Biochemical and Biophysical Research Communications 371 (2008) 132–137

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Transplantation of bone marrow derived cells promotes pancreatic islet repair in diabetic mice

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ARTICLE INFO

Article history:
Received 2 April 2008
Available online 15 April 2008

Keywords:
Bone marrow transplantation
Diabetes
Stem cells

ABSTRACT

The transplantation of bone marrow (BM) derived cells to initiate pancreatic regeneration is an attractive but as-yet unrealized strategy. Presently, BM derived cells from green fluorescent protein transgenic mice were transplanted into diabetic mice. Repair of diabetic islets was evidenced by reduction of hyperglycemia, increase in number of islets, and altered pancreatic histology. Cells in the pancreata of recipient mice co-expressed BrdU and insulin. Double staining revealed β cells were in the process of proliferation. $BrdU^+$ $insulin^-$ $PDX\text{-}1^+$ cells, $Ngn3^+$ cells and $insulin^+$ $glucagon^+$ cells, which showed stem cells, were also found during β-cell regeneration. The majority of transplanted cells were mobilized to the islet and ductal regions. In recipient pancreas, transplanted cells simultaneously expressed CD34 but did not express insulin, PDX-1, Ngn3, Nkx2.2, Nkx6.1, Pax4, Pax6, and CD45. It is concluded that BM derived cells especially $CD34^+$ cells can promote repair of pancreatic islets. Moreover, both proliferation of β cells and differentiation of pancreatic stem cells contribute to the regeneration of β cells.

Nowadays, there are diverse therapeutic strategies for diabetes mellitus including oral medication, insulin injection, and transplantation of pancreas or islets. However, each of these strategies has drawbacks. In seeking a more effective method to cure diabetes, the use of stem cells has been considered. Bone marrow (BM) is an important source of easily procurable adult stem cells that include hematopoietic stem cells, mesenchymal stem cells, and endothelial progenitor cells. BM derived stem cells have pleuripotent differentiation capacity since, following BM derived stem cell transplantation, recipient animals contain cells derived from brain [1], muscle [2], hepatic [3], and epithelial cells [4]. However, the low frequency of such events has tempered enthusiasm over the observations. On the other hand, BM derived cells secrete a variety of cytokines and growth factors such as interleukin (IL)-6, IL-8, IL-12, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) [5,6]. Some cytokines and growth factors play a role in the development of pancreatic β cells, and transplanted BM derived cells can migrate into the injured organ and correct the deficient physiology in living animals [7,8].

BM derived cells are capable of transdifferentiation into pancreatic β cells [9]. In this study, upwards of 3% of pancreatic endocrine β cells were shown to be derived from donor BM derived cells within 6 weeks of the transplantation of BM derived stem cells. Other studies have not found evidence of such transdifferentiation [10–12], although the capacity of BM derived stem cells to initiate pancreatic differentiation has been described [13]. Furthermore, BM transplantation ameliorated hyperglycemia in diabetic mice in several studies [14,15] but not in others [11,16]. So, whether BM derived cells transplantation can improve hyperglycemia and, if so, how pancreatic islets regenerate, are still very much equivocal.

To investigate the problem, we assessed blood glucose levels and pancreatic histology of recipient diabetic mice, and the differentiation and distribution of donor cells after the transplantation of BM derived cells. Using a mouse model of induced diabetes, we presently demonstrate that BM derived cells promote repair of pancreatic islets but are unable to transdifferentiate into islet β cells.

Materials and methods

Animals. C57BL/6J mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Green fluorescent protein (GFP) transgenic mice with the C57BL/6J background were purchased from The Department of Cell Biology, Secondary Military Medical University, Shanghai. All the animals were maintained in the animal facility of Zhongshan Hospital, Fudan University, Shanghai.

