Prospective Isolation of Multipotent Pancreatic Progenitors Using Flow-Cytometric Cell Sorting

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During pancreatic development, neogenesis, and regeneration, stem cells might act as a central player to generate endocrine, acinar, and duct cells. Although these cells are well known as pancreatic stem cells (PSCs), indisputable proof of their existence has not been reported. Identification of phenotypic markers for PSCs leads to their prospective isolation and precise characterization to clear whether stem cells exist in the pancreas. By combining flow cytometry and clonal analysis, we show here that a possible pancreatic stem or progenitor cell candidate that resides in the developing and adult mouse pancreas expresses the receptor for the hepatocyte growth factor (HGF) c-Met, but does not express hematopoietic and vascular endothelial antigens such as CD45, TER119, c-Kit, and Flk-1. These cells formed clonal colonies in vitro and differentiated into multiple pancreatic lineage cells from single cells. Some of them could largely expand with self-renewing cell divisions in culture, and, following cell transplantation, they differentiated into pancreatic endocrine and acinar cells in vivo. Furthermore, they produced cells expressing multiple markers of nonpancreatic organs including liver, stomach, and intestine in vitro. Our data strongly suggest that c-Met/HGF signaling plays an important role in stem/progenitor cell function in both developing and adult pancreas. By using this antigen, PSCs could be isolated prospectively, enabling a detailed investigation of stem cell markers and application toward regenerative therapies for diabetes. *Diabetes* 53:2143–2152, 2004

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Received for publication 16 April 2003 and accepted in revised form 5 May 2004.

CAPC, cell aggregate–producing cell; EC, epithelial-like colony; ECFC, EC-forming cell; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; GLP, glucagon-like peptide; H-CFU-C, hepatic colony-forming unit in culture; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; HS, hormone sensitive; mAb, monoclonal antibody; Pdx, pancreas duodenal homeobox; PP, pancreatic polypeptide; PSC, pancreatic stem cell.

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The pancreas is an organ consisting of three major different structures: the islets of Langerhans, acinar tissue, and ductal epithelia. The islets are composed of neatly arranged endocrine cell populations (glucagon-producing α-cells, insulin-producing β-cells, pancreatic polypeptide [PP]-producing γ-cells, and somatostatin-producing δ-cells). The acinar cells that secrete various enzymes, such as amylase and lipase, into the intestine, comprise a system of terminal or intercalary acini joined by ducts. During pancreatic organogenesis, these endocrine and acinar tissues seem to be developed from a common cell component associated with the pancreatic ductal epithelium (1–4). Thus, pancreatic stem cells (PSCs) responsible for both endocrine and acinar tissue formation are thought to reside in the pancreatic ducts. Identification and isolation of PSCs have generated much interest due not only to their putative developmental importance but also to their therapeutic potential.

Candidate PSC in mouse and human has been reported (5–7). They were derived from pancreatic ductal cell components and maintained in long-term culture, where they could differentiate into multilineage cell types. These cells possess characteristics very similar to those of neural (8,9), epidermal (10,11), mesenchymal (12), myogenous (13), and retinal (14) stem cells that can also propagate in culture. The value of stem cells expanded in vitro is expected to be great not only in conventional studies of their differentiation or self-renewing potential but also in therapy, such as with virus-mediated gene transfer, or as a theoretically unlimited source of cells. Characterization of those PSC candidates, however, has always been carried out retrospectively, after expansion of crudely isolated cells in culture for a relatively long period. Therefore, it has still not been determined which cells possess stem cell activity in vitro as well as in vivo. To distinguish PSCs from other cell types precisely, their prospective identification and single cell–based analysis are required.

The hematopoietic stem cells, probably the best-characterized stem cell population, were prospectively identified and isolated based on expression of cell surface antigens by flow cytometry (15–17). Although their self-renewing ability could not be maintained easily in vitro, prospective identification facilitated rapid progress toward an understanding of these cells' properties and yielded information on genes specifically expressed in this cell population (18,19). Several recent studies using fluorescence-activated cell sorting (FACS) have been conducted to isolate stem cell populations in neural tissue (20,21) and liver (22–24). These prospective studies have not only given us

information about specific characteristics of stem cells, but have also allowed us to separate them selectively from differentiated cells.

