic islets in the vicinity of pancreatic ducts.

We histologically analyzed pancreatic islets in the four groups. With hematoxylin-eosin (HE) staining on day 35, islet number and size were markedly decreased in hyperglycemic (Fig. 2A, b and f), as compared with normoglycemic (Fig. 2A, a and e), controls. While simple BM infusion without pre-irradiation did not reverse the diminished number and size of islets (Fig. 2A, c and g), islet number and size were both restored in STZ+BMT mice (Fig. 2A, d and h). Several islet populations were enlarged as compared with those in normoglycemic controls (Fig. 2A, a and e vs d and h).

Anti-insulin staining of pancreatic specimens is shown in Fig. 2B. In hyperglycemic controls, insulin-positive cells were markedly diminished (Fig. 2B, b) as compared with normoglycemic controls (Fig. 2B, a). In contrast, in STZ+BMT mice, islet numbers were restored and sizes varied with some being enlarged (Fig. 2B, c). Notably, in the large view (Fig. 2B, d), a major population of insulin-positive cells in STZ+BMT mice is located in the vicinity of pancreatic ducts which were stained with anti-keratin/cytokeratin antibody (Fig. 2C).

Next, we performed double-immunostaining using antibodies against insulin and glucagon. In immunofluorescent experiments (Figs. 2C and 2D), to avoid overlapping staining of GFP with FITC, BM cells obtained from wild-type C57BL/6J mice, but not from GFP transgenic mice, were transplanted. Compared with islets of normal and STZ-treated mice (Fig. 2D, a and b), islets in STZ+BMT mice exhibited normal architecture with slightly fewer β cells surrounded by α cells (Fig. 2D, c).

To exclude the possibility that irradiation suppresses inflammation in response to STZ and prevents β cell injury, STZ-treated mice were exposed to lethal irradiation (10 Gy) without subsequent BM cell infusion. Lethal irradiation alone did not lower blood glucose in STZ-treated mice. Pancreatic islets were diminished in size, as in hyperglycemic controls, 9 days after irradiation (mice died 10-14 days after lethal irradiation without BMT in our experiment) (data not shown). Next, to examine prolonged effects of irradiation, mice were sublethally irradiated (5 Gy). Sublethal

irradiation alone likewise did not significantly improve hyperglycemia in STZ-treated mice (data not shown), suggesting that irradiation does not exert protective effects against STZ-induced β cell injury.

To further examine whether these islets in STZ+BMT mice were regenerated or only protected from STZ injury, BrdU staining was performed. In islets of normoglycemic (Fig. 3A) and hyperglycemic (Fig. 3B) controls, there were very few BrdU-positive cells. In contrast, islets of STZ+BMT mice (10 days after BMT) contained substantial numbers of BrdU-positive cells in and around islets, and some were detected among the pancreatic ductal cells (Fig. 3C). In other sections as well, islets containing BrdU-positive cells were mostly located near ducts and blood vessels. Most BrdU-positive cells in islets were insulin-positive, while those outside of the islets, mostly in the ductal structure, did not express insulin. (Fig. 3D). Given reports that pancreatic stem/progenitor cells exist among ductal cells (24-26), BMT following STZ treatment might stimulate the generation of new islets from ductal progenitor cells as well as proliferation of β cells in this model.

We also quantitatively examined the time courses of islet numbers, cell number per islet and the percentage of BrdU-positive cells among islet cells after BMT (Table 1). Though decreased by STZ, islet number was significantly increased 10 and 15 days after BMT. The peak islet number was greater than in normoglycemic control mice by 47%. The islet number and percentage of BrdU-positive cells among islet cells were also increased

through 10 days after BMT, and then fell to normoglycemic control levels. These findings clearly indicate that BMT induces β cell regeneration, resulting in pancreatic islet restoration in this model.

Although no BM-derived insulin-positive cells were detected, the regenerated islets were surrounded by BM-derived CD45-positive cells.

