

Recruitment of Bone Marrow–Derived Endothelial Cells to Sites of Pancreatic β -Cell Injury

Vikram Mathews,¹ Piia T. Hanson,¹ Eric Ford,² Jun Fujita,³ Kenneth S. Polonsky,² and Timothy A. Graubert¹

Endothelial progenitor cells (EPCs) are detectable in the blood and bone marrow throughout life. These cells contribute to new blood vessel formation (neovascularization) in physiological states such as wound healing and in pathological states such as tumor angiogenesis. We hypothesized that bone marrow–derived EPCs could play a role in the response to pancreatic islet cell injury. We used a murine model of experimentally induced β -cell injury followed by transplantation with genetically marked bone marrow cells. Bone marrow–derived cells were detectable throughout the pancreas after transplantation. Whereas the total number of bone marrow–derived cells in the pancreas decreased over time, the frequency of endothelial cells (of both donor and recipient origin) increased after transplantation in the animals in which β -cell injury had been induced. There was no evidence in this model that bone marrow–derived cells differentiated into insulin-expressing cells. This study provides evidence that bone marrow–derived EPCs are recruited to the pancreas in response to islet injury. EPC-mediated neovascularization of the pancreas could in principle be exploited to facilitate the recovery of non–terminally injured β -cells or to improve the survival and/or function of islet allografts. *Diabetes* 53:91–98, 2004

Postnatal neovascularization occurs by remodeling existing blood vessels through local proliferation and migration of endothelial cells (ECs) (1,2). Neovascularization is important both in physiological situations such as wound healing and tissue regeneration after injury and in pathological processes such as diabetic retinopathy and tumor angiogenesis (3,4). Recently, circulating endothelial precursors (endothelial progenitor cells [EPCs]) were identified and implicated in postnatal angiogenesis (5–7). The bone marrow appears to be a reservoir for EPCs in adults (8). Although purification

strategies have not yet been developed for bone marrow EPCs, they may derive from populations enriched for hematopoietic stem cells (9,10) or multipotent adult progenitor cells (11). Several animal models have provided evidence that bone marrow–derived endothelial precursors can contribute to neovascularization (9–11). Mobilization or transplantation of EPCs can enhance neovascularization and is associated with tissue regeneration and functional improvement after injury (12–14).

The dorsal pancreatic bud arises during embryogenesis from the portion of the foregut endoderm that contacts the endothelium of the dorsal aorta and two vitelline veins. Recombination of prepatterned dorsal endoderm with aortic endothelium can induce pancreatic endocrine differentiation, whereas recombination with tissues such as the adjacent notochord or neural tube cannot (15). Conversely, removal of endothelial cell precursors in embryos resulted in failure of endocrine gene expression patterns in pancreatic endoderm (16). These results suggest that EC contact and signaling is necessary and sufficient for endocrine pancreatic development. The pancreatic islets of Langerhans are well vascularized throughout life. This is critical for proper metabolic sensing and homeostatic regulation mediated by these cells. Signals from the endothelium may also have a role in postnatal islet cell proliferation and neogenesis. We hypothesized that neovascularization of the pancreas could be an important component of an adaptive response to β -cell injury.

Bone marrow–derived cells have demonstrated a surprising capacity to contribute to a variety of nonhematopoietic lineages, including tissues of all three germ layers (17). Several hypotheses have been advanced to explain these counterintuitive findings, including persistence of multipotent progenitor cells in adult tissues, itinerant tissue-specific stem cells in bone marrow, intercellular transfer of nuclear material (“fusion”), and transdifferentiation of hematopoietic stem cells to stem cells with alternative fates (“plasticity”) (18). The derivation of functional hepatocytes from bone marrow–derived cells in vivo is one of the most striking demonstrations of apparent lineage plasticity (19). However, recent data suggest that most of the bone marrow–derived hepatocytes in this model are products of cellular fusion (20,21). In vitro, insulin-secreting cells have been derived from hepatic oval cells (22). Taken together, these results and the realization that pancreas and liver share a common developmental origin raise the possibility that bone marrow cells could also potentially generate insulin-secreting cells.

These two lines of reasoning led us to hypothesize that

From the ¹Department of Internal Medicine, Division of Oncology, Stem Cell Biology Section, Washington University, St. Louis, MO; the ²Department of Internal Medicine, Division of Endocrinology, Diabetes and Metabolism, Washington University, St. Louis, MO; and the ³Kyoto Prefecture University of Medicine, 1st Department of Internal Medicine, Kyoto, Japan.

Address correspondence and reprint requests to Timothy Graubert, MD, Washington University School of Medicine, Division of Oncology, Stem Cell Biology Section, Campus Box 8007, 660 South Euclid Ave., St. Louis, MO 63110. E-mail: graubert@medicine.wustl.edu.

Received for publication 11 June 2003 and accepted in revised form 26 September 2003.

BMC, unmanipulated bone marrow cell; DAPI, 4',6-diamino-2-phenylindole; EC, endothelial cell; EGFP, enhanced green fluorescent protein; EPC, endothelial progenitor cell; Lin⁺, lineage positive; Lin[−], lineage depleted; STZ, streptozotocin; vWF, von Willebrand factor.

© 2004 by the American Diabetes Association.

bone marrow cells could be important mediators of the response to β -cell injury. To investigate this question, we used a model of experimentally induced β -cell injury followed by transplantation with genetically marked bone marrow cells.

RESEARCH DESIGN AND METHODS

Animals. Young adult (age 6–10 weeks) C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME) were used as recipients. β -Cell injury was induced with streptozotocin (STZ). STZ (Sigma, St. Louis, MO) was administered intraperitoneally at a dose of 200 mg/kg in a citrate buffer. A proportion of animals that did not become diabetic after 72 h (random blood glucose <300 mg%) received a second injection at the same dose. Blood glucose was monitored twice weekly with a portable glucose monitor (Hemocue, Ängelholm, Sweden). Continuous release insulin (Linshin, Scarborough, ON, Canada) was provided subcutaneously to maintain glycemic control. After transplantation, an additional insulin pellet was inserted if two consecutive blood glucose readings were >400 mg%. Bone marrow donors were young adult (age 6–10 weeks) sex-matched congenic enhanced green fluorescent protein (EGFP) transgenic mice (provided by Dr. M. Okabe, Osaka, Japan) (23). EGFP is under the transcriptional control of the chicken β -actin promoter and the cytomegalovirus enhancer in this strain, resulting in high-level expression in most tissues (23). These animals were generated and have been maintained in a C57BL/6J background by backcross of hemizygous carriers to C57BL/6J for >6 generations. All experiments were conducted with the approval of the Animal Studies Committee at Washington University.

