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Hepatocyte growth factor is constitutively produced by donor-derived bone marrow cells and promotes regeneration of pancreatic β-cells ☆

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Abstract

Recent studies have demonstrated that the transplantation of bone marrow cells following diabetes induced by streptozotocin can support the recovery of pancreatic β-cell mass and a partial reversal of hyperglycemia. To address this issue, we examined whether the c-Met/hepatocyte growth factor (HGF) signaling pathway was involved in the recovery of β-cell injury after bone marrow transplantation (BMT). In this model, donor-derived bone marrow cells were positive for HGF immunoreactivity in the recipient spleen, liver, lung, and pancreas as well as in the host hepatocytes. Indeed, plasma HGF levels were maintained at a high value. The frequency of c-Met expression and its proliferative activity and differentiative response in the pancreatic ductal cells in the BMT group were greater than those in the PBS-treated group, resulting in an elevated number of endogenous insulin-producing cells. The induction of the c-Met/HGF signaling pathway following BMT promotes pancreatic regeneration in diabetic rats.

Keywords: Hepatocyte growth factor; c-Met; Regeneration; β-cell; Bone marrow transplantation

Several recent reports have suggested that adult bone marrow harbors cells with broad developmental plasticity extending beyond the well-established differentiation potential of bone marrow stem cells. After extensive passages in vitro, pluripotential cells derived from bone marrow have been shown to differentiate into endo-, meso-, and ectodermal cells [1]. Nevertheless, other reports have produced conflicting findings [2,3] or attributed the above results to cell fusion [4]. Moreover, growth factor signaling pathways may play a role in post-natal stem cell engraftment and differentiation [5]. Regarding this issue, previous studies on pancreatic regeneration have shown that bone marrow transplantation supports the regeneration of pancreatic β-cells in a mouse model of diabetes mellitus in which the insulin-producing β-cells have been destroyed [6]. This effect does not rely on the transdifferentiation of bone marrow cells; instead, the proposed mechanism stays within the traditional framework of germ layer specification, in

☆ *Abbreviations:* BMC, bone marrow cell; BMT, bone marrow transplantation; PSC, pancreatic stem cell; BMDC, bone marrow-derived cell; STZ, streptozotocin; HGF, hepatocyte growth factor; PDX-1, pancreatic and duodenal homeobox-1; ICC, islet-like cell cluster; PBS, phosphate-buffered saline; BrdU, bromodeoxyuridine; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; DAPI, 4′-6-diamidine-2-phenylindole.

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which bone marrow stem cells differentiate to vascular endothelial cells [7,8]. These cells are thought to secrete inductive factors necessary for the differentiation of stem/progenitor cells into pancreatic lineages [9]. Thus, characterizing the signaling mechanisms involved in the interaction between vascular endothelial cells and endocrine cells is of great importance and may enable novel therapeutic approaches involving the administration of relevant signaling molecules produced by bone marrow-derived cells (BMDC).

Previous investigators have indicated that HGF exerts distinct functions on several types of mesenchyme-originated cells and epithelial cells. HGF acts as a bone marrow stromal-derived factor to stimulate hematopoiesis from progenitor cells. Bone marrow stromal cells have been shown to constitutively produce a significant amount of biologically active HGF that elicits mitogenic, motogenic, and morphogenic activities [10–12]. Moreover, the coupling between HGF and the c-Met receptor integrates complex biological processes, including embriogenesis, angiogengesis, and tissue regeneration [13,14]. Indeed, HGF and c-Met protooncogene are highly expressed during pancreas development [15]. HGF also functions as an insulinotrophic factor and promotes β-cell proliferation and regeneration [16].

In this study, we show for the first time that the c-Met/HGF signaling pathway between engrafted bone marrow-derived cells and c-Met-expressing ductal epithelial cells contributes to the neogenesis of insulin-producing cells from recipient origin.

Materials and methods

Animals and induction of diabetes with streptozotocin. Male Brown Norway rats (95–100 g) were purchased from the Saitama Experimental Animals Supply (Tokyo, Japan) at 5 weeks of age. This study was approved by the Committee on Animal Ethics in the Care and Use of Laboratory Animals of the Showa University Medical School. The animals were allowed free access to standard laboratory chow and water.

The animals were intravenously injected with 50 mg/kg STZ (Sigma–Aldrich, St. Louis, MO, USA) daily for two consecutive days. The STZ was solubilized in sodium citrate buffer, pH 4.5, and injected within 15 min of preparation. Only animals with a plasma glucose level of 350 mg/dL or higher after the STZ treatment had been completed were used.

Isolation of bone marrow cells. Rat BMCs were extracted from the tibias and femurs. The BMCs were isolated using a previously described method [17]. Briefly, the rats were anesthetized with sodium pentobarbital, and the BMCs were obtained by flushing the femurs. The femurs were accessed through a laparotomy to avoid contamination and to increase the cell yield. The cells were suspended in DMEM (Sigma)/10% FBS (Sigma) followed by red cell lysis; after two washes, the pellet was resuspended in culture medium at 4 °C for further use.

Labeling of donor-bone marrow cells. The cells were centrifuged at 400 g for 10 min in a serum-less medium. PKH26 dye (Sigma), a red fluorescent cell linker (4×10^{-6} M), was added, and the cell suspension was incubated for 5 min at 25 °C, periodically inverting the tube. Then, the sample was centrifuged twice at 400*g* for 10 min at 25 °C to remove the cells from the staining solution and the pellet was resuspended in DMEM for transplantation. The cell viability of the stained samples was examined with trypan blue staining before transplantation, showing a uniform BMC viability of over 95% [18].

Transplantation of bone marrow cells to diabetic rats. A rat model of STZ-induced pancreatic damage causing hyperglycemia was used to assess pancreatic repair upon the transplantation of syngenic bone marrow cells without myeloablation [17,19,20]. Seven days after β-cell injury, the recipient rats were prepared for a BMC infusion. The rats were anesthetized (ketamine, 2 g/kg body weight, i.p.), and a BMC suspension (500 μL) containing a median of 6.0 ($5.0–7.0) \times 10^8$ cells was infused using a tuberculin-sterile syringe with a 23 G needle via the tail vein. The control rats were injected with PBS (500 μL).

Plasma glucose, insulin, and HGF level monitoring. Peripheral blood was collected on 0, 7, 14, 28, 35, and 42 days after the BMT, and the blood glucose level was measured between 8:00 and 10:00 AM using a colorimetric glucose assay kit (Sigma, St. Louis, MO, USA). The serum insulin level was quantified at 0, 28, and 42 days after STZ-administration using a radioimmunoassay (Linco Research, St. Charles, Missouri, USA). Plasma HGF levels were measured using rat HGF ELISA kits (Institute of Immunology, Tokyo, Japan) [21].