To investigate whether BM-derived cells transdifferentiated into insulin-producing cells in our model, pancreases from STZ+BMT mice on day 35 were immunostained with anti-insulin antibody, followed by an intensive search for both insulin- and GFP-positive cells using confocal fluorescence microscopy. However, no double-positive cells were detected (Fig. 4A), suggesting that regenerated β cells in STZ+BMT mice are derived from recipient cells. In contrast, intriguingly, GFP-positive, i.e. BM-derived, cells were located around islets (Fig. 4A). In STZ+BM-infused mice, no GFP-positive cells were detected around islets (data not shown). Immunostaining with anti-GFP antibody confirmed that GFP-positive cells exist around islets of STZ+BMT mice (Fig. 4B, black arrows indicate islets). To identify the lineage of BM-derived cells around islets, we used several antibodies to immunostain lineage markers. GFP-positive cells around islets were CD45-positive (Fig. 4C, white arrows indicate islets), although these cells were not positively stained with F4/80, CD68 (macrophage lineage), CD3/CD5 (T cell lineage) or CD20 (B cell lineage) (data not shown), suggesting immature hematopoietic cells. We additionally examined whether these BM-derived cells are positive for an endothelial cell

marker, CD31 (PECAM-1). Although a few GFP-positive cells were positive for CD 31 (Fig. 4D, red arrow), most BM-derived cells in or around islets were not positively stained with this endothelial marker. Taken together, these observations suggest that donor immature hematopoietic cells, which may be expanded and mobilized to peripheral blood after BMT, initiate β cell regeneration.

Mobilization of BM-derived cells is necessary for the glucose-lowering effect of BMT after STZ administration.

To determine whether BM-derived cell mobilization is pivotal in this process, we investigated the effects of BMT on β cell regeneration using eNOS-deficient (*Nos3*$^{-/-}$) mice as a model for impaired BM-derived cell mobilization. In *Nos3*$^{-/-}$ mice, mobilizations of hematopoietic stem cells and endothelial progenitor cells from BM were reportedly impaired after myelosuppression. Deficiency in eNOS reportedly reduces hematopoietic recovery in response to 5-fluorouracil (5-FU) treatment due to impaired progenitor cell mobilization (20). Therefore, we performed similar experiments using *Nos3*$^{-/-}$ mice. First, we compared two BMT groups, i.e. *Nos3*$^{+/+}$ donors to *Nos3*$^{+/+}$ recipients (*Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice) and *Nos3*$^{-/-}$ donors to *Nos3*$^{-/-}$ recipients (*Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice).

Peripheral white blood cells (WBCs) were counted after lethal irradiation and subsequent BM infusion (Fig. 5A). Myelosuppression after irradiation was profound and recovery of peripheral WBC counts was markedly delayed in *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice versus *Nos3*$^{+/+}$ *to*

Nos3$^{+/+}$ mice. Thus, eNOS deficiency impairs hematopoietic reconstitution after not only 5-FU treatment but also BMT. We measured the blood glucose levels after STZ administration followed by BMT (Fig. 5B). In *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice, STZ-induced hyperglycemia was improved to nearly normoglycemic control levels 40 days after the first STZ administration, consistent with the findings shown in Fig. 1C. In contrast, in *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice, BMT did not improve STZ-induced hyperglycemia (Fig. 5B). Thus, eNOS function is essential for improving hyperglycemia after BMT.