Bone marrow transplantation model. Once diabetes had been induced and blood glucose was stabilized with exogenous insulin (consistent values in the range of 100–250 mg%; this was achieved by 1–2 weeks after STZ administration), the animals received bone marrow transplants. Bone marrow cells were obtained by flushing femurs with Hepes-buffered saline buffer followed by red cell lysis. In some cases, the stem/progenitor cells were enriched by depleting the grafts of terminally differentiated hematopoietic cells using a standard cocktail of biotinylated monoclonal antibodies (Ter-119, CD3, Gr-1, CD11b, B220; BD Pharmingen, San Diego, CA) followed by staining with anti-biotin microbeads and purification on a negative selection column (Miltenyi Biotec, Auburn, CA). These lineage-depleted (Lin^-) cells were routinely enriched 8- to 12-fold by the procedure (not shown). Terminally differentiated lineage-positive (Lin^+) cells were recovered from the columns and used in some experiments. Recipients were conditioned with a single dose of irradiation (800 cGy) followed 12–24 h later by transplantation of hematopoietic cells via lateral tail vein injection. Unmanipulated bone marrow cells (BMCs) were transplanted at a dose of $\sim 5.0 \times 10^6$ cells. Lin^- and Lin^+ cells were infused at a dose of $\sim 5.0 \times 10^5$ cells. To rescue lethally irradiated recipients from hematopoietic failure, 1.0×10^5 nontransgenic BMCs were co-infused with the Lin^- and Lin^+ grafts.

Immunohistochemistry. Animals were killed at 1 or 5 months after transplantation. The pancreas was dissected, fixed in 4% formalin overnight, paraffin embedded, and sectioned (4 μm). Formalin fixation and paraffin embedding reduced the intensity of EGFP autofluorescence. Therefore, immunohistochemical staining was used to retrieve the EGFP signal. Slides were de-waxed, hydrated, blocked in 3% BSA and 2% normal goat serum, and then incubated overnight in polyclonal rabbit anti-EGFP antisera (Molecular Probes, Eugene, OR). Slides were then washed twice with 0.05% Triton X-100 in PBS and incubated for 1 h with an alkaline phosphatase conjugated goat anti-rabbit antibody (Sigma). After additional washes with 0.05% Triton X-100 in PBS and detection with a red substrate (Vector Laboratories, Burlingame, CA), the slides were counterstained with hematoxylin.

Immunofluorescence. Two-color immunofluorescence was used to determine the lineage of EGFP-expressing cells. Bone marrow cells were stained with polyclonal (Molecular Probes) or monoclonal anti-EGFP antibodies (BD Clontech, San Diego, CA) followed by the pan-leukocyte marker CD45 (Research Diagnostics, Flanders, NJ) to identify hematopoietic cells, monoclonal anti-human cytokeratin I/II (Cymbus Biotechnology, Chandlers Ford, U.K.) to identify ductal epithelial cells, anti-human von Willebrand factor (vWF) antisera (Dako, Carpinteria, CA) to identify ECs, or antisera to swine insulin (Dako) to identify β -cells. The staining protocol was similar to that used for immunohistochemistry, with the exception that before the use of anti-cytokeratin and anti-vWF, the slides were pretreated with proteinase K (400 $\mu\text{g}/\text{ml}$; Sigma) in PBS for 5 min. Primary mouse antibodies were detected using a Mouse on Mouse kit according to the manufacturer's guidelines (Vector Laboratories). Appropriate secondary antibodies conjugated to fluorochromes were used (Molecular Probes). When using the Mouse on Mouse kit, TX red avidin (Vector Laboratories) was used as the fluorochrome for the

mouse primary antibody. Slides were sealed with Vectashield mounting media (Vector Laboratories) with 4',6 diamino-2-phenylindole (DAPI).

Endothelial morphometry. Slides were viewed on a Nikon Microphot-SA fluorescence microscope, and images were captured using a Colorview camera and analysis software (Soft Imaging System, Lakewood, CO). Five $10\times$ fields chosen at random from at least two slides $>200 \mu\text{m}$ apart were counted, and the result was averaged for each animal. Large vessels, vessels in the periphery of the tissue, and those not enclosed within the pancreatic tissue were excluded. Areas of staining without discrete breaks were counted as single vessels. The presence of a lumen or red cells was not required to be included in the count. Individually highlighted ECs or cell clusters separate from the adjacent microvessels were counted as distinct vessels. For colocalization studies, 200 individual discrete vWF $^+$ cells were studied under oil immersion ($100\times$) and analyzed for coexpression of EGFP. The result was expressed as a percentage for each animal. Results were confirmed by two independent observers.

RESULTS

Donor-derived cells are detectable in the pancreas after transplantation. Young C57BL/6J mice were made diabetic by intraperitoneal injection of STZ. Insulin was administered for the duration of the experiment. Once the animals had stable blood glucose values (1–2 weeks after STZ), they were conditioned with irradiation and received bone marrow cells from sex-matched congenic EGFP-expressing donors. A total of 58 animals were included in this study. All of these animals survived the procedure and were observed for up to 1 year after transplantation. Hematopoietic engraftment was assayed by flow cytometric analysis of peripheral blood samples at 1 month after transplantation. Animals with low hematopoietic chimerism (<50%) due to suboptimal graft preparation or injection technique were excluded from further analysis ($n = 2$ from the Lin^- group). The majority of the remaining animals achieved high hematopoietic chimerism (>85%) (data not shown).

Immunohistochemistry was used to detect EGFP $^+$ cells in formalin-fixed pancreatic sections. This assay was first validated using tissue from nontransplanted wild-type and transgenic EGFP mice. Detection of EGFP $^+$ cells using this technique was sensitive and specific. No background staining was seen in wild-type pancreas (Fig. 1A–C). Exocrine cells in the transgenic animals were uniformly positive with a granular cytoplasmic staining pattern (Fig. 1F). Staining within the islets of transgenic animals was notably heterocellular, with some cells staining brightly and others indistinguishable from the background (Fig. 1E). This finding suggests that donor-derived cells capable of differentiation into insulin-secreting cells could be underestimated in this model.