Pathology examination and histologic staining. The rats were killed at 7, 14, 28 or 42 days after the BMC injection, and tissue specimens were collected for fluorescence immunohistochemistry analysis. For the in vivo detection of proliferating cells in the organs of the recipient rats, transverse sections (about 5 mm thick) were made across the major axes of the liver, lung, kidney, heart, thymus, spleen, and pancreas. Each pancreas was fixed overnight in 10% buffered formalin and embedded in frozen-tissue embedding gel (OTC compound; Miles, Elkhart, IN). Serial sagittal cryosections were cut at a thickness of 5 μm. Three individual sections per animal, each section separated from the others by a distance of at least 200 μm, were selected for each immunostaining analysis. Sections were immunostained with rabbit anti-insulin antibody (diluted 1:200; Santa Cruz Biotechnology), rabbit anti-c-Met antibody (SP 260 1:200; Santa Cruz Biotechnology), rabbit-raised polyclonal anti-HGF antibody (1:50; Immuno-Biological Laboratories, Tokyo, Japan), mouse anti-human cytokeratin 20 antibody (1:100; Dako, Carpinteria, CA), and rabbit anti-human PDX-1 antibody (1:1000; a generous gift from Dr. H. Kaneto, Osaka University, Japan) [22]. FITC-conjugated anti-mouse (1:50; Chemicon International, Temecula, CA), FITC-conjugated anti-rabbit (1:200; Chemicon International), and TRITC-conjugated anti-rabbit antibody (1:100; Sigma) were used as secondary antibodies. Nuclear regions were stained by DAPI counterstaining (Vector, Burlingame, CA). Isotype-matched antibodies and PBS were used as controls for the stained sections. Images were obtained using a fluorescent microscope (Olympus BX51, Tokyo, Japan), and digital images were captured using Axiovision 3.0 software and a confocal laser-scanning microscope (Leica TCS SP2; Heidelberg, Germany); the sections were scanned for fluorescence using the 488-nm line from an argon ion laser and photodetectors (580/30 nm for PKH26/ TRITC fluorescence).

For the replication studies, an intraperitoneal injection of BrdU (50 mg/kg body weight in PBS; Sigma) was given 2 h before pancreas extraction. The labeled cells were visualized using immunostaining with BrdU according to a modification of a previously described method [23]. Briefly, sections were incubated with a mixture of nuclease and mouse anti-BrdU antibody (1:50; Dako) for 2 h at room temperature, washed with Tris–HCl (pH 7.6), incubated with peroxidase anti-mouse immunoglobulin G for 30 min, and stained with 3,3′-diminobenzidine tetrahydrochloride (DAB) using a peroxidase substrate kit (Dako). The number of ductal and islet cells with immunostained nuclei was separately quantified using a microscope (Olympus BX40, Tokyo, Japan); a morphological analysis was performed using NIH image analysis software.

Histomorphometry. A morphometric analysis was performed to assess c-Met receptor and BrdU expression in pancreatic ductal epithelial cells and islet cells, insulin expression in islet-like cell clusters (ICC) and islets, HGF expression, and the population of BMDCs

labeled with PKH26. Tissue sectioning and analyses were performed in 3–5 rats per group. For each of the figures, 10 sections were isolated from each tissue-type in each rat, and a minimum of 3 sections per rat (1st, 5th, and 10th sections) were analyzed according to a blind study design to derive a mean ± SEM value that was then used to draw conclusions.

For each treatment group, the data from each rat were then pooled to calculate the overall mean shown in the figures. Donor-derived cells in three randomly selected fields more than 200 μm apart were counted under a microscope at a magnification of 200×, and the results were averaged for each animal [8]. The number of BMDCs per mm^2 in the pancreas, liver, spleen, lung, kidney, heart, and thymus of the hyperglycemic rat recipients at 7, 14 or 42 days after transplantation was calculated using a laser scanning confocal microscope. Immunofluorescent signals for c-Met receptors on ductal and islet cells and cells with positive DAPI nuclear staining were counted to determine the number of c-Met-positive cells per mm^2. Double staining was used to quantify cells positive for both c-Met and DAPI staining. At least 200–300 nuclei in the ductal and islet cells were analyzed for each sample. Using the same methods, the number of BrdU-positive ductal and islet cells undergoing DNA synthesis was also counted. The number of insulin-positive cells per mm^2 in pancreatic ICC and islet tissues was also calculated; ICCs were defined as clusters of insulin-positive cells less than eight cells across. To measure the number of insulin-producing cells in ICCs and islets, 20 fields per section were counted [15]. The morphological analysis was done using unselected total cell populations by a researcher who was unaware of the identity of the samples.

Statistics. Blood glucose, serum insulin, and HGF concentrations were shown as the mean ± SEM for rats in the transplanted cell or mock (PBS) injection groups. The results were analyzed for significance using a two-tailed Student's *t* test.

Results

Effect of bone marrow cell transplantation on plasma glucose and insulin concentrations

In STZ-treated rats, the morning-non-fast plasma glucose levels were in the hyperglycemic range. The administration of BMCs partially reduced the plasma glucose concentration (BMT group vs. PBS-group, $*p < 0.05$). The effect of the BMT was apparent fourteen days after the BMT. The morning-non-fast plasma insulin concentrations were significantly higher in the BMC-administered rats than in the PBS-treated rats, and the effect of the BMT lasted for 42 days after the BMT (BMT group vs. PBS-group, $*p < 0.05$). Hence, the effect of the BMT did not affect the body weight (Table 1).

Table 1
BMCs or PBS was injected into STZ-induced diabetic rats

	Control	PBS	BMT
Body weight (g)	257.4 ± 3.5	149.2 ± 11.1	167.6 ± 6.4 NS
Plasma glucose (mg/dL)	126 ± 60	748 ± 54	509 ± 23*
Plasma insulin (ng/mL)	1.1 ± 0.2	0.11 ± 0.02	0.40 ± 0.09*

Various parameters were measured at day 42. Plasma glucose and insulin levels were measured under non-fasting conditions.
* $p < 0.05$ vs. PBS-treated group, $n = 5$; data are means ± SEM.

Donor-derived cells generate microchimera in the spleen, liver, lung, and pancreas following transplantation

Rats were made diabetic by IV injection with STZ. Once the animals had stable blood glucose values, they received PKH26-labeled donor-derived bone marrow cells. All of these animals survived the procedure, and no inflammatory episodes of GVHD (graft vs. host disease) were indicated by the clinical and histomorphological findings (data not shown) [19].

