Bone marrow contributes to the population of pancreatic stellate cells in mice

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Bone marrow contributes to the population of pancreatic stellate cells in mice. Am J Physiol Gastrointest Liver Physiol 297: G1138–G1146, 2009. First published October 1, 2009; doi:10.1152/ajpgi.00123.2009.—Activated pancreatic stellate cells (PSCs) play a pivotal role in the development of pancreatic fibrosis. The origin of activated PSCs has been thought to be transformation of quiescent PSCs residing locally in the pancreas. Recent studies have suggested that bone marrow (BM)-derived cells participate in regeneration processes in various organs. This study aimed to clarify the contribution of BM-derived cells to the population of PSCs in mice. We transplanted BM cells from male enhanced green fluorescent protein transgenic mice into female C57BL/6 mice after lethal irradiation. Eight weeks after BM transplantation, chronic pancreatitis was induced by repeated injections of cholecystokinin analog cerulein. Eight weeks after BM transplantation, BM-derived cells accounted for 20.2% of the desmin (a marker of PSCs)-positive cells in the pancreas. We could isolate BM-transplanted BM-derived cells by green fluorescent protein expression and in situ hybridization for the Y-chromosome. Eight weeks after BM transplantation, BM-derived cells accounted for 8.7% of the desmin (a marker of PSCs)-positive cells in the pancreas. We could isolate BM-derived cells, which contained lipid droplets and expressed desmin. They could be transformed to myofibroblast-like cells by culture in vitro, further supporting that BM contributed to the population of quiescent PSCs. After induction of pancreatic fibrosis, BM-derived cells accounted for 20.2% of α-smooth muscle actin-positive activated PSCs. The contribution of BM-derived cells to pancreatic ductal cells (positive for cytokeratin 19) was rare and less than 1%. In conclusion, our results suggested that BM-derived cells contributed to the population of PSCs in mice.

pancreatitis; pancreatic fibrosis; stem cell; pancreatic cancer; green fluorescent protein

PANCREATIC FIBROSIS IS A FUNDAMENTAL feature of chronic pancreatitis (CP) and of desmoplastic reaction associated with pancreatic cancer (2, 5, 24, 31). Over a decade, there is accumulating evidence that activated pancreatic stellate cells (PSCs) play a pivotal role in the development of pancreatic fibrosis in these pathological settings (2, 5, 6, 24, 31, 36). The origin of activated PSCs has been thought to be transformation of quiescent PSCs residing locally in the pancreas (2, 5, 24, 31). In normal pancreas, stellate cells are quiescent and can be identified by the presence of vitamin A-containing lipid droplets in the cytoplasm. In response to pancreatic injury or inflammation, quiescent PSCs undergo morphological and functional changes to become activated PSCs with a myofibroblast-like phenotype, which actively proliferate and migrate, express α-smooth muscle actin (α-SMA), and produce large amounts of extracellular matrix components such as type I collagen. Many of the morphological and metabolic changes associated with the activation of PSCs in animal models of fibrosis also occur when the cells are grown in serum-contain-

MATERIALS AND METHODS

Mice

Wild-type C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). EGFP transgenic mice [C57BL/6 TgN (β-act-EGFP)Osbg] were generously provided by Dr. Masaru Okabe (Genome Research Center, Osaka University, Osaka, Japan) (30). Animals were bred and maintained at the Animal Research Facility of Tohoku University School of Medicine. All animal procedures were performed in accordance with the National Institutes of Health Animal Care and Use Guidelines. The experimental protocol was reviewed and approved by the Animal Experiment Committee of Tohoku University School of Medicine.