EGFP transgenic bone marrow cells were transplanted into wild-type lethally irradiated diabetic recipients. Numerous donor-derived (EGFP $^+$) cells were detectable throughout the pancreas at 1 and 5 months after transplantation (Fig. 1G–L). Donor-derived (EGFP $^+$) cells in five randomly selected $10\times$ fields from at least two slides $>200 \mu\text{m}$ apart were counted, and the result was averaged for each animal. Cells were excluded if they were within lymphoid aggregates or the lumen of vessels, were outside the pancreatic tissue, or were in locations that are prone to staining artifacts (e.g., at the periphery of a section). The level of tissue chimerism peaked at 1 month (mean 205 ± 46 cells per $10\times$ field) and fell thereafter (Fig. 2). At 5 months after transplantation, there were significantly more donor-derived cells in the recipients of grafts en-

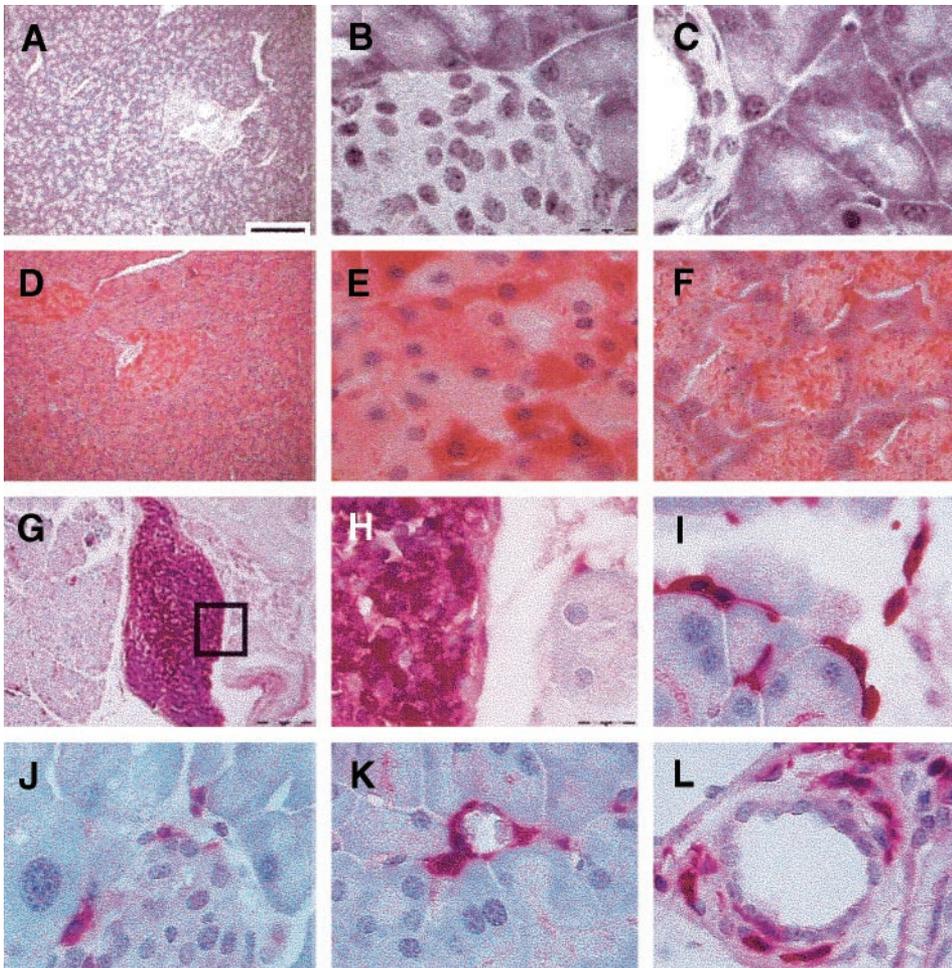


FIG. 1. Immunohistochemical detection of EGFP⁺ cells in the pancreas. Sections from the pancreas of wild-type (A–C) and EGFP transgenic littermate mice (D–F) were stained for EGFP, detected with a red substrate, and counterstained with hematoxylin. In the transgenic animals, EGFP expression is detectable in a diffuse cytoplasmic pattern within islets (E) and in a granular cytoplasmic pattern in acinar cells (F). There is no background staining in wild-type islet (B) or acinar (C) cells. Wild-type diabetic mice were analyzed by immunohistochemistry 5 months after transplantation of bone marrow cells from EGFP⁺ donors (G–L). Peri-pancreatic lymph nodes (G and H) are fully reconstituted with EGFP⁺ donor cells. Within the pancreas, donor cells are scattered throughout the exocrine portion of the organ (I). Donor-derived cells are also seen frequently adjacent to islets (J), blood vessels (K), and ducts (L). The region indicated in G is shown at a higher magnification in H. The scale bar represents 100 μ m (in A, D, and G) or 10 μ m (in the other panels).

riched for hematopoietic stem/progenitors (Lin⁻) compared with the control group that received Lin⁺ grafts (88.7 \pm 25 vs. 35 \pm 13.5 cells per 10 \times field, $P < 0.0001$). β -Cell injury appeared to be a potent stimulus for recruitment of bone marrow-derived cells, because the frequency of these cells was increased more than twofold in the group that received STZ treatment compared with control mice that were transplanted with Lin⁻ grafts

without prior STZ treatment (88.7 \pm 25 vs. 41 \pm 18.5 cells per 10 \times field, $P = 0.005$).

Donor-derived cells in the recipient pancreas include both hematopoietic and nonhematopoietic lineages.