Transplanted chimeric BMDCs were analyzed by the PKH-26 signal. At each time point after the peripheral bone marrow injection, PKH26-labeled BMDCs were detected throughout the spleen liver, lung, and pancreas (Fig. 1). BMDCs were found in the pancreas at a density of 15.2 ± 4.2 cells/mm^2 as well as in the spleen (662.9 ± 230.0 cells/mm^2), liver (117.1 ± 45.2/mm^2), and lung (29.8 ± 13.1/mm^2). In this pancreatic injury model, the majority of donor-derived BMCs were distributed throughout the spleen and liver, and a small population of BMDCs were detected in the host pancreas and lung; the population of engrafted BMDCs in the pancreas, in particular, was quite small compared with that in the spleen and liver ($**p < 0.01$) (Fig. 2). These data demonstrated that BMDCs migrated not only to the injured pancreatic tissue, but to the spleen, liver, and lung. The majority of BMDCs were located in red pulp but some were occasionally seen in the white pulp of the spleen, the sinusoidal area of the liver, around alveolar epithelial cells in the lung and at paraacinar sites in the pancreas (Figs. 1C–E). Remarkably, in the host liver at 7 days after the BMT, the majority of BMDCs were found in zones one and two around the Glisson capsule (Fig. 1A); forty-two days after transplantation, however, the cells had moved and were mainly located in zone three of the liver, near the proximity of hepatic central veins (Fig. 1B). Simultaneously, the majority of BMDCs at 7 days after transplantation were located in the red pulp of the spleen, but the cellularity of BMDCs had increased in the white pulp 42 days after transplantation.

Donor-derived BMDCs and host hepatocytes express HGF immunoreactivity

To assess whether donor-derived cells produce HGF, immunofluorescence colocalization studies were undertaken using anti-HGF antibody (Figs. 3A–I). When spleen, liver, pancreas, lung, kidney, heart, and thymus specimens from recipient rats were observed, the occasional colocalization of HGF and PKH26 signals was noted in the spleen, liver, lung, and pancreas (Fig. 3) [12,24,25]. Overall, $56 \pm 12\%$ of the donor-derived BMDCs were positive for HGF immunostaining in each organ tissue from the recipients collected on day 42, and the proportion of HGF-expressing

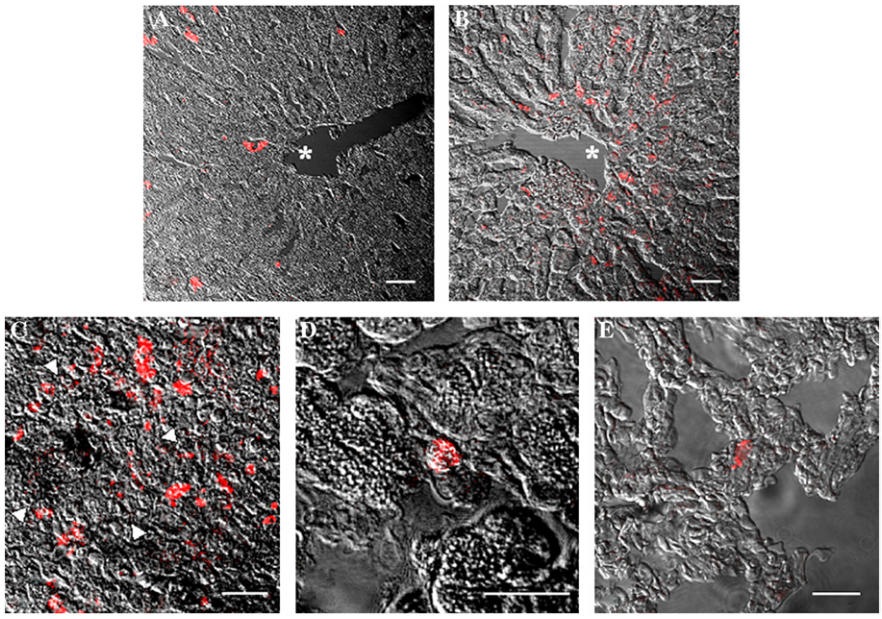

Fig. 1. Laser scanning confocal microscopy images of chimeric BMDCs. PKH26 and phase images were merged. PKH26-labeled BMDCs were detected throughout the liver [day 7: (A), day 42: (B)], spleen (C), pancreas (D), lung (E) [day 42: (C), (D), and (E)], hepatic central vein (*), and white pulp [white triangles in (C)]. Scale bar, 20 μm.

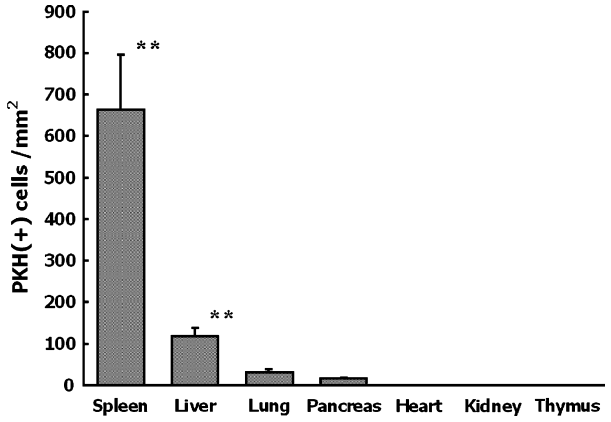

Fig. 2. Morphometric analysis of laser scanning confocal microscopy images of BMDCs. PKH26 signals were observed in host spleen, liver, lung, and pancreas on day 7 (vs. pancreas, $n = 3$, $^{**}p < 0.01$).

donor-derived-BMDCs on day 7 was significantly higher than that in the PBS-treated group (data not shown). In addition, not only the BMDCs, but, remarkably, the hepatocytes in zone three of the liver in proximity of the hepatic central veins expressed an intense HGF immunoreaction. BMDCs engrafted within the sinusoidal area of the liver around the central venous area (zone three) exhibited intense HGF immunoreactivity. The location of the BMDCs coincided with the HGF intensity of the host hepatocytes (Figs. 3A–F).

Plasma HGF level

After BMT, the HGF concentration in the serum of recipients was measured using an ELISA. The constitutive production of HGF was observed in the BMT group (6.0 ± 1.6 ng/mL); the HGF level was 8-fold higher than that in the PBS-treated group and was maintained at an elevated level for up to 42 days after transplantation (Fig. 4; $^{*}p < 0.05$ vs. PBS group).

On day 0 (day 7 after STZ induction), the serum HGF levels in the PBS-treated and BMT groups were elevated because of an STZ-induced inflammatory response.

Characterization of pancreatic ductal epithelial cells by morphometrical analysis

The immunohistochemical morphometry of pancreatic ductal epithelial cells expressing c-Met receptor protein was examined (Fig. 5A), and a strong fluorescent signal specific for c-Met was concentrated at the plasma membrane in PBS and BMT groups. However c-Met receptor expression, which is characteristic of the neonatal state, in the ductal epithelial cells of normoglycemic 7-week (control) rats was extremely low. The number of c-Met-positive pancreatic ductal epithelial cells reached a peak 7 days after BMT with a higher value in the BMT group than in the PBS-treated group significantly (Fig. 5B; $^{**}p < 0.01$.), thereafter it decreased after day 14 and reached the normal level at day 28. Instead, the number of c-Met-positive cells in the islets after BMT was extremely low (Fig. 5B; vs. number of c-Met-positive cells in ducts, $^{***}p < 0.001$).