In the study reported here, we combined monoclonal antibodies and FACS to fractionate cells derived from neonatal and adult mouse pancreas based on surface marker expression. By using an in vitro clonal colony-forming assay system that was established previously (25), we analyzed cells sorted from distinct fractions and attempted to identify pancreatic stem/progenitor cells prospectively to determine their capacity for differentiation and proliferation. In order to enrich the yield of colony-forming cells and thereby permit clonal analysis of this cell class, we attempted in the present study to sort for cells expressing c-Met, the hepatocyte growth factor (HGF) receptor. Interaction between c-Met and HGF, which is mediated by a signal exchange between epithelial and mesenchymal cells (26), plays an essential part in pancreatic development (27,28). This interaction also is active in the regeneration and carcinogenesis of this organ (29–31). In addition, the number of islet-like structures budding out from cultured ductal epithelial cells is increased by HGF (6). These findings, taken together, suggest that c-Met/HGF interaction is critically responsible for growth and differentiation of pancreatic stem and progenitor cells during development, homeostatic cell turnover, and regeneration.

Our current data demonstrate that clonal colonies derived from c-Met–positive cells contained cells expressing several markers for endocrine, acinar, and ductal lineage cells. These results strongly suggest that cells initiating colony formation are defined as PSCs or common progenitors for those cell types. Several c-Met–positive sorted cells continued growing with self-renewing cell divisions, and, only from this cell subpopulation, many cell aggregates budded from monolayer cells emerged in long-term culture. These cell aggregate–producing cells (CAPCs) could differentiate clonally into multiple pancreatic lineage cells in vitro and in vivo. Furthermore, they could generate daughter cells expressing several marker genes for other organs of endodermal origin such as the liver, stomach, and intestine in vitro. These findings indicate that candidate PSCs or progenitors express c-Met, that their numbers can be enriched, and that they can be isolated using flow cytometry.

RESEARCH DESIGN AND METHODS

Dissociation of pancreatic cells. Single-cell suspensions of pancreas-constituting cells were prepared from C57BL/6 mouse neonates (1 day after birth) and adults (12 weeks old) (Clea, Tokyo, Japan). The pancreas was carefully dissected under the microscope, minced by a razor blade, and placed in Ca^{2+}-free Hanks' balanced salt solution (HBSS; Life Technologies, Gaithersburg, MD) containing 5 mmol/l $CaCl_2$ (pH 7.4) and 0.05% or 0.15% collagenase (cat no. C-5138; Sigma Chemical, St. Louis, MO) for either neonatal or adult pancreas. Digestion was carried out by gentle pipetting after incubation for 10–15 min at 37°C. Triturated pancreatic cells were washed three times in the medium before the incubation with antibodies. Cell viability exceeded 85% (neonates) or 70% (adult) as assessed by trypan blue dye exclusion.

Flow cytometry. Dissociated pancreatic cells were incubated on ice for 30 min with biotinylated anti-CD45, TER119, and c-Kit (for adult pancreas) monoclonal antibodies (mAbs) (Pharmingen, San Jose, CA) and c-Met mAb (Upstate Biotechnology, Lake Placid, NY). After three washings with staining medium (PBS containing 3% fetal bovine serum), cells were incubated with fluorescein isothiocyanate–conjugated anti-mouse IgG_{2a} mAb (Pharmingen), allophycocyanin-conjugated anti–c-Kit mAb (Pharmingen) (for neonatal pancreas), phycoerythrin-conjugated anti–Flk-1 mAb (Pharmingen), and streptavidin-labeled Texas Red (Life Technologies) on ice for 30 min. Finally, cells were washed three times and resuspended in staining medium containing propidium iodide (5 μg/ml). The labeled cells were analyzed and separated using FACS Vantage (Becton Dickinson, San Jose, CA). Establishment of the gate was based on the staining profiles of the negative control.