As expected in these lethally irradiated transplanted animals with high peripheral blood chimerism, all hematopoietic organs were reconstituted by donor cells. This was evident in tissue sections by virtually complete repopula-

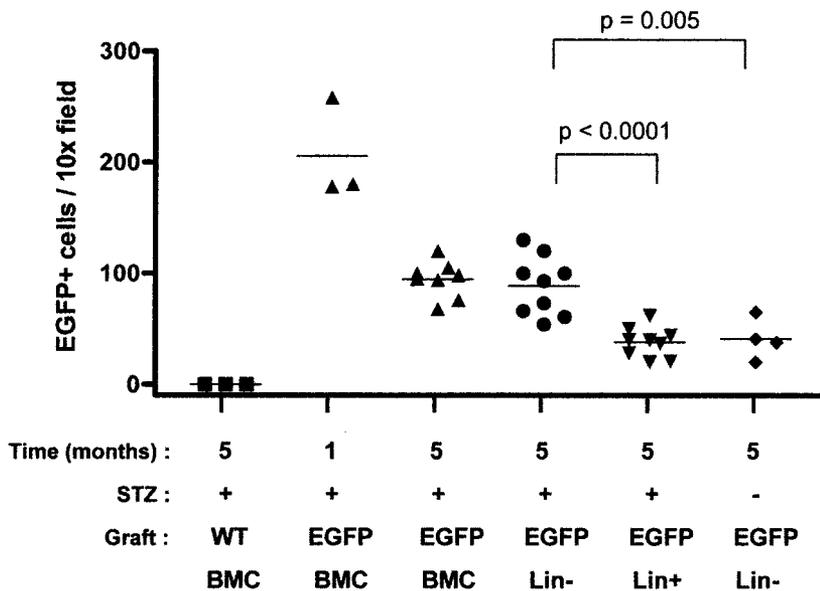


FIG. 2. Enumeration of donor cells in transplanted animals. Bone marrow-derived cells from EGFP⁺ donors were identified in the recipient pancreas by immunohistochemical staining at the indicated times after transplantation. Transplantations were performed with total bone marrow mononuclear cells ($n = 3$ at 1 month, $n = 8$ at 5 months [\blacktriangle]), Lin⁻ cells ($n = 10$ [\bullet]), or Lin⁺ cells ($n = 9$ [\blacktriangledown]). Control animals received nontransgenic bone marrow mononuclear cells ($n = 3$ [\blacksquare]) or Lin⁻ cells without STZ injury ($n = 4$ [\blacklozenge]). The total number of engrafted cells decreased from 1 to 5 months after transplant. The frequency of donor cells was significantly higher in the pancreas of animals receiving lineage-depleted grafts and STZ injury. WT, wild-type.

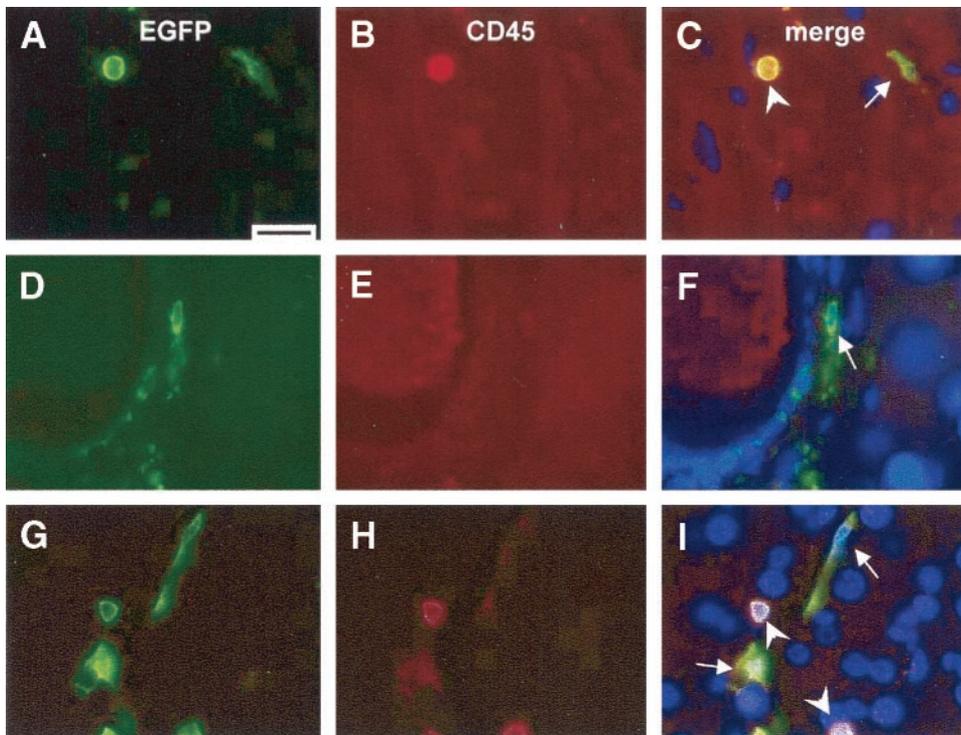


FIG. 3. Immunofluorescence analysis of bone marrow-derived cells in the pancreas. Sections from the pancreas of recipients were stained with anti-EGFP (A, D, and G) and the pan-leukocyte marker CD45 (B, E, and H). Nuclear staining with DAPI (blue) is shown in the merged images (C, F, and I). Representative fields demonstrate that a proportion of donor-derived cells are hematopoietic (arrowheads) and a proportion are nonhematopoietic (arrows). The scale bar represents 10 μ m.

tion of peri-pancreatic lymph nodes by donor-derived cells (Fig. 1G and H). Within the parenchyma of the pancreas, additional donor-derived cells were apparent (Fig. 1I–L). The majority of these cells were distributed throughout acinar tissue (Fig. 1I), especially at early time points. At 5 months after transplantation in animals that received Lin^- or unmanipulated BMC grafts, there was a relative increase in donor-derived cells in a peri-islet and peri-ductal pattern (Fig. 1J and L). Many donor-derived cells were identified in and around blood vessels and had the typical appearance of ECs as evidenced by their peri-vascular location and spindle-shaped morphology (Fig. 1K).