Antibodies. Guinea pig (GP) anti-mouse insulin (Invitrogen, Carlsbad, CA, USA), rabbit anti-GFP (Millipore, Billerica, MA, USA), goat anti-mouse insulin, rabbit anti-mouse glucagon, rabbit anti-mouse insulin, goat anti-mouse nestin, goat anti-mouse PDX-1, goat anti-Pax4, goat anti-Pax6, goat anti-Nkx6.1, goat anti-Nkx2.2, goat anti-Ngn3, goat anti-CD34 (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-BrdU (Sigma–Aldrich, St. Louis,

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doi:10.1016/j.bbrc.2008.04.033

MO, USA), FITC conjugated chicken anti-mouse second antibody (Santa Cruz), rhodamine conjugated donkey anti-GP secondary antibody (Millipore), Cy2 conjugated donkey anti-rabbit secondary antibody, Cy3 conjugated donkey anti-goat secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) and 4′-6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich).

Induction of diabetes. Mice were injected intraperitoneally (i.p.) with 50 mg/kg streptozotocin (STZ; Sigma–Aldrich) daily on days 1–7. STZ was solubilized in sodium citrate buffer, pH 4.5, and injected within 15 min of preparation. Blood glucose was measured by use of a One Touch Sure Step meter (Johnson & Johnson, Shanghai, China) between 9:00 and 11:00 a.m., every three days from days 0 to 10, and then weekly from days 11 to 52.

BM derived cell isolation and transplantation. BM cells from GFP transgenic mice were flushed from the medullary cavities of femurs and tibias. Viability of cells was tested by the exclusion of trypan blue (Sigma–Aldrich) as previously described [14]. Eight hours before transplantation, recipient mice were sublethally irradiated (500cGy) and BM derived cells (6.5×10^6) from GFP transgenic mice were transplanted into the diabetic mice through a tail vein.

BrdU incorporation. Thymidine analogue, 5-bromo-2′-deoxyuridine (BrdU; Sigma–Aldrich), 0.05 g per gram of body weight, was injected intraperitoneally 24 h before harvesting the pancreata. Pancreata were isolated and processed for fluorescent immunohistochemistry studies.

Histological and morphological analyses. Mice pancreata were excised and fixed overnight in 4% paraformaldehyde at 4 °C. Fixed tissues were processed for paraffin embedding and serial 5 μm thick sections were prepared and stained with hematoxylin/eosin (HE; Beyotime Institute of Biology, Suzhou, China) to assess pancreatic islets histology and morphology in the experimental animals. Islets were observed from every tenth, twentieth, and thirtieth section. Histology and morphology of islets were scored with a Carl-Zeiss microscopy Axiovert 200 (Carl-Zeiss, Jena, Germany) and a computer-assisted image analysis program (AxioVision Ver. 4.0; Carl-Zeiss).

Fluorescent immunohistochemistry. Slides were deparaffinized and blocked for 40 min at room temperature in 0.5% bovine serum albumin (BSA) and phosphate buffered saline (PBS). For the staining of insulin, the sections of paraffin-embedded pancreata were incubated overnight with GP anti-mouse insulin antibody (1:400) at 4 °C, and the sections were incubated in secondary antibody with rhodamine (1:500) at 37 °C for 30 min. Twenty fields were examined for each section and the number of islets and insulin-positive cells in each field was determined. For double staining of insulin and glucagon, the sections were incubated overnight at 4 °C with the respective antibody. Antibodies against insulin and glucagon were diluted 1:400 in PBS. Labeled cells were visualized with the matched biotin conjugated secondary antibody with rhodamine and Cy2. For BrdU staining, sections were pretreated with 2 N HCl for 30 min at 37 °C, then incubated overnight with mouse monoclonal anti-BrdU (1:50). Labeled cells were visualized with the matched secondary antibody with FITC (1:200). For the double staining of GFP and insulin, PDX-1, Ngn3, Nkx2.2, Nkx6.1, Pax4, Pax6, and CD34, insulin and Ngn3, sections were incubated overnight at 4 °C with the respective antibody. Antibodies against GFP, insulin, PDX-1, Ngn3, Nkx2.2, Nkx6.1, Pax4, Pax6, and CD34 were diluted 1:100 in PBS. Labeled cells were visualized with the matched biotin conjugated secondary antibody with Cy2 (1:500) and Cy3 (1:500). Isotype-matched antibodies and PBS were used as controls for stained sections. Nuclear regions were stained by DAPI counterstaining. The images were viewed as described earlier in the text.