Cell sorting. After $CD45^+$ or $TER119^+$ hematopoietic cells from neonatal pancreas were gated out, sorting gates were set for c-Met^+ c-Kit^-, c-Met^- c-Kit^+, c-Met^+ c-Kit^+, and c-Met^- c-Kit^- subpopulations. Furthermore, by using antibodies against Flk-1, c-Met^+ c-Kit^- $CD45^-$ $TER119^-$ cells were further fractionated. Similarly, cells positive for CD45, TER119, and/or c-Kit in adult pancreas were gated out. After exclusion of Flk-1^+ cells from the c-Kit^- $CD45^-$ $TER119^-$ cell subpopulation, sorting gates were set for c-Met^+ Flk-1^- c-Kit^- $CD45^-$ $TER119^-$ and c-Met^+ Flk-1^+ c-Kit^- $CD45^-$ $TER119^-$ cell subpopulations. For low-density culture analysis, sorted cells were plated on 35-mm tissue culture dishes (Becton Dickinson) at a density of 200 cells/cm^2. For single-cell culture analysis, sorted cells were plated on 96-well tissue culture plates (Becton Dickinson). The nozzle size of the FACS Vantage is 70 μm. Sort speed is <1,000 cells/s. Residual erythrocytes, debris, doublets, and dead cells were excluded by forward scatter, side scatter, and propidium iodide gating. Viability of sorted cells exceeded 90% as assessed by trypan blue exclusion.

In vitro colony assay. Sorted cells from neonatal pancreas were cultured in our fresh standard medium (23). Our standard culture medium is a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 (Sigma) with 10% fetal bovine serum (Serologicals, Norcross, GA), γ-insulin (1 mg/ml; Wako, Tokyo, Japan), dexamethasone (1×10^{-7} mol/l; Sigma), nicotinamide (10 mmol/l; Sigma), L-glutamine (2 mmol/l; Life Technologies), β-mercaptoethanol (50 mmol/l; Sigma), HEPES (5 mmol/l; Wako), and penicillin/streptomycin (Life Technologies). For single-cell culture analysis of adult pancreatic cells, we used 50% standard medium supplemented with medium conditioned by 7-day culture with STO cells. Human recombinant HGF (50 ng/ml; Sigma) and epidermal growth factor (EGF) (20 ng/ml; Sigma) were added 24 h after culture initiation. EGF was used in combination with HGF because several reports have represented a mitogenic effect of EGF for pancreatic cells (32–34). In this series of experiments, we routinely added both of them into the culture medium. To induce differentiation, glucagon-like peptide (GLP)-1 (1-37) or GLP-1 (7-36) (Sigma) was supplemented into single-cell cultures of adult pancreatic cells. During the culture period, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. The number of epithelial-like colonies (ECs) was determined after 8 days of culture.

RT-PCR analysis. Detection of lineage-specific gene expression in each clonal colony by RT-PCR was conducted as described (23). Primer sequences are as follows (many have been presented already in our previous work [23,24]; those that have not are given in parentheses): α-cell marker preproglucagon and β-cell markers preproinsulin I, preproinsulin II, islet amyloid polypeptide (*IAPP*), and glucose transporter-2 (*glut-2*) (5′-ACA GAG CTA CAA TGC AAC GTG G-3′ and 5′-CAA CCA GAA TGC CAA TGA CGA T-3′); γ-cell marker PP (5′-CTC CCT GTT TCT CGT ATC CA-3′ and 5′-AGA GCA GGG AAT CAA GCC AA-3′); δ-cell marker preprosomatostatin, acinar cell markers amylase-2 and hormone-sensitive (HS) lipase, and ductal cell markers cytokeratin 19 and carbonic anhydrase II (5′-AAC GTT GAG TTT GAT GAC TCT-3′ and 5′-AGT TGT CCA CCA TCG CTT CTT-3′); and a miscellaneous gene nestin (5′-AGA AGAGCA GAA CTT AGA AT-3′ and 5′-TAG AGG TTT CAC AAT TCT CT-3′). Hepatocyte-related markers included albumin, α-fetoprotein, and glucose-6-phosphatase. Intestine/stomach-related markers included intestinal fatty-acid binding protein (*fabp-2*), gastric inhibitory peptide (*GIP*), cholecystokinin (*CCK*), tryptophan hydroxylase (*TPH*), gastrin (35), and pepsinogen F. Primers for transcriptional factors were also used as follows: pancreas duodenal homeobox (*Pdx*)-1, neurogenin 3 (*ngn3*) (5′-AGT GCT CAG TTC CAA TTC CAC-3′ and 5′-AAG AAG TCT GAG AAC ACC AGT-3′), hepatocyte nuclear factor (HNF)-1, HNF-3α, HNF-3β, HNF-3γ, HNF-4, and HNF-6 (5′-ATG ACC ATG GCC TGT GAA ACT-3′ and 5′-ATT CAG GTG GGC ATG AGG AT-3′). As a positive control, primers for hypoxanthine phosphoribosyltransferase (*HPRT*) were also prepared. PCR cycles were as follows: initial denaturation at 95°C for 4 min, followed by 35–45 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. PCR products were separated in 2% agarose gel.