To exclude the possibility that all the donor-derived cells seen in the pancreas were hematopoietic cells (e.g., neutrophils, lymphocytes, or macrophages) trafficking through the organ in these unperfused animals, two-color

immunofluorescence was performed using an antibody to the pan-leukocyte marker CD45. Colocalization studies revealed that many of the EGFP^+ cells in the early posttransplant period did indeed express this hematopoietic marker (Fig. 3). However, nonhematopoietic donor-derived cells were also clearly detectable (Fig. 3). A total of 200 EGFP^+ cells per animal were analyzed under oil immersion ($100\times$), and the number of CD45^- cells was expressed as a percentage. Only cells clearly within the parenchyma of the pancreas were included. Donor-derived cells (EGFP^+) on the periphery of the section, within lymph aggregates or located within the lumen of blood vessels, were excluded. A substantial proportion of donor-derived cells detectable in the pancreas were nonhematopoietic at 1 month after transplantation (Fig. 4A). This proportion did not change significantly between 1 and 5

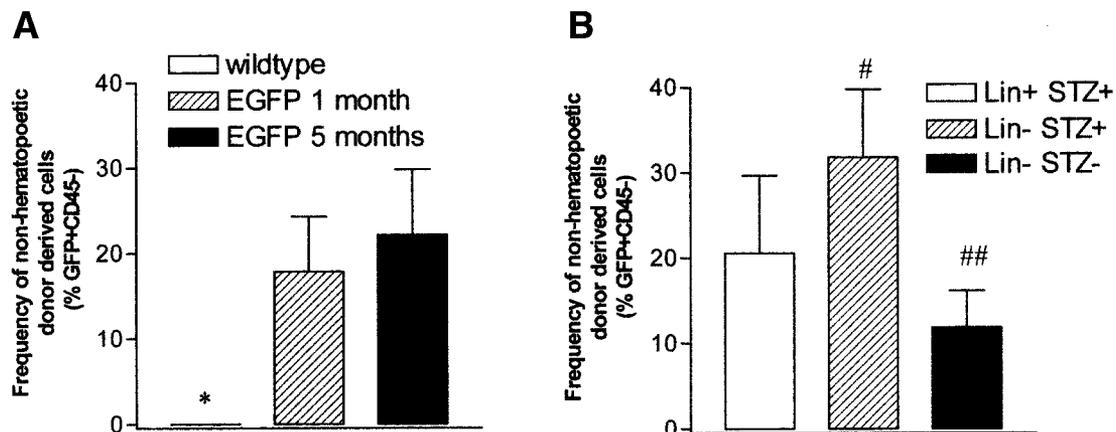


FIG. 4. Quantification of nonhematopoietic donor-derived cells in the transplant recipients. The frequency of nonhematopoietic cells of donor origin ($\% \text{EGFP}^+ \text{CD45}^-$) was counted in the recipients of EGFP^+ BMCs at 1 and 5 months after transplantation (A). The proportion of $\text{EGFP}^+ \text{CD45}^-$ cells did not differ significantly between 1 ($n = 3$ mice) and 5 ($n = 8$ mice) months after transplantation. The frequency of $\text{EGFP}^+ \text{CD45}^-$ cells as a function of graft manipulation (Lin^- vs. Lin^+) and β -cell injury (STZ^+ vs. STZ^-) was determined (B). The proportion of donor-derived nonhematopoietic cells detectable in the pancreas was highest after transplantation with Lin^- cells and after islet injury with STZ. *Undetectable; # $P = 0.01$ compared with the Lin^+ group; ## $P < 0.001$ compared with the STZ^+ group.

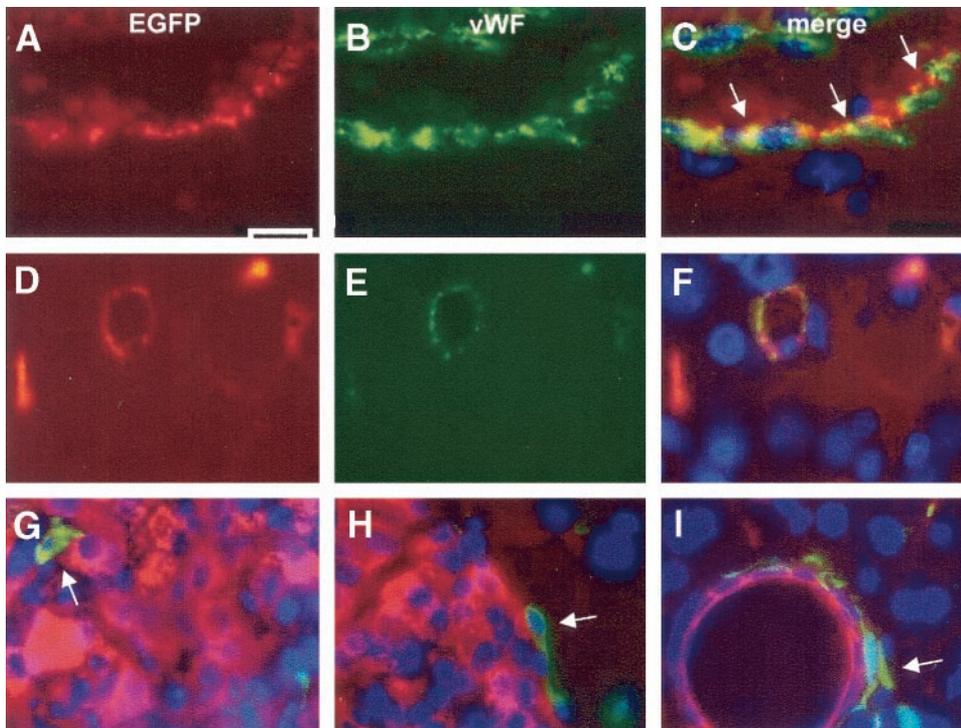


FIG. 5. Immunofluorescence colocalization analysis of bone marrow-derived cells. Donor-derived cells are identified by EGFP staining (A and D), ECs by staining with anti-vWF (B and E), and nuclei with DAPI on the merged images (C and F). A–C: A medium-sized vessel that is chimeric for donor (arrows) and recipient-derived ECs. D–F: A capillary consisting of donor-derived ECs surrounding a central lumen. Merged images of representative sections stained with anti-EGFP (green) and DAPI (blue) are shown in G–I. Donor-derived cells (arrows) are occasionally seen adjacent to and within islets, identified by insulin-expressing β -cells (red, G and H). Insulin and EGFP signals do not colocalize. Cytokeratin staining (red, I) similarly demonstrates that donor-derived cells were frequently in close proximity but were generally distinct from ductal epithelial cells. The scale bar represents 10 μ m.

months (17.8 ± 6.5 vs. $22 \pm 7.6\%$, $P = 0.4$). There was a significantly higher proportion of nonhematopoietic donor-derived cells in the recipients of Lin^- compared with Lin^+ grafts (31.7 ± 8 vs. $20.5 \pm 9\%$, $P = 0.01$) (Fig. 4B). The cell dose in the Lin^- grafts was 10-fold lower than that in the unmanipulated grafts and yet resulted in a significantly higher number of nonhematopoietic cells in the pancreas after transplantation (31.7 ± 8 vs. $22 \pm 7.6\%$, $P = 0.02$). This suggests that the cells capable of long-term engraftment within the pancreas are enriched in the Lin^- fraction. Furthermore, STZ injury was associated with a higher frequency of nonhematopoietic cell engraftment (Fig. 4B). Therefore, STZ injury increases recruitment of bone marrow cells in absolute number and disproportionately for the fraction that lacks the hematopoietic marker CD45.