As shown in Fig. 5E, the mitogenic activity in the pancreatic ducts at day 7 after BMT increased the frequency of BrdU-positive ductal cells undergoing DNA

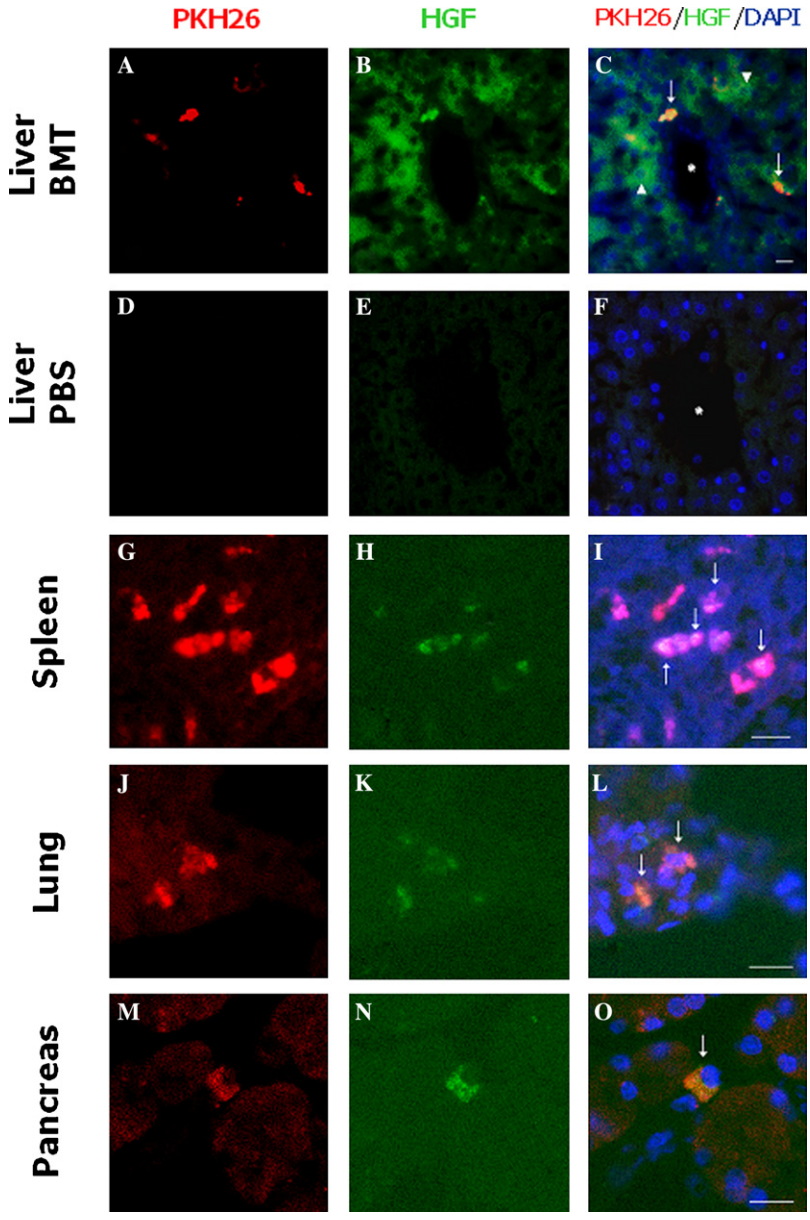

Fig. 3. Triple immunofluorescent detection of HGF, PKH26, and DAPI using fluorescent microscopy. Sections of recipient liver (A–C), spleen (G–I), lung (J–L), and pancreas (M–O) from BMT rats and recipient liver from PBS-treated diabetic rats (D–F). PKH26 (red), HGF (green), merged images (yellow), and nuclear staining with DAPI (blue) are shown. Donor-derived BMCs labeled with PKH26 were positive for HGF (arrows). Host hepatocytes expressing HGF (arrowheads), hepatic central vein (asterisk). Scale bar, 10 μm.

synthesis by two-fold, compared to that in the PBS-treated group ($*p < 0.05$). The number of proliferating cells in the ducts after BMT was 3.4-fold greater than that in the PBS-treated group (Fig. 5F; $**p < 0.01$). These data directly indicated that the proliferating activity of the host ductal epithelial cells was significantly enhanced after BMC transplantation. In contrast, the number of BrdU-positive cells in the islets was extremely low after BMT (Fig. 5F; $***p < 0.001$).

From the aspect of differentiation, the potency of pancreatic and PDX-1 in inducing differentiation into insulin-producing cells was demonstrated in host pancreatic ductal epithelial cells seven days after BMT. In Fig. 5C, the staining of the pancreas with anti-PDX-1 and anti-cytokeratin 20 antibodies revealed PDX-1/cytokeratin 20 double-positive cells in the pancreatic ducts. Note that the ducts of the BMT-treated rats expressed PDX-1 at a high frequency, but PDX-1 expression in the ducts of the PBS-treated group was extremely low seven days after BMT (14 days after STZ-induction.) This indicated that the BMT induced differentiation in the pancreatic ductal epithelial cells to a higher degree than in the PBS-treated group in this model.

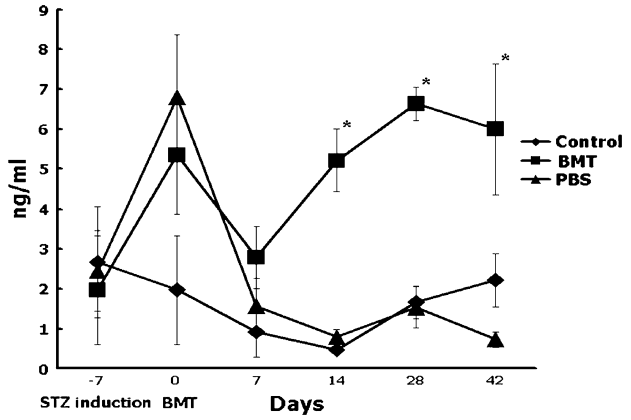

Fig. 4. Quantitative determination of serum HGF level using an ELISA. STZ was administered seven days before the BMT. On the day of the BMT, the serum HGF concentrations in the BMT and PBS-treated groups were high because of an inflammation following STZ-treatment. Data are presented as means ± SEM, $n = 5$, $*p < 0.05$ vs. PBS-treated group.

Immunohistochemical analysis revealed that endogenous pancreatic insulin-producing cells were present in the islet cells (Fig. 6A) and ICC (Fig. 6B); and these had a tendency to exhibit amplified mitotic activity (Figs. 5E and F), resulting in a 3.5-fold greater number of insulin-producing cells per mm^2, with a significant difference between the BMT group and the PBS-treated group ($**p < 0.01$) (Fig. 6C). The number of insulin-positive cells in the islets was 10.5-fold greater than that in the PBS-treated group ($**p < 0.01$) (Fig. 6D), while that in the ICC was 3-fold greater than in the PBS-treated group ($*p < 0.05$) (Fig. 6E).