Antibodies

Antibodies used were as follows: FITC-labeled anti-GFP (goat polyclonal; Abcam, Cambridge, UK), anti-desmin (mouse monoclonal; Dako, Buckinghamshire, UK), Alexa Fluor546-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR), Alexa Fluor546-labeled goat anti-rabbit IgG (Molecular Probes), anti-α-SMA (rabbit polyclonal, Abcam), anti-type I collagen (rabbit polyclonal, Abcam), anti-cytokeratin-19 (CK19) (rabbit polyclonal, Abcam), anti-amylnase (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-von Willebrand factor (rabbit polyclonal, Abcam).
Isolation and Transplantation of BM Cells

Sex-mismatched BM transplantation was performed using 7-wk-old female C57BL/6 mice as recipients and 7-wk-old male EGFP mice as donors (Fig. 1). Whole BM was harvested from male EGFP mice by flushing femurs, tibias, and pelvises with PBS supplemented with 2% FBS (MP Biomedicals, Irvine, CA) using 29-gauge needles. Cells were filtered through 130-μm cell strainers (Becton Dickinson, Franklin Lakes, NJ), collected by centrifugation, washed, counted, and resuspended in PBS supplemented with 2% FBS. Wild-type female C57BL/6 mice were lethally irradiated (at 7 Gy) using a PANTAK irradiator (Shimadzu, Kyoto, Japan) and intravenously transplanted with GFP-positive BM cells (1 × 10^7 cells/mouse) via tail vein immediately after the irradiation.

For some experiments, wild-type male C57BL/6 mice received BM transplantation from male EGFP mice (male-to-male transplantation) in a similar manner.

Assessment of BM Reconstitution

Eight weeks after BM transplantation, we evaluated the hematopoietic reconstitution by fluorescence in situ hybridization (FISH) for the Y-chromosome in the spleen and by flow cytometry for GFP expression in peripheral blood leukocytes.

**FISH for the Y-chromosome.** The spleen was removed and fixed by immersion in 4% paraformaldehyde overnight at 4°C. The specimens were embedded in regular paraffin wax and cut into 5-μm sections, sealed under glass with rubber cement, and heated to 60°C air dried. The FITC-labeled Y-chromosome paint was added to the sections, sealed under glass with rubber cement, and heated to 60°C for 10 min at 37°C. The protease was quenched in 0.2% glycine, refixed with 4% paraformaldehyde, dehydrated through graded alcohols, and air dried. The FITC-labeled Y-chromosome paint was added to the sections, sealed under glass with rubber cement, and heated to 60°C for 10 min, followed by overnight incubation at 37°C. The sections were subjected to washes of decreasing stringency with standard sodium citrate and analyzed for fluorescence using an all-in-one type fluorescent microscope (BioZero BZ-8000; Keyence, Osaka, Japan) with Z-stack function and BZ analyzer software (Keyence).

**Flow cytometry.** Peripheral blood cells were prepared from the recipient mice, and red blood cells were lysed with the red blood cell lysis buffer. The peripheral blood leukocytes were analyzed for GFP expression by flow cytometry using a FACScan flow cytometer (Becton Dickinson) after gating out propidium iodide-positive cells.

Immunofluorescent Staining

Immunofluorescent staining was performed as previously described (21). Briefly, tissue sections were deparaffinized and rehydrated. Following antigen retrieval with the target retrieval solution (Dako), the slides were blocked with 3% BSA and incubated with the mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA) for 1 h. The slides were incubated with mouse anti-desmin antibody (at 1:200 dilution) and FITC-labeled goat anti-GFP antibody (at 1:200 dilution) overnight at 4°C. After washes, the slides were incubated with Alexa Fluor 546-labeled goat anti-mouse IgG antibody (at 1:200 dilution) for 1 h. After washes, the slides were analyzed for fluorescence using a fluorescent microscope. Expression of α-SMA and CK19 was examined in a similar manner using the appropriate pairs of primary and secondary antibodies. For some experiments, we performed immunofluorescent staining following FISH for the Y-chromosome.

Isolation of Quiescent PSCs

Quiescent PSCs were isolated using the Nycodenz solution (Nycomed Pharma, Oslo, Norway) as previously described for the isolation of rat PSCs (2, 5, 19). Briefly, the pancreata were removed, minced with scissors, and incubated with 0.02% pronase (Roche, Mannheim, Germany), 0.02% pronase (Roche), and 0.1% DNase I (Roche) in Hank’s balanced salt solution for 30 min at 37°C. Digested tissue was pipetted through narrow orifices, filtered through a 150-μm mesh, and centrifuged. Cells were washed and resuspended in 8 ml of Gey’s balanced salt solution supplemented with 0.3% BSA. The cell suspension was mixed with 9 ml of 28.7% (wt/vol) of the Nycodenz solution. The Nycodenz gradient was prepared by layering the cell suspension in Nycodenz underneath 6 ml of Gey’s solution with BSA. The gradient was centrifuged at 1400 g for 20 min. The fuzzy band just above the interface of the Nycodenz solution and the aqueous buffer was harvested, and the cells were washed and resuspended in Ham’s F-12/DMEM (1:1) supplemented with 10% FBS. For some experiments, cells were maintained in Ham’s F-12/DMEM (1:1) supplemented with 10% FBS, penicillin sodium, and streptomycin sulfate.