Donor-derived cells in the pancreas do not coexpress markers of ductal epithelium or β -cells. To assess whether donor-derived cells within the pancreas acquired the phenotype of mature pancreatic lineages, immunofluorescence colocalization studies were undertaken using markers that identify ductal epithelium (cytokeratin) and β -cells (insulin). Between 1 and 5 months after transplantation, the number of donor-derived cells adjacent to ducts and islets increased (Fig. 5G–I). Occasional colocalization of cytokeratin and EGFP signals were noted, particularly in the animals that received STZ treatment followed by transplantation with Lin^- cells. This result suggests that some donor-derived cells may have the capacity to differentiate into ductal epithelium, although this appears to be a rare event and could be accounted for by cellular fusion. We next asked whether insulin-expressing donor-derived cells could be detected. Approximately 7,000 insulin-positive cells per animal were examined for insulin and EGFP coexpression in sections from mice that did not receive STZ treatment. Because the size and number of islets is dramatically reduced by STZ injury, fewer cells ($\sim 1,500$ per animal) were analyzed in these sections. Donor-de-

rived cells were frequently seen adjacent to and within islets (Fig. 5G and H). Using strict colocalization criteria (nucleus and adjacent cytoplasm had to be clearly visualized), donor-derived cells (EGFP⁺) immunostained for insulin were not seen in any of the transplant groups. This analysis should be sufficiently sensitive to identify bone marrow-derived insulin-expressing cells at a frequency as low as 0.06%.

Bone marrow cell transplantation did not alter the natural history of STZ-induced diabetes. Among the group of animals that received STZ treatment and were transplanted, there was no significant difference in blood glucose or insulin requirement for up to 1 year in comparison to control animals that were not transplanted but received STZ treatment. The transplanted animals gained significantly less weight over the course of a year than STZ-treated mice that were not transplanted (mean 1.5 vs. 7.8 g, $P < 0.01$). This was likely a consequence of pretransplant total body irradiation. Although weight gain was highest in recipients of Lin^- grafts (107.2% of starting weight), this was not significantly different from weight gain in recipients of BMC grafts (94.3%, $P = 0.21$). Removal of insulin pellets from transplanted STZ-treated mice inevitably resulted in the return of hyperglycemia. Although this analysis could miss subtle improvement in islet function, by these criteria, there was no significant alteration in the course of diabetes after bone marrow transplantation in this model.

Increased numbers of ECs of both donor and recipient origin are present after β -cell injury and bone marrow transplantation. A proportion of donor-derived cells in the pancreas after transplantation acquired an endothelial phenotype, as demonstrated by morphology and costaining for EGFP and vWF (Fig. 5C and F). The majority of these donor-derived ECs were scattered in the interstitium of the pancreas. EGFP⁺vWF⁺ cells were occasionally seen in clusters of 10–15. The cells frequently

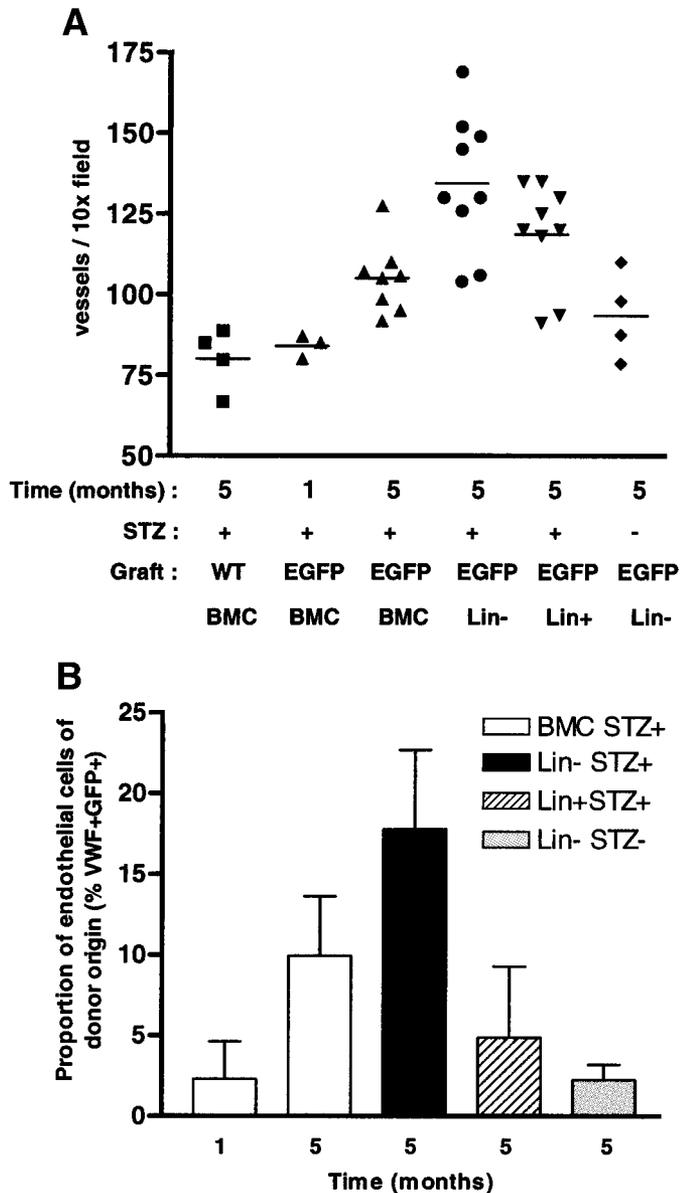


FIG. 6. Quantification of ECs within the pancreas after transplantation. Transplants were performed as indicated in the legend to Fig. 2. The total number of blood vessels increased in the pancreas after transplantation of bone marrow cells (A). The frequency of vessels was highest after transplantation with Lin⁻ grafts and after STZ injury. The increase in vessels was accounted for by a rise in both the donor and recipient ECs (B). The proportion of donor-derived ECs increased from 1 to 5 months after transplantation and was highest after transplantation with Lin⁻ grafts and after STZ injury.