In *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice, not only mobilization of BMT-derived progenitor cells, but also pancreatic endothelial function may be impaired due to systemic eNOS deficiency. Therefore, we performed an additional BMT, i.e. *Nos3*$^{-/-}$ donors to *Nos3*$^{+/+}$ recipients (*Nos3*$^{-/-}$ *to Nos3*$^{+/+}$ mice), whose eNOS is intact in pancreatic blood vessels. In *Nos3*$^{-/-}$ *to Nos3*$^{+/+}$ mice, myelosuppression was profound and subsequent recovery of the WBC count was delayed as compared to *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice, but this delay in recovery was significantly less severe than that seen in *Nos3*$^{-/-}$ recipients (Fig. 5A). In *Nos3*$^{-/-}$ *to Nos3*$^{+/+}$ mice, blood glucose levels also reached mid-range values; the glucose-lowering effects of BMT did occur but were significantly blunted (Fig. 5B). These findings indicate that the lack of hyperglycemia improvement in *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice is not attributable solely to the impaired pancreatic endothelial function of recipients. The glucose-lowering effect of BMT inversely correlates with the severity of myelosuppression and delayed recovery which apparently reflects impaired mobilization of BM cells to peripheral blood.

BMT-induced β cell regeneration was impaired in STZ-treated Nos3$^{-/-}$ mice.

To quantify BMT-induced β cell regeneration in *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ and *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice, pancreatic insulin contents 40 days after STZ (30 days after BMT) were measured (Fig. 5C). Compared to *Nos3*$^{+/+}$ controls without STZ treatment, STZ-treated *Nos3*$^{+/+}$ mice had markedly lower pancreatic insulin contents. In *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice, BMT partially restored pancreatic insulin contents, consistent with our findings that plasma insulin levels were partially restored by BMT (Fig. 1D). In contrast, in *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice, BMT effects on pancreatic insulin contents were very limited; pancreatic insulin contents were significantly lower in *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice than in *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice (Fig. 5C).

Next, changes in islet numbers and percentage of BrdU-positive cells among islet cells in response to BMT were compared between *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice and *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice. While islet numbers were increased in STZ-treated *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice during the period 7-15 days after BMT, islet numbers were significantly less in STZ-treated *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice (Fig. 5D). In addition, while percentages of BrdU-positive cells among islet cells were markedly increased in STZ-treated *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice 7-10 days after BMT, there were significantly fewer such cells in STZ-treated *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice (Fig. 5E). These results suggest that impaired BM-derived cell mobilization in *Nos3*$^{-/-}$ mice suppresses BMT-induced β cell regeneration after acute injury.

BM-derived CD45-positve cells around islets are important for β cell regeneration

induced by BMT.

To examine whether impaired mobilization of BM-derived cells in *Nos3*$^{-/-}$ mice affects hematopoietic cell assembly around islets and β cell regeneration, we compared pancreases from *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ and *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice using anti-insulin and CD45 antibodies (Fig. 5F). In *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice, substantial numbers of CD45-positive cells were detected in and around the regenerated islets (*red arrows* in Fig. 5F, c-e). No such cells were detected around islets in *Nos3*$^{+/+}$ or *Nos3*$^{-/-}$ mice treated with STZ alone (Fig. 5F, b and g), suggesting that STZ-induced inflammation alone is not responsible for recruiting these cells. In pancreases from *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice, regenerated islets were located near pancreatic ducts and blood vessels (*white arrows* in Fig. 5F, c-e). In contrast, β cell regeneration was markedly impaired in *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice (Fig. 5F , h-j) and far fewer CD45-positive cells were present in and around islets in *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ than in *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice. These results support the notion that BMT-induced BM-derived cell mobilization is critical for regeneration of recipient β cells from stem/progenitor cells in pancreatic ducts.

DISCUSSION

Recently, considerable research attention has focused on pancreatic β cell regeneration. In particular, several previous studies examined the role of BM-derived cells in β cell regeneration using BMT (10-18), but no definitive conclusions have yet been reached. In this study, we clearly demonstrate that BMT can regenerate recipient β cells under certain conditions. Our data supported those of a previous report (14) showing BMT to improve hyperglycemia in STZ-induced diabetic mice via regeneration of recipient pancreatic β cells. Herein, we attempted to elucidate the mechanisms whereby BMT induces β cell regeneration.