**AdipoRed staining.** Intracellular lipid accumulation was assessed using the AdipoRed reagent (Lanza, Basel, Switzerland), which specifically partitions into the lipid droplets and fluoresces at 572 nm. Isolated PSCs were directly plated on μ-slides (Ibidi, Munich, Germany) and incubated at 37°C for 4 h in a humidized incubator under 5% CO₂. The cells were treated for 15 min with the AdipoRed assay reagent and analyzed for fluorescence using a fluorescent microscope. For some experiments, PSCs isolated from male mice, which had received BM transplantation from male EGFP mice, were stained with the AdipoRed reagent, followed by immunofluorescent staining for GFP and FISH for the Y-chromosome.

Induction of Pancreatic Fibrosis

Pancreatic fibrosis was induced by repeated injections of cholecystokinin analog cerulein as previously described (28) (Fig. 1). Eight weeks after BM transplantation, recipient female mice received six intra-abdominal injections of cerulein (50 μg/kg body wt) at 1-h intervals, 3 days per week, for the total of 6 wk. Control recipient female mice received 0.9% saline in a similar manner. Groups of six mice were euthanized under anesthesia at 2, 4, 6, 9, and 12 wk following the initial injection of cerulein, and the pancreata were immediately removed for further analyses.

Hematoxylin-Eosin Staining and Sirius Red Staining

Pancreata were fixed by immersing in 4% paraformaldehyde at 4°C overnight. The specimens were embedded in regular paraffin wax and cut into 4-μm sections. On these sections, hematoxylin-eosin staining and Sirius Red staining, which preferentially labels collagen fibrils with red color (12),
Statistics

Total number of desmin- or α-SMA-expressing cells, total number of GFP-positive cells, and total number of overlapped cells between immunopositive cells and GFP-positive cells were each counted in 10 randomly-selected fields at ×400 magnification per section in six mice at the respective time points. The presence or absence of the Y-chromosome was also checked at ×800 magnification with Z-stack function. Vascular cells and immunofluorescence-expressing cells, which did not include nuclei, were excluded from the count. Morphometric analyses were done independently by two observers (A. Masamune and K. Kikuta), who had not been informed of the identity of the specimens. Data are shown as means ± SE. Differences between the groups were evaluated by ANOVA, followed by Fisher’s test for post hoc analysis. A P value of <0.05 was considered to be statistically significant. All statistical analyses were performed using the SPSS version 13.0 statistical analysis software (SPSS, Chicago, IL).

RESULTS

Hematological Reconstitution in the Recipient Mice

We first evaluated the hematological reconstitution following lethal irradiation and BM transplantation. Eight weeks after the BM transplantation, the spleen of recipient mice showed almost complete reconstitution by Y-chromosome-positive donor male cells (Fig. 2A). Flow cytometry analysis showed that more than 98% of the peripheral leukocytes of the recipient mice expressed GFP (Fig. 2B), indicating that almost all of the BM-derived cells in the recipient mice were successfully replaced with those of GFP-positive donor male mice.

BM-Derived Cells Were Present in the Pancreas Before the Induction of CP

We examined the contribution of BM-derived cells in the pancreas before the induction of CP. At 8 wk after the BM transplantation, the pancreata of the recipient mice showed a normal gross appearance compared with those of nontransplanted control mice. On histology, the structure of the pancreas tissue appeared normal, with little evidence of inflammation (data not shown). BM-derived cells were tracked in the pancreas sections appeared normal, with little evidence of inflammation (data not shown). BM-derived GFP cells were tracked by fluorescence in situ hybridization (FISH) for the Y-chromosome in the spleen (A and B) or GFP expression by flow cytometry (C). A: almost all of the cells in the spleen of the recipient female mice contained Y-chromosome. B: spleen of the male and female mice served as positive and negative controls, respectively. Nuclei were counterstained with DAPI (blue). Original magnification: ×800. C: more than 98% of the peripheral leukocytes of the recipient mice [BM transplant, BMT(+) ] expressed GFP. Peripheral leukocytes of the female mice that had not received BM transplantation [BMT(−) ] served as a negative control.