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

Results

STZ-induced diabetes in mice

The diabetogenic action of STZ results primarily from its highly specific cytotoxic action on the β-cells of the Islets of Langerhans [17]. Presently, recipient mice were treated daily for seven consecutive days with 50 mg/kg STZ, which induced pancreas injury and

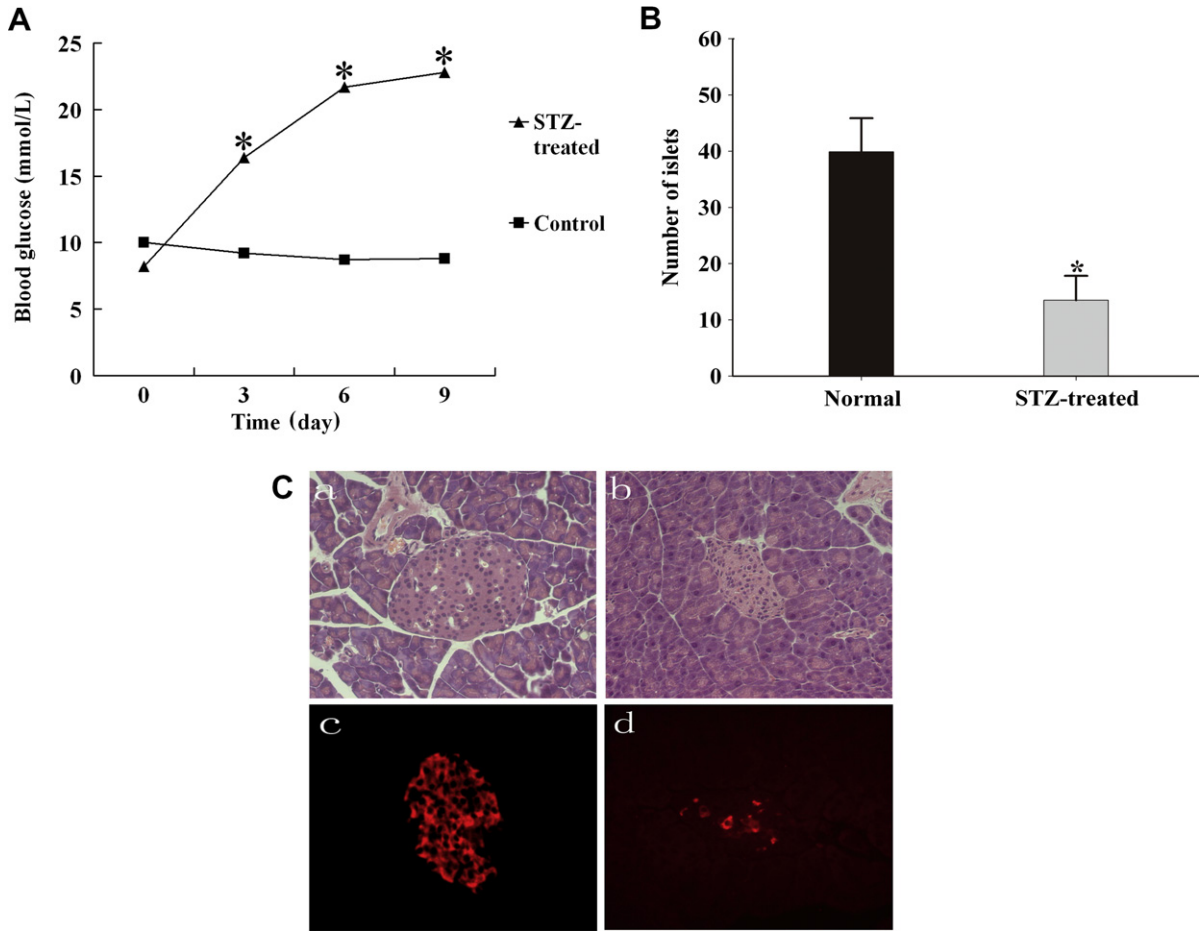

Fig. 1. Pancreatic damage and induction of hyperglycemia after STZ treatment in C57BL/6J. (A) Blood glucose levels in STZ-treated ($n = 53$) and control ($n = 50$) mice at days 3, 6, and 9 were significantly elevated, $^{*}p < 0.01$; (B) compared with the control mice ($n = 12$), the number of islets (day 9) decreased rapidly in STZ-treated mice ($n = 12$), $^{*}p < 0.01$; HE stained (day 9) pancreatic sections of control mice (C-a) and STZ-treated mice (C-b), insulin immunofluorescent (day 9) of pancreatic sections in control mice (C-c) and STZ-treated mice (C-d), When examined at a magnification of 400×, compared with control mice, the islet size was smaller and the number of insulin-expressing cells was significantly reduced.