Immunocytochemistry. Cells in each colony were cultured for 12 days. They were then washed three times with PBS and fixed first with 4% phosphate-buffered paraformaldehyde for 5 min at room temperature and then with 25% acetone in methanol for 1 min at room temperature. After the fixation, cells were washed in PBS containing 0.05% polyoxyethylene (20) sorbitan monolaurate (Tween 20) (Wako) and treated with 0.2% Triton X-100 (Sigma) for 1 h at room temperature. Nonspecific binding was blocked with 10% nonimmune serum of the species from which the secondary antibody had been obtained. Fixed cells were incubated with primary antibody goat anti-insulin (Santa

Cruz Biotechnology, Santa Cruz, CA), mouse anti-glucagon (Sigma), rabbit anti-somatostatin (Affiniti Research Products, Exeter, U.K.), or rabbit anti-amylase (Sigma) in a moist chamber for 16 h at 4°C. After washing in PBS-Tween 20 and blocking, cells were incubated with Alexa 488–conjugated donkey anti-goat IgG (Molecular Probes, Eugene, OR), Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), or Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) for 3 h at 4°C, respectively. After final washing, cells were viewed using a Zeiss LSM510 laser scanning microscope.

Immunohistochemistry. Neonatal and adult pancreatic tissues were fixed in 4% phosphate-buffered paraformaldehyde overnight at 4°C, dehydrated overnight in 20% sucrose at 4°C, and then embedded in OCT compound. Cryostat sections of these tissues were dried and fixed secondarily by acetone for 10 min at room temperature. Sections were dried at −20°C overnight, washed with PBS-Tween 20, and treated with 0.2% Triton X-100 for 1 h at room temperature. For CA II and Flk-1 staining, tissues were treated with 10 mmol/l citric buffer (pH 6.0) for 1 h at 37°C between washing and Triton treatment. After washing in PBS-Tween 20 and blocking, tissues were incubated with the following primary antibodies in a moist chamber for 16 h at 4°C: goat anti-insulin, mouse anti-glucagon, goat anti-amylase (Santa Cruz Biotechnology), rabbit anti-Met (Santa Cruz Biotechnology), sheep anti-CAII (The Binding Site, Birmingham, U.K.), or goat anti-Flk-1 (Santa Cruz Biotechnology). As secondary antibodies, we used Alexa 488–conjugated goat anti-rabbit IgG (Molecular Probes), Alexa 488–conjugated donkey anti-goat IgG, Alexa 488–conjugated donkey anti-sheep IgG, Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), and Cy3-conjugated donkey anti-rabbit IgG. Stained tissues were viewed using a Zeiss LSM510 laser scanning microscope.

Retroviral infection. Production of a retroviral vector is described elsewhere (24). For marking of a CAPC clone with enhanced green fluorescent protein (EGFP), concentrated virus supernatant was added to 6-well plate cultures in which cells had grown up to 40–50% confluence in 2.5 ml of standard medium with 5 μg/ml protamine sulfate (Sigma) followed by "spinoculation" (36). Residual virus was eliminated by washing the cells with PBS and changing the medium after 24 h. EGFP-positive cells were identified by FACS Vantage.