localized around ducts. The total number of blood vessels (donor or recipient) increased over time in the posttransplant animals (Fig. 6A). This was similar in the groups that received Lin⁻ or Lin⁺ grafts (134.6 ± 21.4 vs. 118.7 ± 16 vessels per $10\times$ field, $P = 0.09$) and was significantly higher in animals that had STZ-induced injury in comparison to a similar group that did not receive STZ (134.6 ± 21.4 vs. 93 ± 13.5 vessels per $10\times$ field, $P = 0.005$). The proportion of individual ECs of donor origin also increased with time and was maximal in mice that received a Lin⁻ graft and had STZ-induced injury ($17.8 \pm 5\%$). This value was significantly higher in comparison to animals transplanted with Lin⁻ cells without prior STZ-induced

injury ($2.2 \pm 0.9\%$, $P < 0.0001$) and in comparison to animals that received STZ followed by transplantation with a Lin⁺ graft ($4.9 \pm 4.4\%$, $P < 0.0001$) (Fig. 6B). The results suggest that the frequency of ECs of both donor and recipient origin increased in the pancreas. The proliferation and/or migration of recipient ECs into the pancreas is a response to β -cell injury rather than to irradiation, because animals that were irradiated and transplanted without prior STZ injury did not have a significant increase in recipient-derived ECs (Fig. 6B).

DISCUSSION

There is increasing evidence that the bone marrow may serve as a reservoir for cells that contribute to the repair of organs after cellular injury. The present experiments were undertaken to determine the extent to which the bone marrow can provide precursors for cellular repair after injury to pancreatic β -cells. The model we used involved the administration of the pancreatic β -cell toxin STZ followed by bone marrow transplantation with EGFP⁺ cells to assess the capacity of bone marrow cells to respond to this form of β -cell injury. These experiments demonstrate that bone marrow-derived cells readily home to the injured pancreas. At early time points, the majority of donor-derived cells were scattered interstitially throughout the pancreas. Many of these cells express the pan-leukocyte marker CD45 and likely represent an inflammatory response composed of lymphocytes and/or macrophages. Over time, donor-derived cells appeared more frequently in a peri-islet and peri-ductal distribution. Although the total number of donor-derived cells decreased over time, the proportion that lacked CD45 expression (and are therefore nonhematopoietic cells) was stable. Rare donor-derived cells expressed a marker of ductal epithelium, but convincing evidence of EGFP and insulin coexpression was not seen. A significant proportion of the donor-derived nonhematopoietic cells had the morphology of ECs, localized to small blood vessels, and expressed the endothelial marker vWF. Transplantation without prior β -cell injury resulted in significantly fewer donor-derived cells in the pancreas and a significantly lower proportion accounted for by ECs. We further demonstrated that the cells in the bone marrow that give rise to ECs in the injured pancreas are enriched in the fraction that contains hematopoietic stem/progenitor cells.

There was no evidence in this model of detectable insulin expression by bone marrow-derived cells within the pancreas. While this article was in preparation, Ianus et al. (24) reported that insulin-expressing cells could be generated from bone marrow cells in vivo. In this model, an EGFP reporter was activated in bone marrow-derived cells via Cre recombinase expression under the control of a rat insulin II promoter (25). The frequency of insulin-expressing bone marrow-derived cells was low (one to two cells per islet) as assessed by immunofluorescence, but was estimated to be 1.7–3% of total islet cells by flow cytometric analysis (24). Although this experimental design was similar in some respects to the transplantation protocol used for the animals in our study that did not receive STZ treatment, there were subtle differences in the pretransplant conditioning, timing of analysis, and cell doses used. Other differences between the models, includ-

ing the promoters driving EGFP expression (ROSA26/insulin vs. pCAGGS) and the assays used (flow cytometry vs. immunofluorescence) may have affected the sensitivity for detecting EGFP⁺insulin⁺ bone marrow-derived cells. Conversely, the experimental strategy used by Ianus et al. did not allow them to assess the importance of bone marrow-derived EPCs and whether these cells could have contributed to their findings. Hess et al. (26) recently reported that transplantation of GFP⁺ bone marrow cells into diabetic mice resulted in modest improvement in hyperglycemia associated with an increase in PECAM-1⁺ pancreatic ECs. Although GFP and insulin colocalized in some islet cells in these animals (3/1,653 cells without STZ injury, 11/433 cells with STZ injury), the authors concluded that the major benefit of transplantation could be attributed to recovery of endogenous β -cells facilitated by bone marrow-derived ECs. Differences in mouse strain, STZ dose, and time of analysis may account for the functional improvement noted by Hess et al. but not by our group. Taken together, our results and the studies of Ianus et al. and Hess et al. support the notion that bone marrow cells could contribute to the recovery of injured pancreatic β -cells.

Neovascularization of the pancreas is likely to be an adaptive response to β -cell injury. Our analysis may have underestimated the contribution of bone marrow EPCs to the endothelial population in the injured pancreas for several reasons. First, a significant proportion of ECs in the donor strain used in these studies do not express detectable levels of EGFP by immunohistochemical analysis (data not shown). In addition, vWF Ag staining underestimates the frequency of ECs in the microvasculature (27). Therefore, the total population of ECs that appear in the injured pancreas as well as the proportion that are bone marrow-derived may be higher than that reported here.

Tissue injury and ischemia are potent stimuli for neovascularization. Bone marrow EPCs have been shown to contribute to neovascularization in several models, including retinal, myocardial, and hind limb ischemia (13,28,29). Although we were able to demonstrate that ECs of bone marrow origin populate the injured pancreas, this was not accompanied by evidence of functional improvement in our model. However, improved β -cell function would be difficult to assess because exogenous insulin was required throughout the experiment. It is conceivable that strategies that further enhance pancreatic neovascularization could lead to β -cell neogenesis, proliferation, or improved function. This might be achieved through optimization of the dose, timing, or route of EPC delivery and/or coadministration of growth factors such as vascular endothelial growth factor, which stimulate the proliferation and/or homing of ECs (30,31).