hyperglycemia. During the seven-day regimen, blood glucose levels increased from normal to become severely hyperglycemic (Fig. 1A) and the number of islets rapidly declined (Fig. 1B). Histological and morphological assessment of pancreatic sections stained with HE and for insulin showed that the hyperglycemia was associated with destruction of pancreatic islets, especially the damage of β cells in islets (Fig. 1C).

Effect of transplantation of BM derived cells on diabetic mice

Diabetic mice were randomly chosen and divided into two groups at day 10. One group (n = 18) received BM derived cells from GFP mice and the other group (n = 18) received PBS. The blood glucose level of BM derived cell transplanted diabetic mice decreased significantly at days 38, 45, and 52, although the decline did not restore the normal blood glucose level (Fig. 2A). The weight of mice STZ-treated mice and STZ+BM-treated mice were similar at days 38, 45, and 52 (data not shown). At day 52, we analyzed and counted the islets and β cells in the two groups. The numbers of islets and β cells were partly restored in BM derived cell transplanted mice. In contrast, the number of islet and β cells was still very low in the control group (Fig. 2B–D). Notably, there were three small islets around the pancreatic ducts in BM derived cell transplanted mice (Fig. 2C-a).

Influence of BM derived cells on pancreatic engraftment and pancreatic stem cell differentiation

To characterize pancreatic engraftment, we performed GFP fluorescent immunohistochemical analysis of tissue sections. As illustrated by fluorescent immunohistochemistry, donor GFP$^+$ cells were detected in the pancreas. The majority of GFP$^+$ cells were in and around islets (Fig. 3A) and ductal regions (Fig. 3B). Staining for BrdU and insulin revealed that no proliferating (BrdU$^+$) cell was detected in islets of STZ-treated mice (Fig. 3C). Both proliferating β cells and non-β cells were found in islet of STZ+BM-treated mice (Fig. 3D). And we noticed that some insulin$^-$ BrdU$^+$ cells stained positive for PDX-1 (Fig. 3E). In order to assay for putative pancreatic progenitor cells during pancreatic β cells regeneration, we performed double staining for insulin and endocrine progenitor cell marker neurogenin 3 (Ngn3) [18]. In STZ-treated mice, no Ngn3$^+$ cell was found (Fig. 3F). In contrast, we detected Ngn3$^+$ cells in islet of STZ+BM-treated mice (Fig. 3G). In addition, we performed double staining using antibodies against insulin and glucagon. Compared with islets of normal and STZ-treated mice, cells co-expressing insulin and glucagon were found only in STZ+BM-treated mice; however, we could not find these cells in other groups (Fig. 3H).

Nature of BM derived cells in pancreas of recipient diabetic mice

To investigate whether donor BM derived cells were capable of differentiation into islet β cells, we obtained pancreatic sections from mice that received BM derived cells and double stained the sections for GFP and insulin. No double positive cells were detected (Fig. 4A). To further confirm this result, we double stained sections for GFP and either PDX-1, Ngn3, Nkx2.2, Nkx6.1, Pax4, or Pax6, all of which are expressed in different periods of islet β cell development. Again, no double staining cells were evident (Fig. 4B–G).