Cell transplantation. After the initiation of clonal culture, we maintained EGFP-tagged cells in culture by replating them every 7 days. Donor cells were usually prepared from cells obtained at these passage points. EGFP-tagged cells were trypsinized, washed, and resuspended at a concentration of 2×10^6 cells in 100 μl standard medium. They were then injected into pancreata of recipient mice through the common bile duct (C57BL/6, 4 weeks old, $n = 5$; Clea) under anesthesia. We also injected standard medium without cells as a negative control ($n = 5$).

RESULTS

Flow-cytometric isolation and enrichment of cells with high growth capacity in the neonatal pancreas. To examine the growth and differentiation potential of c-Met–positive cells in the pancreas, neonatal pancreata were dissociated and fractionated into four subpopulations by FACS, using antibodies to c-Met and c-Kit (stem cell factor receptor) after excluding hematopoietic cells identified by the expression of CD45 (leukocyte common antigen) and TER119 (a molecule resembling glycophorin and exclusively expressed on immature erythroid cells). Because Rachdi et al. (37) showed that c-Kit is expressed in some mature β-cells residing in islets, we attempted to eliminate such cells by using an antibody against c-Kit. Nonhematopoietic CD45$^-$ TER119$^-$ cells comprised 37.8 ± 4.11% of the initial population (mean ± SD). These cells were further subfractionated into the following groups: *1*) c-Met$^+$ c-Kit$^+$ cells (0.01 ± 0.02%), *2*) c-Met$^+$ c-Kit$^-$ cells (0.99 ± 0.11%), *3*) c-Met$^-$ c-Kit$^+$ cells (0.81 ± 0.07%), and *4*) c-Met$^-$ c-Kit$^-$ cells (35.9 ± 2.97%) (Fig. 1*A*). The proliferation of cells in each subpopulation was then examined in vitro.

We previously set up culture conditions (25) in which single cells proliferated to form clusters of up to several hundred cells. Sorted cells adhered to the culture dishes and formed clonal colonies as culture proceeded (Fig. 1*B–E*). Cells were initially cultured at 200 cells/cm^2, but only 20–30% of cells adhered to the culture dish; nonadherent cells were removed by changing the medium. This clonal density of the culture allowed colonies to form separately from each other. At day 8 of culture, colonies formed by morphologically identified epithelial-like cells (ECs) were seen (Fig. 1*F* and *G*). The cell that had initiated the formation of these colonies was provisionally designated as an EC-forming cell (ECFC). In addition to epithelial-like cells, fibroblast-like cells can grow, but not extensively. Fibroblast-like cells, however, do not express any marker genes for endocrine and exocrine pancreatic cells.

In in vitro colony assays of FACS-sorted cells, few ECs or none were found in CD45$^+$, TER119$^+$, c-Met$^+$ c-Kit$^+$ CD45$^-$ TER119$^-$, c-Met$^-$ c-Kit$^+$ CD45$^-$ TER119$^-$, and c-Met$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell populations. Most were found in c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells. Sorting for c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells achieved 13.1- and 9.4-fold enrichment of ECFCs compared with total neonatal pancreatic cells and CD45$^-$ TER119$^-$ cells, respectively (Fig. 1*H*).

Since c-Met is also expressed in vascular endothelial cells and HGF works as an angiogenic factor (38,39), we conducted further fractionation of the c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulation into the following two distinct subpopulations by using an antibody against the vascular endothelial cell marker Flk-1: *1*) c-Met$^+$ Flk-1$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells (0.26 ± 0.03%) and *2*) c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells (0.59 ± 0.01%) (Fig. 1*I*). In cultures with a clonal density of 200 cells/cm^2, ECFCs were identified mostly from the c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulation (Fig. 1*J*).