Discussion

There is evidence that a trophic growth factor, HGF, is produced by bone marrow stromal cells and that HGF secretion acts on the marrow to maintain the

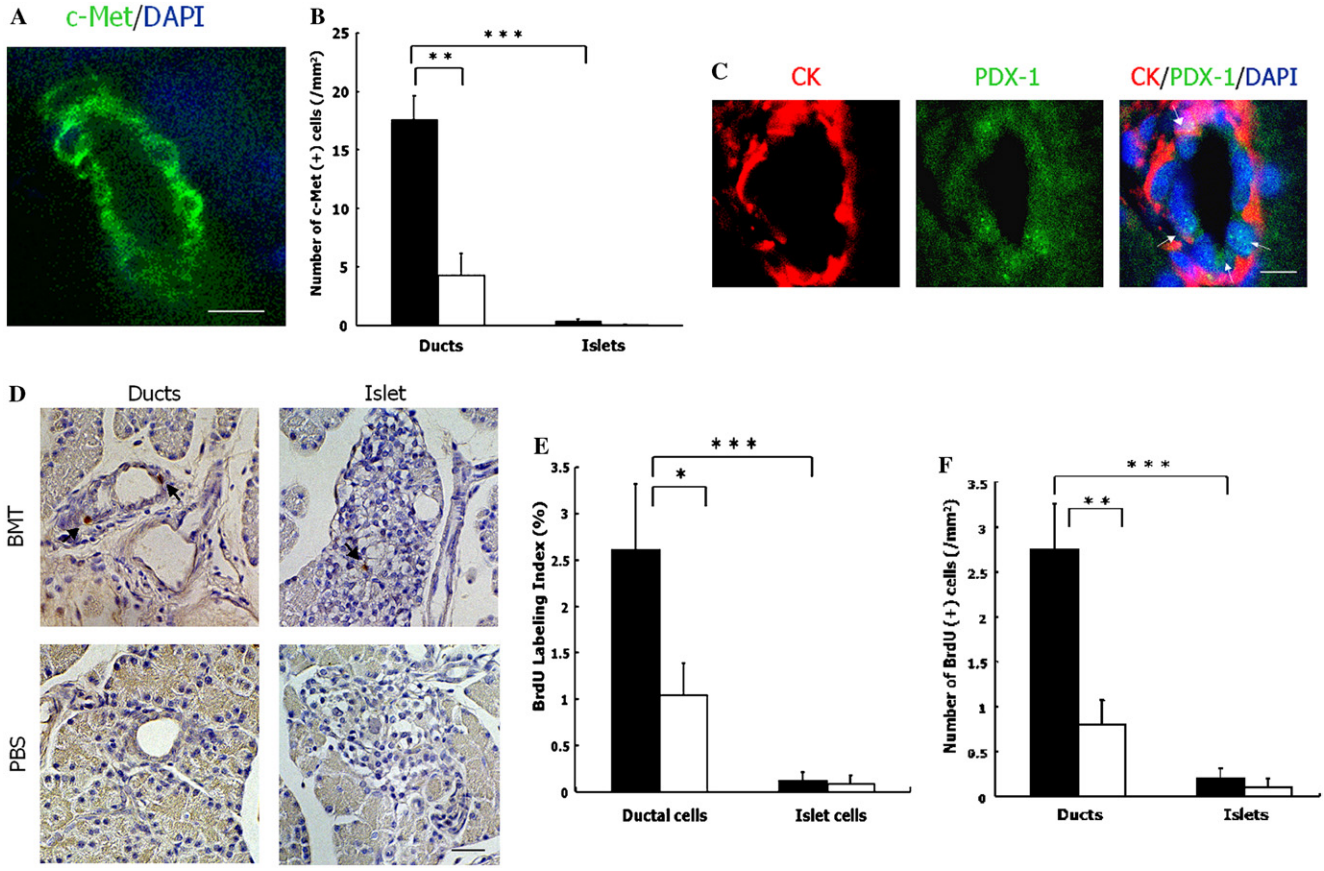

Fig. 5. Morphometric analysis of c-Met receptor expression, proliferative activity, and proliferative response in pancreatic ductal cells. Immunofluorescent detection of c-Met receptor (green) on ductal cells in pancreas and DAPI (blue) on day 7 (A). Scale bar, 20 μm. The numbers of c-Met-positive cells per mm^2 (B), pancreatic ductal cells expressed PDX-1 (green)/cytokeratin (red) double signals on day 7 (arrow). Nuclei were stained with DAPI (blue) (C). Scale bar, 10 μm. BrdU immunolabeled nuclei (brown signals pointed by arrows) indicating those cells that incorporated BrdU into newly synthesized DNA at day 7 (D). Scale bar, 30 μm. The BrdU-labeling index (E) and the number of BrdU-positive cells in pancreatic ductal cells and islet cells per mm^2 (F) were counted in the BMT group (black squares) and the PBS-treated group (white squares), Values represent means ± SEM, $n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