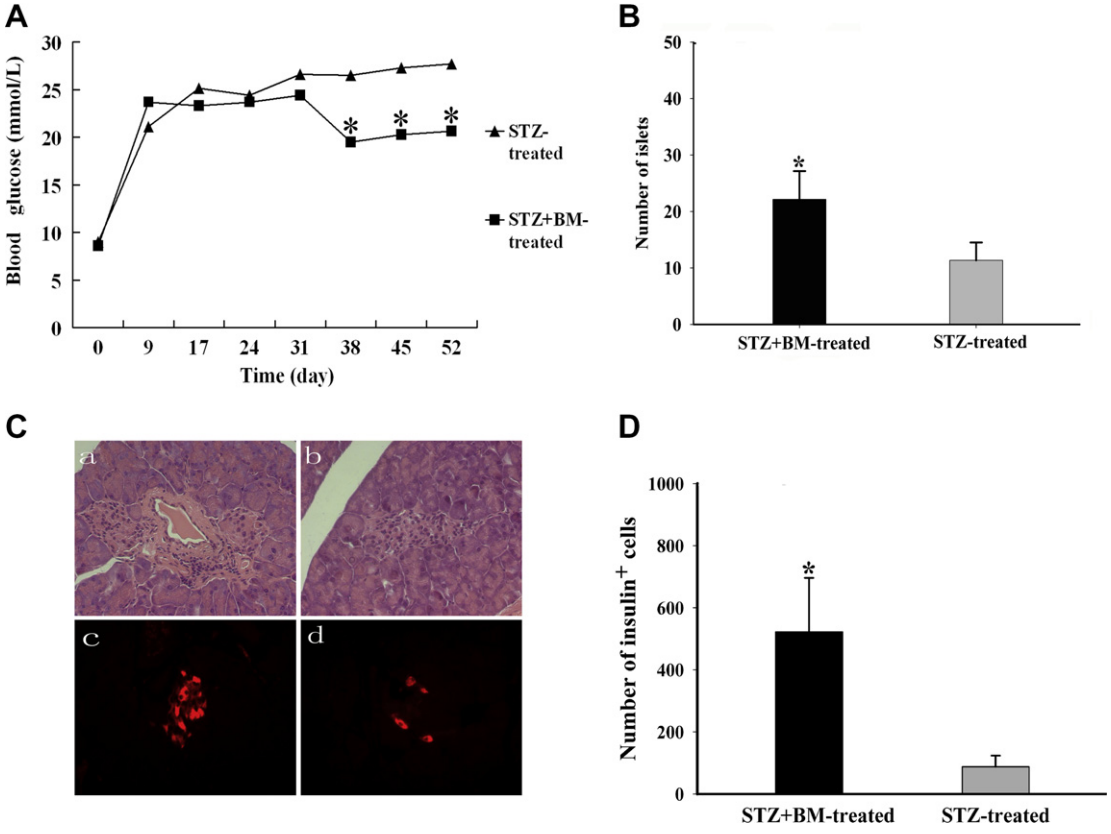

Fig. 2. Effect of transplantation of BM derived cells on diabetic mice. (A) Compared with STZ-treated mice injected with PBS (n = 18), blood glucose of STZ-treated mice transplanted with BM derived cells (n = 18) were significantly reduced at days 38, 45, and 52, $^*p < 0.01$; (B) the number of islets was significantly increased in STZ+BM-treated mice, $^*p < 0.01$; HE stained (day 52) pancreatic sections of STZ+BM-treated mice (C-a) and STZ-treated mice (C-b), insulin immunofluorescent (day 52) of pancreatic sections in STZ+BM-treated (C-c) and STZ-treated mice (C-d), magnification 400×; after BM derived cells transplantation, the histology and morphology of islets and the number of insulin positive cells were partly restored. (D) The number of insulin positive cells in STZ++BM-treated mice (n = 18) was significantly larger than that in STZ-treated mice (n = 18), $^*p < 0.01$.