Differentiation potential of ECFCs. We next examined the differentiation potential of ECFCs. Cells in each EC were analyzed by RT-PCR to identify the expression of genes encoding several markers for pancreatic endocrine (α-cell, preproglucagon; β-cell, preproinsulin I and II; γ-cell, PP; and δ-cell, preprosomatostatin), acinar (amylase 2 and HS lipase), and ductal (cytokeratin 19 and carbonic anhydrase II) cells. Although it was difficult to find cells expressing these markers, aside from cytokeratin 19, before day 5 of culture, ECFCs exhibited various differentiation patterns at day 12 (Table 1). Most colonies contained two (21.4%; $n = 28$ colonies assessed), three (32.1%), four (28.6%), or five (17.9%) lineage cell types. Eleven colonies (39.3%) contained cells that were differentiating into all three major cell types: endocrine, acinar, and ductal. Immunocytochemical analysis also showed that ECs contained cells positive for insulin, glucagon, somatostatin, or amylase at day 12 (Fig. 2). These results indicate the multipotency of ECFCs.

Localization of c-Met–positive cells in the pancreas. In order to determine where c-Met–positive cells localize in the developing mouse pancreas, we performed immunohistochemical staining of neonatal pancreas with an anti-Met antibody. Positive cells were detected in acinar tissues (Fig. 3*A–C*) and also in some ducts (Fig. 3*D–F*) and vascular endothelia (Fig. 3*G–K*). The percentage of c-Met–positive cells that we estimate is <0.5% in acinar cells, <1% of ducts, and <10% of blood vessels. c-Met–positive ductal or vascular endothelial cells coexpressed either carbonic anhydrase II or Flk-1 (Fig. 3*D–F* and *I–K*). In