BM-Derived Cells Contributed to the Population of Quiescent PSCs

The presence of BM-derived desmin+ cells in the pancreas, before the induction of CP, suggested the contribution of BM-derived cells to the population of quiescent PSCs. However, it should be noted that desmin alone is not a specific marker of quiescent PSCs; 20 to 40% of PSCs still express desmin after the activation (2, 5, 24, 31). In addition to desmin, quiescent PSCs are characterized by the presence of lipid droplets containing vitamin A in the cytoplasm (2, 5, 24, 31). Therefore, we examined whether these cells contained lipid droplets. We found GFP+ cells containing lipid droplets in the cytoplasm in the pancreas of the recipient mice before the induction of CP (Fig. 4A). To further address this issue, we isolated PSCs from the recipient mice after the BM transplantation. We found GFP+ cells containing lipid droplets in the cytoplasm (Fig. 4, B and C). A percentage of 12.8 ± 5.4% (n = 4 preparations) of the isolated AdipoRed-stained cells expressed GFP. Because AdipoRed-stained GFP+ cells might be macrophages, we performed immunofluorescent staining for desmin (a marker of PSCs, but not macrophages) and GFP in...
isolated cells. The expression of desmin was shown in GFP/H11001 cells (Fig. 4D). Quiescent PSCs are known to be transformed to myofibroblast-like cells when the cells are grown in serum-containing medium in culture on plastic (2, 5, 24, 31). By culture in serum-containing medium in vitro, BM-derived cells were transformed to myofibroblast-like cells, which expressed -SMA, an index of activated PSCs (Fig. 4E). Collectively, our results suggested that BM-derived cells contributed to the population of quiescent PSCs.

Mechanisms Underlying the Generation of BM-Derived PSCs

Conversion of BM stem cells into highly specialized cells of distinct organs is either due to transdifferentiation or cell fusion in the target organ (34). Therefore, it was possible that contribution of BM-derived cells to quiescent PSC population could be attributable to transdifferentiation or spontaneous cell fusion of BM-derived cells with the resident PSCs. To address this issue, we isolated PSCs from male mice that had received BM transplantation from male GFP mice (male-to-male transplantation) for the combined analyses of GFP, lipid droplets, and the presence of multiple Y-chromosomes. As shown in Fig. 5, all 80 GFP/H11001/AdipoRed cells examined had only one Y-chromosome. Therefore, it was unlikely that BM-derived quiescent PSCs were generated by spontaneous cell fusion.

BM-Derived Cells Contributed to the Population of Activated PSCs in the Fibrotic Pancreas