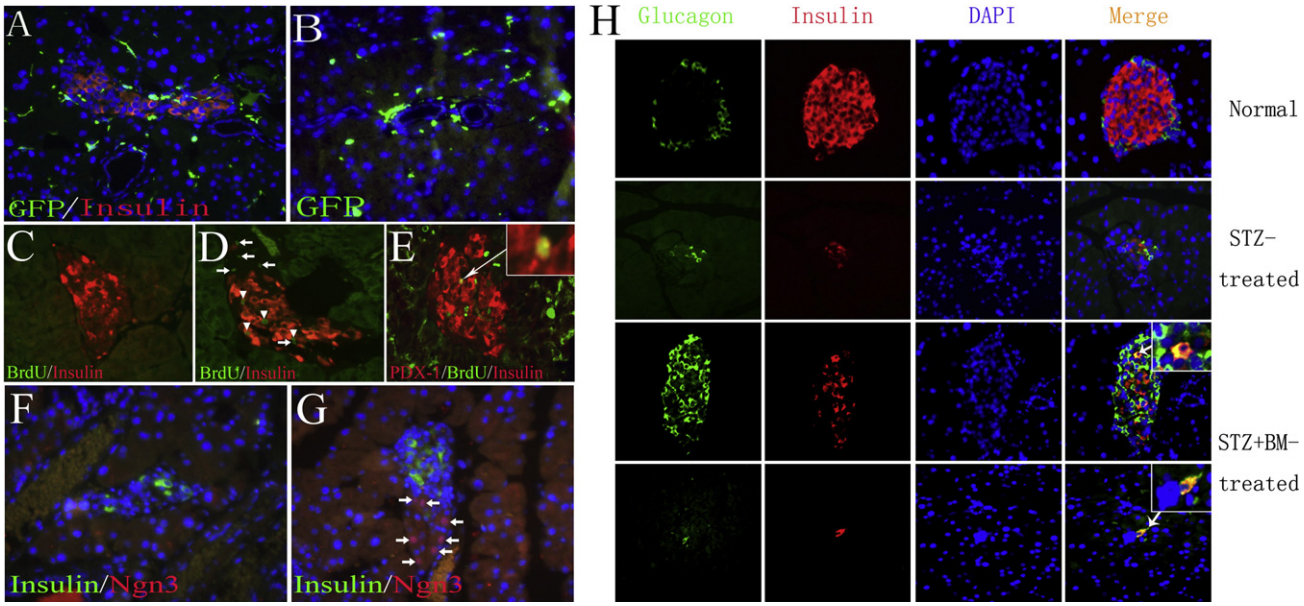

Fig. 3. Double staining results. Transplanted BM derived cells engrafted islet region (A) and ductal region (B), magnification 400×. Visualization of BrdU-labeled proliferation cells in islet of STZ-treated mice (C) and STZ+BM-treated mice (D), magnification 400×, arrowhead-pointed cells are insulin$^+$BrdU$^+$ cells, arrow-pointed cells are insulin$^-$ BrdU$^+$ cells. Representative visualization of insulin negative, BrdU- and PDX-1-positive cells (arrow indicated) in islet of STZ+BM-treated mice (E), magnification 400×. Representative analysis of Ngn3 expressing cells in islet of STZ-treated mice (F) and STZ+BM-treated mice (G), magnification 400×, arrow-pointed cells are Ngn3$^+$ cells. (H) The double stain of pancreatic sections with anti-insulin and anti-glucagon antibodies, pancreatic sections were drawn from normal (top), STZ-treated (upper middle) and STZ+BM-treated (lower middle and bottom), yellow (arrow) indicates insulin and glucagon double positive cells, magnification 400×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CD34 is an *o*-sialylated glycoprotein expressed in some stem cells such as hematopoietic stem cells and endothelial progenitor cells. To further investigate the existing types of BM derived cells in recipient mice, sections were double stained for GFP and CD34. Simultaneously expressed GFP and CD34 cells were evident in some pancreas sections (Fig. 4H). Donor derived CD45$^+$ cells have been found in the pancreas of diabetic mice after BM derived cell transplantation [19]. However, we presently did not find cells that simultaneously expressed GFP and CD45 (Fig. 4I).