Islet transplantation as a therapeutic option for diabetes is hampered by limited graft availability, lifelong immunosuppression, and high graft rejection rates. Newer immunosuppressive regimens have decreased the complications of such therapy and have significantly improved the rejection rates (32). Neovascularization is required to ensure the viability and function of the allograft. Transplanted islets derive their microvasculature at least in part from the recipient (33). In rodent models, the vascularity of

transplanted islets is reduced in comparison to normal islets (34). The resulting ischemia is associated with graft hypoxia (35). Techniques that improve the ability of the transplanted islets to rapidly establish vascular communication with the recipient could decrease the required dose of allograft material and improve its function. Cotransplantation of islets with bone marrow grafts enriched for EPCs is a potential platform for achieving this goal and merits further study.

ACKNOWLEDGMENTS

This work was supported by Pilot and Feasibility grants to T.A.G. from the Diabetes Research and Training Center (5 P60 DK20579) and the Beta Cell Biology Consortium (U19 DK42502).

NOTE ADDED IN PROOF

While this manuscript was under review, Choi et al. (Choi JB, Uchino H, Azuma K, Iwashita N, Tanaka Y, Mochizuki H, Migita M, Shimada T, Kwamori R, Watada H: Little evidence of transdifferentiation of bone marrow-derived cells into pancreatic beta cells. *Diabetologia* 46:1366–1374, 2003) reported results that are consistent with our findings. This group transplanted pCAGGS-GFP transgenic bone marrow cells into STZ-treated congenic C57BL/6 mice and found numerous donor-derived cells in the pancreas 5 weeks later. GFP⁺ donor cell coexpressed vWF in some cases, but no insulin⁺ or pdx1⁺ donor-derived cells were found in the islets. This study supports our conclusion that bone marrow may provide endothelial precursors in response to β -cell injury, but has little capacity to give rise to insulin-producing cells under these conditions.

REFERENCES

1. Risau W: Differentiation of endothelium. *FASEB J* 9:926–933, 1995
2. Folkman J, Shing Y: Angiogenesis. *J Biol Chem* 267:10931–10934, 1992
3. Folkman J: Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 29:15–18, 2002
4. Auricchio A, Behling KC, Maguire AM, O'Connor EM, Bennett J, Wilson JM, Tolentino MJ: Inhibition of retinal neovascularization by intraocular viral-mediated delivery of anti-angiogenic agents. *Mol Ther* 6:490–494, 2002
5. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schattman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275:964–967, 1997
6. Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MA, Storb RF, Hammond WP: Evidence for circulating bone marrow-derived endothelial cells. *Blood* 92:362–367, 1998
7. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W: In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood* 95:3106–3112, 2000
8. Murayama T, Tepper OM, Silver M, Ma H, Losordo DW, Isner JM, Asahara T, Kalka C: Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. *Exp Hematol* 30:967–972, 2002
9. Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R: Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med* 8:403–409, 2002
10. Grant MB, May WS, Caballero S, Brown GA, Guthrie SM, Mames RN, Byrne BJ, Vaught T, Spoerri PE, Peck AB, Scott EW: Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med* 8:607–612, 2002
11. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM: Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest* 109:337–346, 2002
12. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M,

- Isner JM, Asahara T: Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 5:434–438, 1999
13. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S: Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 7:430–436, 2001
 14. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM: Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 106:3009–3017, 2002
 15. Lammert E, Cleaver O, Melton D: Role of endothelial cells in early pancreas and liver development. *Mech Dev* 120:59–64, 2003
 16. Lammert E, Cleaver O, Melton D: Induction of pancreatic differentiation by signals from blood vessels. *Science* 294:564–567, 2001
 17. Krause DS: Plasticity of marrow-derived stem cells. *Gene Ther* 9:754–758, 2002
 18. Verfaillie CM, Pera MF, Lansdorp PM: Stem cells: hype and reality. *Hematology (Am Soc Hematol Educ Program)* 369–391, 2002
 19. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M: Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med* 6:1229–1234, 2000
 20. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M: Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 422:897–901, 2003
 21. Vassilopoulos G, Wang PR, Russell DW: Transplanted bone marrow regenerates liver by cell fusion. *Nature* 422:901–904, 2003
 22. Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peck AB: In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. *Proc Natl Acad Sci U S A* 99:8078–8083, 2002
 23. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y: 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 407:313–319, 1997
 24. Ianus A, Holz GG, Theise ND, Hussain MA: In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. *J Clin Invest* 111:843–850, 2003
 25. Gannon M, Shiota C, Postic C, Wright CV, Magnuson M: Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. *Genesis* 26:139–142, 2000
 26. Hess D, Li L, Martin M, Sakano S, Hill D, Strutt B, Thyssen S, Gray DA, Bhatia M: Bone marrow-derived stem cells initiate pancreatic regeneration. *Nat Biotechnol* 21:763–770, 2003
 27. Offersen BV, Borre M, Sorensen FB, Overgaard J: Comparison of methods of microvascular staining and quantification in prostate carcinoma: relevance to prognosis. *APMIS* 110:177–185, 2002
 28. Otani A, Kinder K, Ewalt K, Otero FJ, Schimmel P, Friedlander M: Bone marrow-derived stem cells target retinal astrocytes and can promote or inhibit retinal angiogenesis. *Nat Med* 8:1004–1010, 2002
 29. Ikenaga S, Hamano K, Nishida M, Kobayashi T, Li TS, Kobayashi S, Matsuzaki M, Zempo N, Esato K: Autologous bone marrow implantation induced angiogenesis and improved deteriorated exercise capacity in a rat ischemic hindlimb model. *J Surg Res* 96:277–283, 2001
 30. Young PP, Hofling AA, Sands MS: VEGF increases engraftment of bone marrow-derived endothelial progenitor cells (EPCs) into vasculature of newborn murine recipients. *Proc Natl Acad Sci U S A* 99:11951–11956, 2002
 31. Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA, Rafii S: Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J Exp Med* 193:1005–1014, 2001
 32. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230–238, 2000
 33. Vajkoczy P, Olofsson AM, Lehr HA, Leiderer R, Hammersen F, Arfors KE, Menger MD: Histogenesis and ultrastructure of pancreatic islet graft microvasculature: evidence for graft revascularization by endothelial cells of host origin. *Am J Pathol* 146:1397–1405, 1995
 34. Mattsson G, Jansson L, Carlsson PO: Decreased vascular density in mouse pancreatic islets after transplantation. *Diabetes* 51:1362–1366, 2002
 35. Carlsson PO, Palm F, Mattsson G: Low revascularization of experimentally transplanted human pancreatic islets. *J Endocrinol Metab* 87:5418–5423, 2002