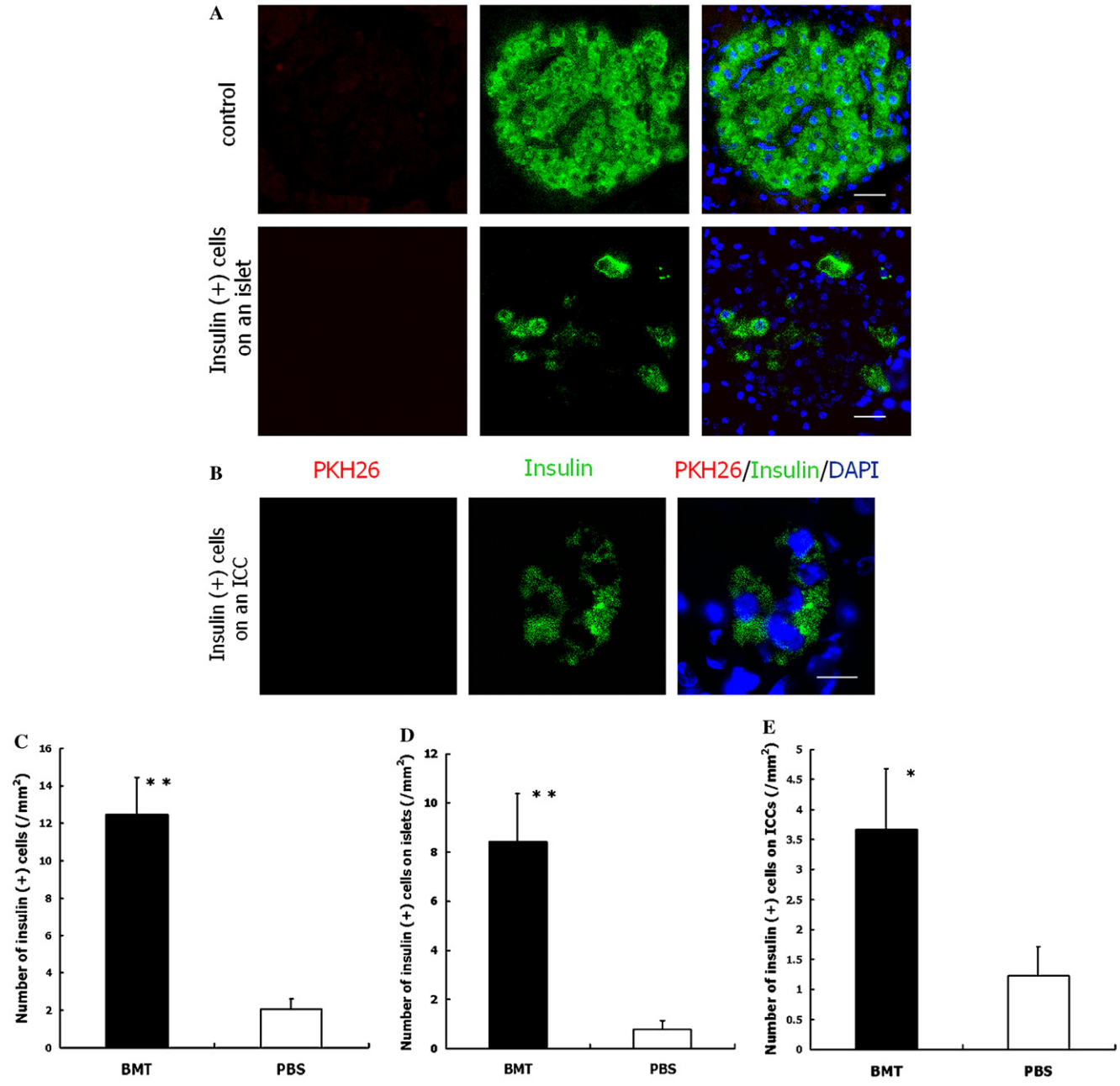

Fig. 6. Morphometric analysis of endogenous insulin-producing cells Histomorphological sections from diabetic rats at day 42 were stained with insulin. Insulin immunofluorescent signals in islet cells (A, bar, 30 μm) and ICC (B, bar, 20 μm) in the pancreas were detected by a fluorescent microscope. Insulin (green), PKH26 (red), DAPI (blue), and insulin/PKH26/DAPI (merged). A quantitative analysis of insulin-producing cells is shown in (C–E). The number of insulin-positive cells per mm^2 (C). The number of insulin-positive cells per mm^2 in islets (D) and in ICC (E) in the pancreas was counted in the BMT group (black squares) and the PBS-treated group (white squares), $n = 5$, $^*p < 0.05$, $^{**}p < 0.01$ vs. PBS-treated group.

self-renewal of hematopoiesis [26] and stimulates DNA synthesis in rat hepatocytes in primary cultures [12]. Furthermore, transplanted marrow cells express HGF activity under "diabetic niche." The location of donor-derived bone marrow cells and that of intense HGF immunoreactivity were nearly identical in this study (Fig. 3C, arrows). These results indicate the possible secretion of HGF from BMDCs. Remarkably, BMDCs were also engrafted within the sinusoid of the liver around the central venous area (zone three), and intense HGF immunoreactivity was also detected in this same region (Fig. 3C, arrowheads). This result might indicate the existence of an "HGF secretion pathway" involving the direct interaction of BMDCs and hepatocytes, and/or paracrine interactions with unknown factors secreted from BMDCs [27].

HGF acts in vivo in a paracrine fashion, but the presence of circulating HGF and the induction of its transcription in distant organs during tissue injury (such as partial hepatectomy) also support an endocrine mechanism [28]. Rats that received bone marrow cells showed a partial reversal of their diabetes status: lower blood glucose levels, an increased concentration of serum insulin, and a number of insulin-producing cells in their pancreas. This result may have derived from the high concentration of plasma HGF, which affected the host c-Met-positive ductal epithelial cells in an endocrine and/or paracrine fashion. HGF is known to induce DNA synthesis in ductal pancreatic cancer cell lines in a dose-dependent manner, leading to a rapid induction of HGF receptor tyrosine phosphorylation and a subsequent time- and dose-dependent increase in c-fos expression [29]. Indeed, exogenous HGF (~8 ng/mL) protected β-cells from death and promoted their proliferation in an HGF gene transfected mouse model of STZ-induced diabetes [30], and HGF (3–30 ng/mL) induced DNA synthesis in a dose-dependent manner in pancreatic cancer cell lines with c-Met expression in vitro [29]. Moreover, the concentration of plasma HGF in our study was 6.0 ± 1.6 ng/mL, and several lines of evidence support an insulinotrophic effect of HGF in this study.

Regarding diabetic status, the activation of c-Met expression in pancreatic ductal epithelial cells should provide insights into the mechanism of the up-regulation of this factor in the presence of high glucose levels [31]. As observed in the PBS-treated group (Fig. 5B), c-Met was expressed in pancreatic ductal epithelial cells in the presence of a high glucose status. Interleukin-1 (IL-1), IL-6, and HGF were also shown to up-regulate c-Met expression in a previous study [32]. Together, these results demonstrated increases in the expression of HGF and its receptor, c-Met, suggesting an endocrine and/or paracrine growth effect in injured β-cells. The number of c-Met-positive pancreatic ductal epithelial cells increased after BMT in the present study (Fig. 5B). These data are based on the theory that HGF secretion from BMDCs acts in a paracrine fashion, and that the intense concentration of plasma HGF acting in an endocrine fashion following the administration of BMCs could cause the ductal cells to amplify c-Met receptor expression, compared to the PBS-treated condition.

Several lines of evidence have indicated that progenitor cells continue to exist in adult pancreas, located in the pancreatic ducts or in the vicinity of the ducts, and that these cells may convert to endocrine cells in response to differentiation signals [33,34]. Moreover, the ductal cells in this model were also positive for c-Met, which was expressed in the adult pancreas in regions similar to those seen in neonatal tissues [15]. c-Met-positive cells in the adult pancreas would possess stem/progenitor cell characteristics similar to those of neonatal c-Met-positive cells [35]. The present study supports the traditional stem cell hypothesis and the new pancreatic stem cell theory. The number of ductal epithelial cells expressing c-Met increased after BMT, and the proliferative activities of the ductal epithelial cells also increased after the BMC administration, as shown by the histomorphometric BrdU-incorporation study (Figs. 5E and F). Marrow transplantation also promotes the differentiation of ducts through the expression of PDX-1 (Fig. 5C).