We next examined the contribution of BM-derived cells in the fibrotic pancreas. We induced CP in mice by repeated intra-abdominal injections of cerulein. As previously reported (28), the three-time weekly regimen of cerulein treatment induced pancreatic fibrosis, whereas the effects were not evident in saline-treated mice (Fig. 6, A and B, data not shown). -SMA (a marker of activated PSCs)-positive cells were abundantly observed in the periacinar fibrotic areas and vascular walls in the pancreas of the recipient mice (Fig. 6C). BM-derived GFP+ cells appeared in the periacinar fibrotic areas (Fig. 6D). We examined the contribution of BM to activated PSCs by combined expression of -SMA and Y-chromosome or GFP. We could identify -SMA-positive activated PSCs, which contained Y-chromosome in the nucleus (Fig. 6E). After repeated injections of cerulein for 6 wk, BM-derived activated PSCs accounted for 20.2 ± 2.5% of the total population of activated PSCs (Fig. 6E). GFP analysis showed similar results to that of FISH for the Y-chromosome. On the other hand, BM-derived activated PSCs were not evident in the pancreatic tissue of control saline-treated mice (data not shown). After the cerulein treatment was stopped, pancreatic injury began to resolve and the number of -SMA-positive PSCs markedly fell. During the resolution period of pancreatic fibrosis, the contribution of BM to activated PSCs was slightly decreased (Fig. 6F). The proportion of BM-derived cells in desmin-positive cells was not decreased at 9 wk (P = 0.35) but decreased at 12 wk compared with that at 6 wk (P = 0.004). Because 20 to 40% of activated PSCs still expressed desmin (2, 5, 24, 31), the...
decrease of BM-derived desmin-positive cells might be affected by that of activated PSCs. To show that BM-derived cells produced collagen in the pancreas, we additionally performed immunofluorescent staining for GFP along with the AdipoRed staining. Original magnification: ×800. The arrow denotes GFP⁺ cells, which contained AdipoRed⁺ lipid droplets in the cytoplasm. B–E: PSCs were isolated using the Nycodenz gradient, directly plated on μ-slides, and incubated for 4 h at 37°C. B and C: cells were treated with the AdipoRed assay reagent and analyzed for fluorescence using a fluorescent microscope. There were BM-derived cells (auto-fluorescence in green) and non-BM-derived cells (GFP⁻, B) possessing intracellular lipid droplets (red) in the cytoplasm. Merged photographs (B and the bottom of C) are also presented. D: expression of GFP (green) and desmin (red) was examined by immunofluorescent staining. E: after 7-day incubation in serum-containing medium, GFP⁺ BM-derived cells were transformed to myofibroblast-like cells, which expressed α-smooth muscle actin (α-SMA) (red). Nuclei were counterstained with DAPI (blue). Original magnification: ×800 (B–E).

BM-Derived Cells Contributed to Some Ductal, But Not Acinar or Endothelial Cells in the Pancreas

Finally, we examined whether BM-derived cells contributed to other cell populations in the pancreas. In mice that had received injections of cerulein for 6 wk, we could find some, but rare, GFP⁺ ductal cells, identified by positive staining for CK19 (Fig. 7A). BM contribution to some ductal cells was also shown by FISH for the Y-chromosome (Fig. 7B). To make sure that these findings were not attributable to low-frequency artifacts, we performed FISH for the Y-chromosome, followed by double immunofluorescent staining against GFP (a marker of BM herein) and CK19. As shown in Fig. 7C, we could find CK19-positive cells that were positive for both of the two BM markers, GFP and Y-chromosome. BM-derived cells accounted for 0.8 ± 0.2% of the total pancreatic ductal cells. However, we could not find acinar (positive for amylase) or vascular endothelial cells (positive for von Willebrand factor), which were positive for GFP or contained Y-chromosome in the nucleus (data not shown). Thus BM-derived cells contributed to ductal, but not acinar or endothelial, cells in the pancreas.
further supporting that BM-derived cells contributed to the population of quiescent PSCs. BM-derived PSCs seen in male-to-male transplants revealed only one Y-chromosome, suggesting that these cells resulted from transdifferentiation but not from cell fusion. Although the turnover of stellate cells in normal pancreas is unknown, it seems unlikely that substantial repopulation from BM occurs in the absence of pancreatic injury or a specific stimuli to the recruitment of BM cells. However, it should be noted that contribution of BM-derived cells to the population of quiescent PSCs herein occurred in the context of lethal irradiation and BM transplantation and, therefore, a degree of radiation injury to all organs including the pancreas. Along this line, Wang et al. (37) illustrated the crucial role of lethal irradiation to act as a selection pressure and encourage engraftment of BM-derived cells after sex-mismatched BM transplantation. Similar engulfment of the BM-derived cells to the population of stellate cells has been shown in the liver; BM-derived (quiescent) hepatic stellate cells could be detected at 13–31.7% at 8 wk after BM transplantation in the absence of additional liver damage (4, 32). The mechanisms by which BM-derived cells are recruited to the pancreas remain largely unknown and require further investigation.