Discussion

Both type 1 and type 2 diabetes result from an inadequate mass of functioning β cells. It is important for the diabetic to restore pancreatic islets function by islet β cell regeneration. BM derived cells may have a role in pancreatic regeneration [13,15], but the mechanism of pancreatic regeneration is still unclear. Our results demonstrate that the transplantation of BM derived cells significantly reduces the blood glucose level of diabetic mice after 4 weeks, although the reversal of hyperglycemia is not complete. The blood glucose alteration was accompanied with the detection of cells in the islet that co-expressed insulin and BrdU. It indicated that BM derived cells transplantation initiated β-cell proliferation. We also found that some BrdU tagged cells were insulin-negative. And some of these cells expressed PDX-1 which has been labeled the master regulator of pancreas development [20]. Previous studies showed that BrdU positive, insulin negative, and PDX-1 positive cells were β cells progenitors [21]. In addition, cells that expressed Ngn3 were detected in islets of STZ+BM-treated mice. Expression of Ngn3 revealed an islet cell precursor population in the islet [22]. We also found the cells in the adult pancreas that co-expressed insulin and glucagon. Such simultaneous expression of different hormones is a hallmark of stem cells. Moreover, double staining experiments revealed that the stem cells were still in the process of differentiation. Currently, there is considerable controversy regarding the existence and location of pancreatic stem cells [23]. However, our results confirm that pancreatic stem cells exist in the islet of adult pancreas. Based on the discovery of BrdU$^+$ insulin$^-$ PDX-1$^+$ cells, Ngn3$^+$ cells and insulin$^+$ glucagon$^+$ cells only in BM derived cell transplanted mice, we suggest that BM derived cells can activate the differentiation of pancreatic stem cells.

Previous studies have shown that pancreatic β cells arise only from self-duplication of preexisting β cells and not from pancreatic stem cells [24]. However, several lines of evidence support that differentiation of pancreatic stem cells contribute to the regeneration of β cells in our model. First, BrdU$^+$ insulin$^-$ PDX-1$^+$ cells were found in the islets of STZ+BM-treated mice. Previous studies have demonstrated that BrdU$^+$ insulin$^-$ PDX-1$^+$ cells could differentiate into β cells [21]. Second, Ngn3 was activated during the course of β cells regeneration. Ngn3 is a key transcription factor for differentiation of the endocrine pancreas [25]. Previous studies have shown that Ngn3 activation resulted in significantly increased insulin transcripts [26,27]. Third, insulin and glucagon double stained cells only found in the process of embryonic stem cells [28–31] and pancreatic ductal cells [32] differentiated into islet β cells *in vitro*. And we also found the same double stained cells during the process of β cells regeneration. Therefore, we consider that BM derived cells transplantation can trigger differentiation of pancreatic stem cells into β cells. In conclusion, we suggest that, after transplantation of BM derived cells, both proliferation of β cells and differentiation of pancreatic stem cells contribute to the regeneration of β cells.

To our knowledge, this is the first report of cells co-expressing insulin and glucagon *in vivo*, and also the first report that BM derived cells can activate pancreatic stem cells differentiation.

In sections of recipient pancreata, we did not find insulin positive, BM derived cells. PDX-1, Ngn3, Nkx2.2, Nkx6.1, Pax4, and Pax6 are important transcription factors which are expressed in different developmental stages of pancreatic β cells [33]. We also did not find these factors and GFP co-expressed cells in recipient mice. These observations strongly support the suggestion that BM derived cells do not differentiate into pancreatic β cells to con-