The regeneration of β-cells reflects both mechanisms: replication from existing differentiated β-cells and the recruitment of new β-cells from a precursor pool. The pancreatic duct was not a major source of islet stem cells in STZ-treated mice on day 5 after β-cell injury [36]. However, the relative contribution of the replication of the existing β-cells to the total β-cell growth is less important in this model seven days after the BMT (fourteen days after STZ-treatment). One may infer that HGF from BMDCs preferentially modulated the β-cell neogenesis pathway induced by PSCs. This is supported by our results concerning the number of ICCs. Neogenesis is considered to consist of β-cell differentiation from PSCs located in ductal epithelial cells, which differentiate into β-cells and migrate to form new islets or replenish existing islets [23]. Actually, we found a significantly higher number of ICCs associated with the ducts as PSC and β-cells in the islets of the pancreas in BMT-treated rats, compared with that in the PBS-treated group. Moreover, the higher mitotic activity of the ductal cells in the BMT group also supports the hypothesis of more active neogenesis from PSCs (Figs. 5E and F). This phenomenon was similar to the model of β-cell regeneration after insulin therapy [37]. These observations raise the possibility that BMDCs may also promote an increase in the number of local pooled β-cell progenitors, which when modulated through the c-Met/HGF signaling pathway lead in turn to an increase in insulin-producing cells, further enhancing the number of pooled β-cell precursor cells under diabetic conditions.

The interaction between c-Met and HGF, which is mediated by a signal exchange between epithelial and mesenchymal cells [38], plays an essential part in pancreatic development [15,39]. On binding to its high-affinity c-Met receptor, HGF acts on a variety of epithelial cells in multiple ways: as an activator of mitogenesis [40], motogenesis [14], and morphogenesis [28]. The number of ICCs budding out from cultured ductal epithelial cells is increased by HGF [41]. These findings, taken together, suggest that the c-Met /HGF interaction is critically responsible for the growth and differentiation of pancreatic stem and progenitor cells during development, homeostatic cell turnover, and regeneration [23,41]. These results suggest that some BMDCs may play a role in synthesizing exogenous HGF and secretagogues targeted at organs to enhance HGF release, possibly

promoting c-Met-positive pancreatic ductal epithelial cells as PSCs that differentiate into β-cells. Concerning the expression of the surface marker "c-Met," the c-Met/HGF interaction may be critical for the growth and differentiation of pancreatic stem and progenitor cells [35]. These findings permit us to speculate that the c-Met/HGF signaling pathway between BMDCs and PSCs is critically responsible for the development of pancreatic stem cells into adult β-cells in this model.

Further studies are needed to identify the specific cells within the bone marrow that are responsible for the secretion of HGF or various growth factors and cytokines required to initiate regenerative activity in PSCs and to explore whether c-Met- or other specific cell surface marker-positive "PSCs" can proliferate and differentiate into mature insulin-producing cells to restore damaged pancreatic endocrine function. The differentiation and proliferation of β-cells induced by HGF from BMDCs might be an effective approach for ameliorating type 2 diabetes; these physiological actions have prompted us to create bone marrow cells capable of producing a more intense HGF concentration for translational investigations.

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References

[1] D.S. Krause, N.D. Theise, M.I. Collector, O. Henegariu, S. Hwang, R. Gardner, S. Neutzel, S.J. Sharkis, Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell, Cell 105 (2001) 369–377.

[2] Y. Jiang, B.N. Jahagirdar, R.L. Reinhardt, R.E. Schwartz, C.D. Keene, X.R. Ortiz-Gonzalez, M. Reyes, T. Lenvik, T. Lund, M. Blackstad, J. Du, S. Aldrich, A. Lisberg, W.C. Low, D.A. Largaespada, C.M. Verfaillie, Pluripotency of mesenchymal stem cells derived from adult marrow, Nature 418 (2002) 41–49.

[3] R.F. Castro, K.A. Jackson, M.A. Goodell, C.S. Robertson, H. Liu, H.D. Shine, Failure of bone marrow cells to transdifferentiate into neural cells in vivo, Science 297 (2002) 1299.

[4] G. Vassilopoulos, P.R. Wang, D.W. Russell, Transplanted bone marrow regenerates liver by cell fusion, Nature 422 (2003) 901–904.

[5] O. Cleaver, D.A. Melton, Endothelial signaling during development, Nat. Med. 9 (2003) 661–668.

[6] D. Hess, L. Li, M. Martin, S. Sakano, D. Hill, B. Strutt, S. Thyssen, D.A. Gray, M. Bhatia, Bone marrow-derived stem cells initiate pancreatic regeneration, Nat. Biotechnol. 21 (2003) 763–770.

[7] E. Lammert, O. Cleaver, D. Melton, Induction of pancreatic differentiation by signals from blood vessels, Science 294 (2001) 564–567.

[8] V. Mathews, P.T. Hanson, E. Ford, J. Fujita, K.S. Polonsky, T.A. Graubert, Recruitment of bone marrow-derived endothelial cells to sites of pancreatic beta-cell injury, Diabetes 53 (2004) 91–98.

[9] E. Lamment, O. Cleaver, D. Melton, Role of endothelial cells in early pancreas and liver development, Mech. Dev 120 (2003) 59–64.

[10] F. Galimi, G.P. Bagnara, L. Bonsi, E. Cottone, A. Follenzi, A. Simeone, P.M. Comoglio, Hepatocyte growth factor induces proliferation and differentiation of multipotent and erythroid hemopoietic progenitors, J. Cell Biol. 127 (1994) 1743–1754.

[11] I.S. Weimar, D. de Jong, E.J. Muller, T. Nakamura, J.M. van Gorp, G.C. de Gast, W.R. Gerritsen, Hepatocyte growth factor/scatter factor promotes adhesion of lymphoma cells to extracellular matrix molecules via alpha 4 beta 1 and alpha 5 beta 1 integrins, Blood 89 (1997) 990–1000.

[12] K. Takai, J. Hara, K. Matysumoto, G. Hosoi, Y. Osugi, A. Tawa, S. Okada, T. Nakamura, Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis, Blood 89 (1997) 1560–1565.

[13] D.S. Grant, H.K. Kleinman, I.D. Goldberg, M.M. Bhargava, B.J. Nickoloff, J.L. Kinsella, P. Polverini, E.M. Rosen, Scatter factor induces blood vessel formation in vivo, Proc. Natl. Acad. Sci. USA 90 (1993) 1937–1941.

[14] R. Montesano, K. Matsumoto, T. Nakamura, L. Orci, Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor, Cell 67 (1991) 901–908.

[15] T. Otonkoski, V. Cirulli, M. Beattie, M.I. Mally, G. Soto, J.S. Rubin, A. Hayek, A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth, Endocrinology 137 (1996) 3131–3139.