Upon culture on plastic in serum-containing medium in vitro, BM-derived cells containing lipid droplets were transformed to myofibroblast-like cells, which expressed α-SMA, an index of activated PSCs (2, 5). Our findings suggest a path via the activation of BM-derived quiescent PSCs as a source of BM-derived α-SMA + myofibroblasts in the pancreas. In addition, recent studies have suggested that there are other sources of myofibroblasts, namely fibrocytes (8) and epithelial and perhaps endothelial cells via epithelial- and endothelial-mesenchymal transitions (16, 17). Fibrocytes are BM-derived circulating collagen-producing cells that express CD45 or other hematopoietic markers but are negative for α-SMA (8). Fibrocytes have been suggested to represent an alternative source for myofibroblasts in lung, liver, and kidney fibrosis (8). To support the contribution of fibrocytes to myofibroblasts in the pancreas, the accumulation of CD34 + fibrocyte-like cells has been shown in the stromal tissues of patients with CP (7). Because the proportion of BM-derived cells in α-SMA-positive cells was increased during the progression of pancreatic fibrosis, it was likely that BM-derived cells were additionally recruited from the circulation during the pancreatic injury. However, it was difficult to differentiate fibrocytes from activated PSCs in our experimental settings because fibrocytes recruited from the peripheral circulation ultimately develop an α-SMA + phenotype (8). On the other hand, the proportion of BM-derived ductal cells was less than 1%, and BM-derived vascular endothelial cells could not be detected in this study. Therefore, contribution of BM to myofibroblasts via epithelial- and endothelial-mesenchymal transitions is, if any, a very rare event in the pancreas.

Although repeated injections of cerulein are a model of pancreatic fibrosis in CP (28), it is increasingly accepted that activated PSCs are also responsible for pancreatic fibrosis associated with pancreatic cancer (6, 36). In this context, it is likely that BM contributes to stellate cell population in pancreatic fibrosis associated with pancreatic cancer. Direkze et al. (11) reported that BM-derived cells accounted for about 25% of tumor-associated myofibroblasts and fibroblasts, and they

DISCUSSION

Using sex-mismatched BM transplantation from male EGFP mice to female mice, we examined the contribution of BM-derived cells to the population of PSCs, the major profibrogenic cell type in the pancreas. We here showed that BM-derived cells contributed to the population of desmin + quiescent PSCs and of α-SMA + activated PSCs before and after cerulein treatment, respectively. BM-derived cells accounted for 8.7% of the desmin (a marker of PSCs)-positive cells in the pancreas at 8 wk after BM transplantation. After induction of pancreatic fibrosis, BM-derived cells accounted for about 20% of total α-SMA-positive activated PSCs. During the resolution of pancreatic fibrosis, the proportion of BM-derived stellate cells fell markedly, suggesting an intrapancreatic source of renewal and/or preferential loss of BM-derived activated PSCs. It has been proposed that loss of activated PSCs might result from apoptosis or differentiation to a quiescent state (24, 28, 31). The development of pancreatic fat-containing cells during recovery is a feature of this CP model, but the accumulation of activated PSCs during pancreatic injury was not accounted for by an equally extensive accumulation of fat-containing cells (28). Thus both apoptosis and differentiation to a quiescent state might explain the loss of BM-derived activated PSCs. Although it is not within the scope of this study, it would be of interest to see whether BM-derived PSCs undergo these processes in different manners from non-BM-derived PSCs.

One interesting finding of this study is the contribution of BM-derived cells to the population of quiescent PSCs. Although we initially used intermediate filament protein desmin as a marker of PSCs, desmin alone is not a specific marker of quiescent PSCs, and 20 to 40% of PSCs still express desmin after the activation (2, 5, 24, 31). Therefore, desmin-positive cells might include both quiescent and some, but not all, of activated PSCs. To further establish that BM contributed to the population of quiescent PSCs, we examined the presence of lipid droplets by the AdipoRed staining. We found GFP + cells, which contained lipid droplets in the cytoplasm in the pancreas sections from mice without cerulein treatment. In addition, we could isolate GFP + cells that contained lipid droplets in the cytoplasm and expressed desmin in vitro. These cells could be transformed to myofibroblast-like cells upon culture in vitro,
were concentrated toward the edge of the tumor in a murine model of pancreatic insulinoma. Interestingly, it has been shown that human BM-derived mesenchymal stem cells became activated and resembled carcinoma-associated myofibroblasts on prolonged exposure to conditioned medium from human breast cancer cells (25). Thus cancer microenvironment is capable of differentiating mesenchymal stem cells into myofibroblasts. The question remains as to whether the BM contribution to tumor stroma represents a host defense mechanism or creates a tumor-supportive environment.