First, we demonstrated that BMT, but not simple BM cell infusion without pre-irradiation, promotes β cell regeneration after STZ-induced injury. What are the differences between these procedures? BMT involves lethal irradiation and subsequent BM cell infusion. We confirmed, using FACS analysis, that recipient BM is essentially replaced with that of donor mice after BMT. In contrast, mice receiving BM cell infusion alone without pre-irradiation showed no BM replacement with donor-derived cells. Myelosuppression and subsequent expansion of donor BM cells take place in BMT. During this process, donor BM cells home to the BM microenvironment and progenitor cells mobilize and expand in the peripheral blood (27). In contrast, simple BM cell-infusion does not induce homing or expansion of donor BM cells. Therefore, expansion of immature BM cells, which are rarely detected in peripheral blood in normal circumstances, is likely to be

important for β cell regeneration after BMT. We ruled out the possibility that irradiation suppresses inflammation in response to STZ administration and prevents β cell injury. STZ-treated mice were exposed to lethal (10 Gy) and sublethal (5 Gy) irradiation without subsequent BM cell infusion. Irradiation alone had no effect on hyperglycemia or β cell number in STZ-treated mice. Furthermore, in STZ+BMT mice on day 2 after BMT, islet numbers and cell numbers per islet were both significantly decreased by STZ, but were restored by day 10. Thus, it is unlikely that irradiation itself protects β cells.

Next, we found that a major population of post-BMT islets were located near pancreatic ducts and blood vessels. This observation raises possibilities regarding the origins of post-BMT islets. In general, multipotent adult stem cells are located in somatic tissues, which maintain and regenerate impaired tissues (28, 29). However, there is considerable controversy regarding the existence and location of 'pancreatic tissue stem cells' (30, 31). Previous studies have shown pancreatic stem/progenitor cells in ductal epithelium (24-26). However, recent reports suggest that β cells arise only from self-duplication of preexisting β cells, i.e. β cells cannot be derived from non-β cell progenitors (32, 33). In this study, post-BMT islets were located near pancreatic ducts. In addition, BrdU-positive cells were detected in the vicinity of pancreatic ducts in STZ+BMT mice. After islet numbers had been decreased by STZ, a rise above normoglycemic control levels was seen, indicating new islet formation. In addition, BM-derived cells accumulated in and around post BMT-islets. Thus, BM-derived cells are likely to stimulate proliferation and

differentiation of pancreatic stem/progenitor cells in ductal epithelium, resulting in new islet formation. Given the observation that BrdU-positive cells in islets expressed insulin, these cells must still have been proliferative after differentiation into pancreatic β cells. However, further studies, focusing on the origin of newly generated islets, are needed to support this speculation. While BM-derived cells which accumulated around the islets in STZ+BMT mice were CD45-positive, immunohistochemical studies revealed that these cells do not express mature T or B lymphocyte or macrophage markers. Taken together with the finding that simple BM infusion without pre-irradiation induced neither β cell regeneration nor accumulation of BM-derived cells (data not shown), we speculate that these immature BM-derived cells send signals triggering proliferation and differentiation of stem/progenitor cells into β cells. Our next goal is identification of these signals.

To examine the causal relationship between BM-derived cell mobilization and BMT-induced β cell regeneration, we performed similar experiments using a model of impaired BM-derived cell mobilization. Mechanisms underlying mobilization of hematopoietic and endothelial progenitor cells from BM after myelosuppression have been studied in detail (34). After myelosuppression, secreted cytokines/chemokines, such as granulocyte-colony stimulating factor, stromal cell-derived factor and vascular endothelial growth factor, activate matrix metalloproteinase (MMP)-9 in the BM microenvironment. Activated MMP-9 processes membrane-bound KitL, releases it as sKitL, followed by binding of sKitL to c-kit on the stem cell surface and stimulation of