[16] A. Hayek, G.M. Beattie, V. Cirulli, A.D. Lopez, C. Ricordi, J.S. Rubin, Growth factor/matrix-induced proliferation of human adult beta-cells, Diabetes 44 (1995) 1458–1460.

[17] I. Avital, D. Inderbitzin, T. Aoki, D.B. Tyan, A.H. Cohen, C. Ferraresso, J. Rozga, W.S. Arnaout, A.A. Demetriou, Isolation, characterization, and transplantation of bone marrow-derived hepatocyte stem cells, Biochem. Biophys. Res. Commun. 288 (2001) 156–164.

[18] C.M. Traycoff, S.T. Kosak, S. Grigsby, E.F. Srour, Evaluation of ex vivo expansion potential of cord blood and bone marrow hematopoietic progenitor cells using cell tracking and limiting dilution analysis, Blood 85 (1995) 2059–2068.

[19] R. Renkonen, P. Hayry, Bone marrow transplantation in the rat. I. Histologic correlations and quantitation of cellular infiltrates in acute graft-versus-host disease, Am. J. Pathol. 117 (1984) 462–470.

[20] C. Ricordi, N. Murase, C. Rastellini, R. Behboo, A.J. Demetris, T.E. Starzl, Indefinite survival of rat islet allografts following infusion of donor bone marrow without cytoablation, Cell Transplant. 5 (1996) 53–55.

[21] T. Nakamura, S. Mizuno, K. Matsumoto, Y. Sawa, H. Matsuda, T. Nakamura, Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF, J. Clin. Invest. 106 (2001) 1511–1519.

[22] T. Miyatsuka, H. Kaneto, Y. Kajimoto, S. Hirota, Y. Arakawa, Y. Fujitani, Y. Umayahara, H. Watada, Y. Yamasaki, M.A. Magnuson, J. Miyazaki, M. Hori, Ectopically expressed PDX-1 in liver initiates endocrine and exocrine pancreas differentiation

but causes dysmorphogenesis, Biochem. Biophys. Res. Commun. 310 (2003) 1017–1025.
[23] S. Bonner-Weir, L.A. Baxter, G.T. Schuppin, F.E. Smith, A second pathway for regeneration of adult exocrine and endocrine pancreas, A possible recapitulation of embryonic development, Diabetes 42 (1993) 1715–1720.
[24] C.C. Fehlner-Gardiner, H. Cao, L. Jackson-Boeters, T. Nakamura, B.E. Elliott, S. Uniyal, B.M. Chan, Characterization of a functional relationship between hepatocyte growth factor and mouse bone marrow-derived mast cells, Differentiation 65 (1999) 27–42.
[25] I.S. Weimar, N. Miranda, E.J. Muller, A. Hekman, J.M. Kerst, G.C. de Gast, W.R. Gerritsen, Hepatocyte growth factor/scatter factor (HGF/SF) is produced by human bone marrow stromal cells and promotes proliferation, adhesion and survival of human hematopoietic progenitor cells (CD34+), Exp. Hematol. 26 (1998) 885–894.
[26] G. Hartmann, L. Naldini, K.M. Weidner, M. Sachs, E. Vigna, P.M. Comoglio, W. Birchmeier, A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the c-Met receptor and induces cell dissociation but not mitogenesis, Proc. Natl. Acad. Sci. USA 89 (1992) 11574–11578.
[27] M. Satoh, H. Mioh, N. Konishi, I. Takahashi, T. Tamaoki, Characterization of the molecules involved in the hematopoietic microenvironment provided by mouse stromal cell line MC3T3-G2/PA6 using a unique reporter system that analyzes the direct cell-to-cell interaction, Acta Haematol. 98 (1997) 95–103.
[28] J.S. Rubin, D.P. Bottaro, S.A. Aaronson, Hepatocyte growth factor/scatter factor and its receptor, the c-Met proto-oncogene product, Biochim. Biophys. Acta. 1155 (1993) 357–371.
[29] K. Kiehne, K.H. Herzig, U.R. Folsch, c-Met expression in pancreatic cancer and effects of hepatocyte growth factor on pancreatic cancer cell growth, Pancreas 15 (1997) 35–40.
[30] C. Dai, Y. Li, J. Yang, Y. Liu, Hepatocyte growth factor preserves beta cell mass and mitigates hyperglycemia in streptozotocin-induced diabetic mice, J. Biol. Chem. 278 (2003) 27080–27087.
[31] Y. Liu, E.M. Tolbert, A.M. Sun, L.D. Dworkin, In vivo and in vitro evidence for increased expression of HGF receptor in kidney of diabetic rat, Am. J. Physiol. Renal. Physiol. 271 (1996) F1202–F1210.
[32] A. Mohul, L. Lin, A. Beedle, A. Kanbour-Shkir, M.C. DeFrances, Y. Liu, R. Zarnegar, Modulation of c-Met protooncogene (HGF receptor) mRNA abundance by cytokines and hormones: evidence for rapid decay of the 8 kb c-Met transcript, Oncogene 9 (1994) 2045–2052.
[33] D. Gu, N. Sarvetnick, Epithelial cell proliferation and islet neogenesis in IFN-γ transgenic mice, Development 118 (1993) 33–46.
[34] I. Swenne, Pancreatic beta-cell growth and diabetes mellitus, Diabetologia 35 (1993) 193–201.
[35] A. Suzuki, H. Nakauchi, H. Taniguchi, Prospective isolation of multipotent pancreatic progenitors using flow-cytometric cell sorting, Diabetes 53 (2004) 2143–2152.
[36] A. Fernandes, L.C. King, Y. Guz, R. Stein, C.V. Wright, G. Teitelman, Differentiation of new insulin-producing cells is induced by injury in adult pancreatic islets, Endocrinology 138 (1997) 1750–1762.
[37] J. Movassat, C. Saulnier, B. Portha, Insulin administration enhances growth of the beta-cell mass in streptozotocin-treated newborn rats, Diabetes 46 (1997) 1445–1452.
[38] E. Sonnenberg, D. Meyer, K.M. Weidner, C. Birchmeier, Scatter factor/hepatocyte growth factor and its receptor, the c-Met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development, J. Cell Biol. 123 (1993) 223–235.
[39] T. Furukawa, W.P. Duguid, M. Kobari, S. Matsuno, M.S. Tsao, Hepatocyte growth factor and Met receptor expression in human pancreatic carcinogenesis, Am. J. Pathol. 147 (1995) 889–895.
[40] F. Roos, A.M. Ryan, S.M. Chamow, G.L. Bennett, R.H. Schwall, Induction of liver growth in normal mice by infusion of hepatocyte growth factor/scatter factor, Am. J. Physiol. 268 (1995) G380–G386.
[41] V.K. Ramiya, M. Maraist, K.E. Arfors, D.A. Schatz, A.B. Peck, J.G. Cornelius, Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells, Nat. Med. 6 (2000) 278–282.