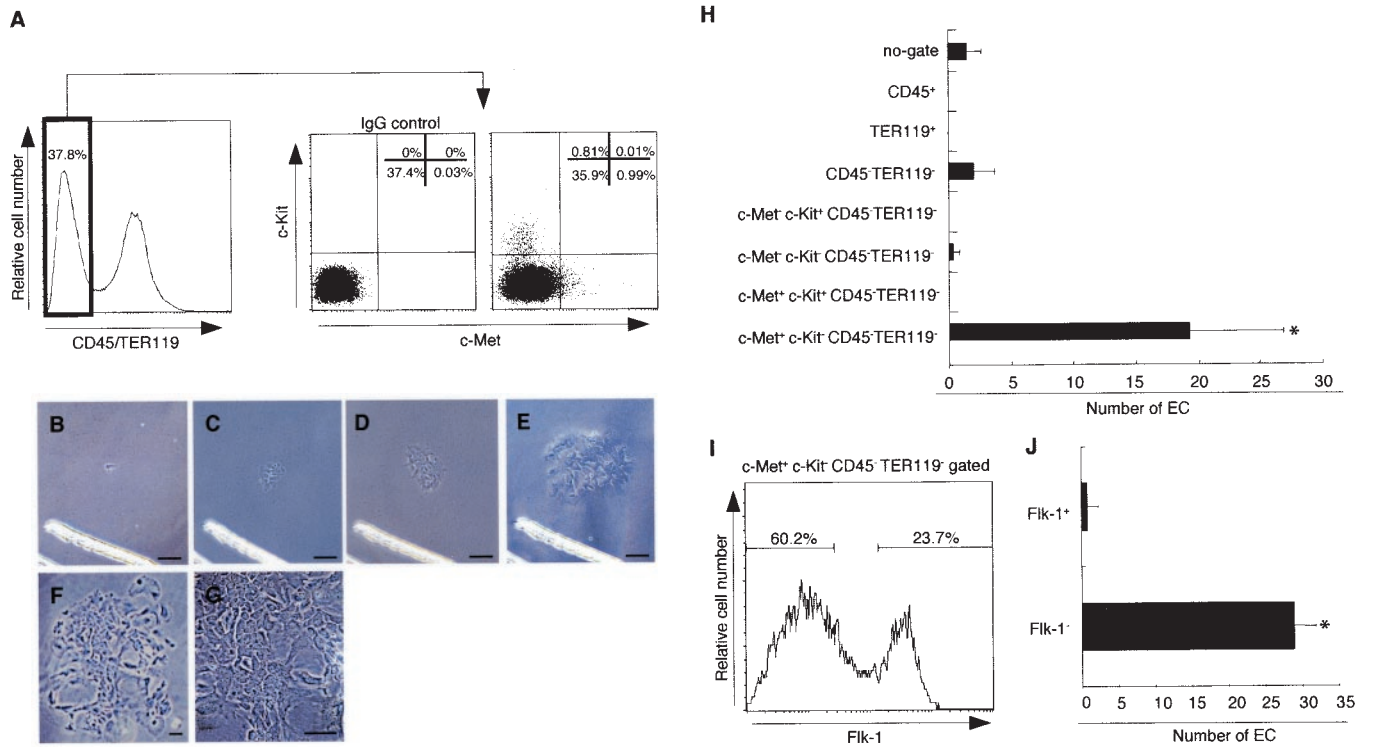

FIG. 1. Flow cytometric isolation and enrichment of colony-forming cells in the neonatal pancreas. *A*: CD45$^+$ or TER119$^+$ hematopoietic cells were gated and removed from the initial pancreatic tissue specimen. Nonhematopoietic (CD45$^-$ TER119$^-$) cells were fractionated based on c-Met and c-Kit expression. Percentages of fractionated cells are shown at the top of each panel. For in vitro colony assay, sorting gates were set for the c-Met$^-$ c-Kit$^+$, c-Met$^-$ c-Kit$^-$, c-Met$^+$ c-Kit$^+$, and c-Met$^+$ c-Kit$^-$ subpopulations. Establishment of the gate was based on the staining profiles of the negative control (IgG control). Representative data are shown for six independent experiments. *B–G*: Formation of a colony derived from a sorted cell in culture. Under the clonal density culture (200 cells/cm^2 in each 35-mm tissue culture dish), a single sorted cell attached itself to the culture dish after 12 h (*B*). The cell then began to divide and formed a colony after 48 (*C*), 72 (*D*), and 120 h (*E*). Scratches in the substrate serve to identify the field. *F* and *G*: A colony continued to grow, generating an EC at day 8. *H*: The number of ECs was counted after 8 days in the clonal density culture (200 cells/cm^2). *I*: We further fractionated the c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulation into two subpopulations by using antibodies against a vascular endothelial cell marker Flk-1. For in vitro colony assay, sorting gates were set for the c-Met$^+$ Flk-1$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$ and c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulations. *J*: The number of ECs was counted after 8 days in the clonal density culture (200 cells/cm^2). *H* and *J*: The graphs show the average of 18 dishes for each cell subpopulation in six independent experiments. *Mann-Whitney *U* test: $P < 0.001$. Scale bar: 100 μm.

contrast, c-Met–expressing cells in acinar tissues were not positive for any markers of pancreatic lineage cells (Fig. 3*C*, data not shown). In the human pancreas, it has been reported (27) that c-Met is expressed in β-cells, for which HGF acts as a mitogen. We detected some weakly c-Met–positive cells in mouse islets, but its expression was not consistent; it appeared to vary from location to location. Alternatively, c-Met expression within β-cells may have been specific to a particular strain or cellular developmental stage, occurring only during growth, differentiation, or functional maturity.

In the adult pancreas, stem/progenitor cells are thought to reside in the pancreatic ducts. Our present study shows that ductal cells, as well as some acinar cells, in the neonatal pancreas express c-Met and that they can be sorted using FACS. Therefore, we speculated that some

TABLE 1
Expression of various marker genes in independent neonatal epithelial colonies

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Preproglucagon (α-cell)	+	+	−	+	+	+	−	+	−	−	−	+	−	+	+	+	+	−	+	+	+	−	+	−	−	−	+	−
Preproinsulin I (β-cell)	+	+	−	−	+	+	−	+	−	−	+	−	+	−	+	−	+	−	+	+	+	−	−	+	−	+	+	+
Preproinsulin II (β-cell)	+	+	−	−	+	−	−	+	−	−	+	+	−	−	+	−	+	−	−	+	+	−	−	−	−	+	+	+
PP (γ-cell)	−	+	+	−	+	−	−	+	+	+	−	+	+	+	−	+	+	+	+	−	−	−	−	+	−	−	+	−
Preprosomatostatin (δ-cell)	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	−	+	−	−	−	−	−	+	+	−	−	−	−
Amylase 2 (exocrine cell)	−	−	+	+	−	−	+	−	−	−	−	+	+	+	−	−	−	−	−	+	+	+	+	+	−	+	+	−
HS lipase (exocrine cell)	−	−	+	−	−	−	+	−	−	−	−	+	−	−	−	−	−	+	−	+	−	+	+	−	+	−	−	+
Cytokeratin 19 (ductal cell)	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	−	+
Carbonic anhydrase II (ductal cell)	−	−	+	−	−	−	+	−	−	−	−	+	+	−	−	−	−	−	+	+	−	+	+	−	+	−	−	−
c-Met	−	+	+	−	+	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
HPRT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lineages included in a colony	3	3	3	3	5	3	2	4	2	2	2	5	5	5	4	3	5	3	4	4	4	2	4	4	2	3	4	3