To our knowledge, this study is the first to confirm the recent report by Marrache et al. (18) showing that BM-derived cells contributed to the population of myofibroblasts and of some ductal cells after the induction of CP with cerulein, but some differences existed. First, contribution of BM-derived cells to the population of quiescent PSCs was not evident before the cerulein treatment in the previous study. Second, the proportion of BM-derived cells in the population of PSCs was different. In the previous study, BM contributed to about 5% of desmin-positive cells in mice treated with cerulein for 6 wk, and the proportion remained unchanged up to 45 wk. BM reportedly contributed to about 6% of α-SMA-positive cells in mice treated with cerulein for 45 wk, but no results were presented at other time points. The reasons for these differences remain unclear, but they might reflect different experimental protocols. In the previous study, lethal irradiation was performed at 6 Gy, twice, 4 h apart, and a total of 5 × 10^6 BM cells were transplanted. At 4 wk after BM transplantation, mice received cerulein once an hour for seven consecutive hours, twice a week for 10 wk, and once a week for additional 35 wk.
Different protocol of lethal irradiation suggests different degrees of radiation injury to all organs including the pancreas, resulting in the different degree of recruitment of BM-derived cells. As shown by culture in vitro, BM-derived quiescent PSCs had the ability to undergo transformation to \( \alpha \)-SMA\(^+\) cells. This activation process is likely to occur by repeated injections of cerulein in vivo, leading to the increased proportion of BM-derived cells in the population of \( \alpha \)-SMA\(^+\) activated PSCs. Second explanation is the different efficiency of BM engraftment; nearly 100% BM replacement was accomplished herein, whereas the level was 73.7% in the previous study (18). It is likely that less efficiency results in underestimation of the BM contribution.

One limitation of this study is that we performed the transplantation of whole BM but not a specific subset of BM cells. Because adult BM contains both hematopoietic stem cells and mesenchymal stem cells, it is unclear which type (or both) of stem cells contributes to the population of PSCs. Contribution of mesenchymal stem cells is likely (3, 32), but recent studies have shown that fibroblasts and myofibroblasts including hepatic stellate cells and glomerular mesangial cells in the kidney originate from hematopoietic stem cells in mice (26, 29). BM may provide fibrogenic cells, but, on the other hand, autologous BM (33) and marrow-derived endothelial progenitor cells (27) could be antifibrotic. It remains unclear which types of cells in the BM contribute to fibrogenesis and which might be antifibrotic and/or what mediators regulate these apparently divergent activities of BM-derived cells. This is an important issue because recent studies have suggested a therapeutic application of BM-derived cells for the treatment of fibrosis in several organs including the liver (35). The homing of myelogenic cells in the injured pancreas might have a positive impact on the resolution of pancreatic fibrosis because these cells express matrix metalloproteinases, which augment the degradation of fibrotic extracellular matrix (2, 5, 14). Elucidation of the roles of BM-derived cells in the development and/or

![Fig. 7. BM-derived cells contributed to ductal cells in the pancreas. The pancreata were removed at 6 wk after the beginning of cerulein treatment. Immunofluorescent staining for cytokeratin-19 (CK19) (red) was performed, along with immunofluorescent staining for GFP (A) or FISH for the Y-chromosome (B). Nuclei were counterstained with DAPI (blue). The arrows indicate CK19\(^+\)/GFP\(^+\) (A) and CK19\(^+\)/Y\(^+\) (B) cells. Enlarged image is also shown as an insert. Original magnification: \( \times 800 \). C: immunofluorescent staining for CK19 (red) and FISH for the Y-chromosome were performed (left), followed by immunofluorescent staining for GFP. Nuclei were counterstained with DAPI (blue). The arrows indicate CK19\(^+\)/GFP\(^+\)/Y\(^+\) cells. Original magnification: \( \times 800 \).](http://ajpgi.physiology.org/)

resolution of pancreatic fibrosis will facilitate better understanding and rational approaches for the treatment of CP as well as pancreatic cancer.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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