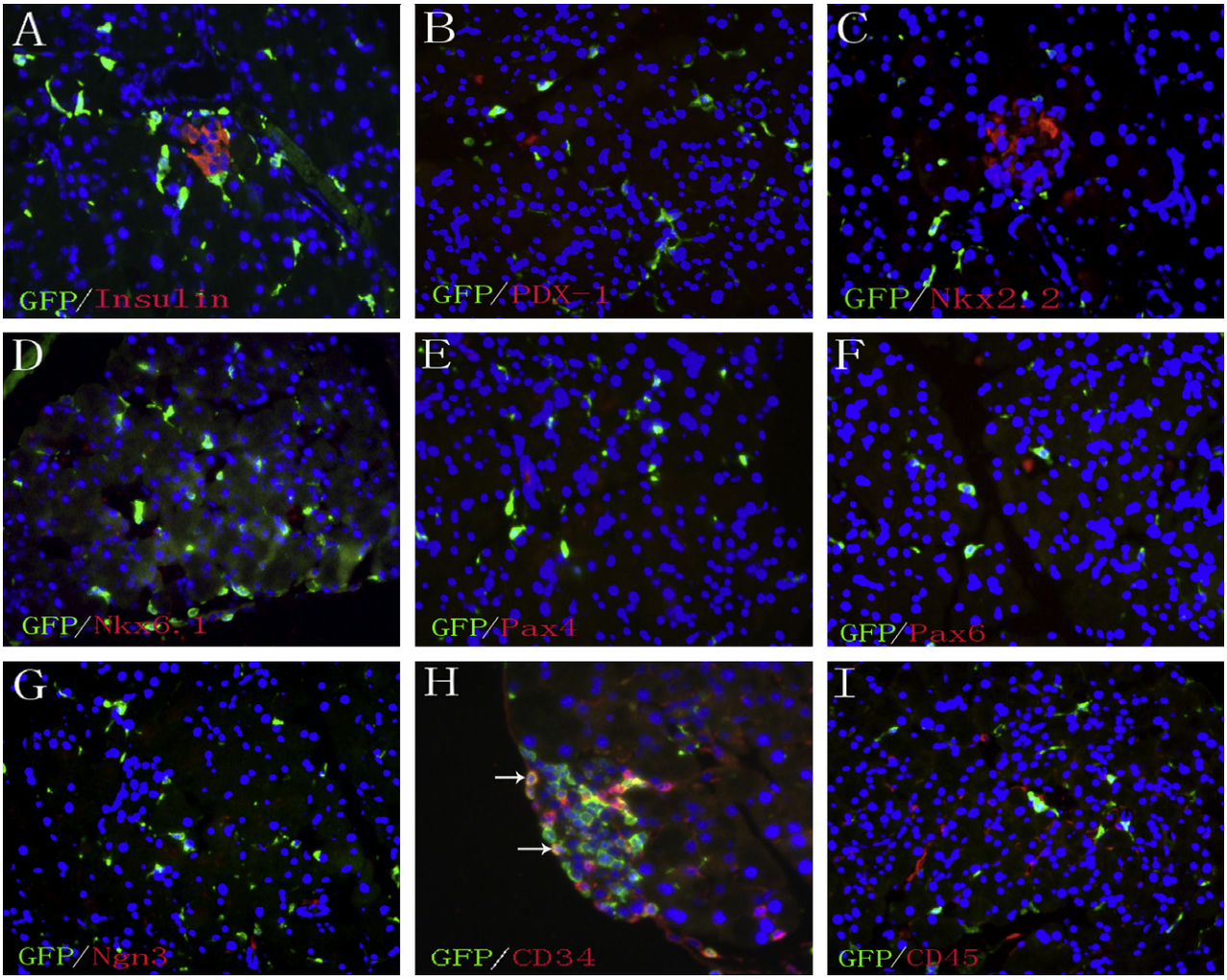

Fig. 4. Expression of several cell markers by GFP$^+$ cells in pancreatic sections of STZ+BM-treated mice. There were no cells simultaneously expressed GFP and insulin (A), PDX-1 (B), Nkx2.2 (C), Nkx6.1 (D), Pax4 (E), Pax6 (F), Ngn3 (G) and CD45 (I), magnification 400×, cells expressed GFP and CD34 (H) simultaneously were found in recipient pancreas (arrows indicated).

tribute to the reduction of hyperglycemia. But, we do not rule out the possibility that BM derived cells can differentiate into pancreatic β cell *in vitro*.

Which kinds of cells are involved in pancreatic regeneration is still unknown. Some studies have implied a role in pancreatic regeneration of endothelial cells [13] and CD45$^+$ cells [19]. Our results indicate that GFP$^+$ cells express CD34 but not CD45 in the recipient pancreata. Thus, we conclude that CD34$^+$ cells, but not CD45$^+$ cells, may play a role in pancreatic regeneration.

The present findings raise the possibility that BM derived cells may be useful to treat hyperglycemia. Elucidation of the mechanism by which BM derived cells, especially CD34$^+$ cells, activate pancreatic stem cells may shed new light on diabetes therapy.

Acknowledgments

This research was supported by a grant from National Nature Science Foundation (30371388), a grant from Research Fund for the Doctoral Program of Higher Education (20040246057) and a grant from Shanghai Municipal Government (04DZ19505).

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