its mobilization from the BM. Since nitric oxide from BM is necessary for MMP-9 activation, sKitL production and the resultant mobilization of BM-derived cells are impaired in *Nos3*$^{-/-}$ mice (20). Therefore, using *Nos3*$^{-/-}$ mice, we examined the effects of BMT on blood glucose levels and glucose β cell regeneration. We first confirmed that recovery of the WBC count after BMT was significantly delayed in *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice as compared to *Nos3*$^{+/+}$ *to Nos3*$^{+/+}$ mice. Judging from the doubling time of hematopoietic cells, a one week delay in WBC recovery indicates marked impairment of BM cell mobilization by approximately two orders of magnitude. In *Nos3*$^{-/-}$ *to Nos3*$^{-/-}$ mice, BMT had virtually no effects on blood glucose levels, pancreatic insulin contents, islet numbers or percentage of BrdU-positive cells among islet cells. In addition, far fewer CD45-positive cells were detected in and around islets. These results support the notion that BMT-induced BM-derived cell mobilization plays a pivotal role in β cell regeneration from ductal progenitor cells.

Neovascularization in ischemic regions is also impaired in *Nos3*$^{-/-}$ mice, because of decreased mobilization of BM-derived endothelial progenitor cells (20). The microvasculature is well-developed in pancreatic islets (35). Endothelial signals are reportedly important for islet development (36), insulin gene expression and β cell proliferation (37). In the present study, a small population of BM-derived cells around regenerated islets was positively stained with CD31, although these cells were largely CD31-negative. This obeservation is consistent with the results of previous report (14).

Therefore, in addition to hematopoietic progenitor cells, endothelial progenitor cells mobilized from BM may contribute to β cell regeneration after BMT by promoting islet microvasculature formation. In addition, BM-derived endothelial progenitor cells have been shown to contribute to neovascularization in impaired tissues, including myocardial (38) and hind limb ischemia (39). Recruitment of these cells reportedly occurs in response to acute injury of β cells (19). Taken together with the finding that, when BMT was performed 30 days after STZ treatment, hyperglycemia-improving effects were far smaller, acute STZ injury might trigger migration of immature BM-derived cells to the injured pancreas.

We do not rule out the importance of eNOS in pancreatic blood vessels for BMT-induced β cell regeneration. However, in *Nos3*$^{-/-}$ *to* *Nos3*$^{+/+}$ mice, which have decreased BM eNOS (because of BM replacement with eNOS-deficient cells) with intact pancreatic eNOS, the blood glucose lowering effects of BMT were significantly blunted as compared to *Nos3*$^{+/+}$ *to* *Nos3*$^{+/+}$ mice. Thus, glucose-lowering effects correlated inversely with the severity of myelosuppression and delayed recovery of the peripheral WBC count, suggesting the importance of BM-derived cell mobilization, rather than eNOS activity in pancreatic blood vessels, in BMT-induced β cell regeneration.

In summary, BMT promotes β cell regeneration after STZ-induced injury. A series of BMT experiments using *Nos3*$^{-/-}$ mice demonstrated BM-derived cell mobilization to be essential for BMT-induced β cell regeneration. Acute injury with STZ treatment may

trigger recruitment of immature BM-derived cells to the injured pancreas. Recruited BM-derived cells may then stimulate stem/progenitor cells located in the recipient pancreas, resulting in islet regeneration. To our knowledge, this is the first report showing mobilization of BM-derived cells to be involved in β cell regeneration in diabetic animals. From the view point of clinical application, it is important to study the effects of myelosuppression-inducing reagents, such as anti-tumor drugs, on pancreatic islet regeneration.

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Abbreviations

BM, bone marrow; BMT, bone marrow transplantation; STZ, streptozotocin; GFP, green fluorescence protein; eNOS, endothelial nitric oxide synthase; BrdU, bromo-deoxyuridine; PBS, phosphate buffered saline; FACS, fluorescence-activated cell sorting

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