HPRT, hypoxanthine phosphoribosyltransferase.

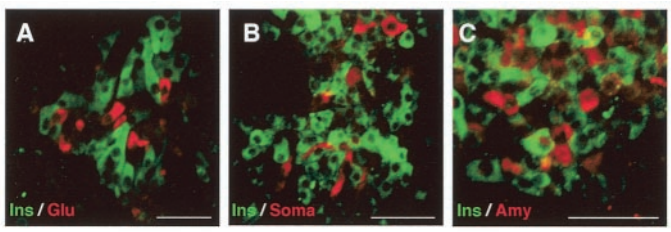

FIG. 2. In vitro multilineage colony formation from sorted c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells. c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells were sorted, cultured for 12 days, and allowed to form ECs. Then, immunocytochemical double staining was conducted on EC constituent cells. *A*: Insulin (green) and glucagon (red). *B*: Insulin (green) and somatostatin (red). *C*: Insulin (green) and amylase (red). Scale bar: 50 μm (*A–C*).

ductal cells in the adult pancreas would also be positive for c-Met. Expectedly, immunohistochemical analysis revealed that c-Met is expressed in adult pancreas in similar regions as seen for neonatal tissues, including ductal, acinar, and vascular endothelial cells (Fig. 3*L–Q*). These data show that the expression of c-Met is maintained in some restricted regions from developing through the adult stage, suggesting that c-Met–positive cells in the adult pancreas would possess stem/progenitor cell characteristics, similar to neonatal c-Met–positive cells.

Isolation and characterization of c-Met–positive cells in adult mouse pancreas. We next tried to isolate c-Met–positive cells from the adult pancreas and examine the growth and differentiation potential of these cells. Cells obtained from the adult pancreas were fractionated by FACS using antibodies against c-Met, Flk-1, c-Kit, CD45, and TER119 (Fig. 4*A*). Similar to neonates, sorting of c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells in the adult pancreas achieved high enrichment of adult ECFCs (Fig. 4*B*). Although a few cells exhibited autofluorescence, they do not form colonies in culture. High enrichment of ECFCs permitted efficient culture of clone-sorted c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells for analyses of their differentiation potential. Cells identified upon clone sorting by flow cytometry were cultured in individual wells of 96-well plates. Each well was examined under a microscope after clone sorting to confirm the deposition of a single cell. Once a cell sorter is adjusted for optimal setting before the experiment, we seldom find wells with more than two cells after clone sorting. When we found these wells, we excluded them from samples for analysis.

To examine the differentiation potential of adult ECFCs, immunocytochemistry and RT-PCR were performed on EC constituent cells. They strongly express the ductal cell marker cytokeratin 19; however, no insulin, glucagon, somatostatin, or amylase expression was detected (Fig. 4*C* and *D*, data not shown). RT-PCR analysis also revealed that all examined ECs contained cells positive for CK19 and c-Met, but none expressed any endocrine cell markers (Table 2). However, *Pdx-1*, expressed in differentiating ductal cells, and an acinar cell marker, HS lipase, were expressed in cells from several ECs. These results suggest that supplemental factors would be required for such cells to differentiate into functional cells. Therefore, we sought to induce lineage marker expression in isolated cells using GLP-1, especially GLP-1 (7-36) and GLP-1 (1-37), which can induce differentiation of β-cells in the pancreas (40) or activate insulin gene expression in intestinal epithelial cells (41), respectively. In cultures with these peptides,

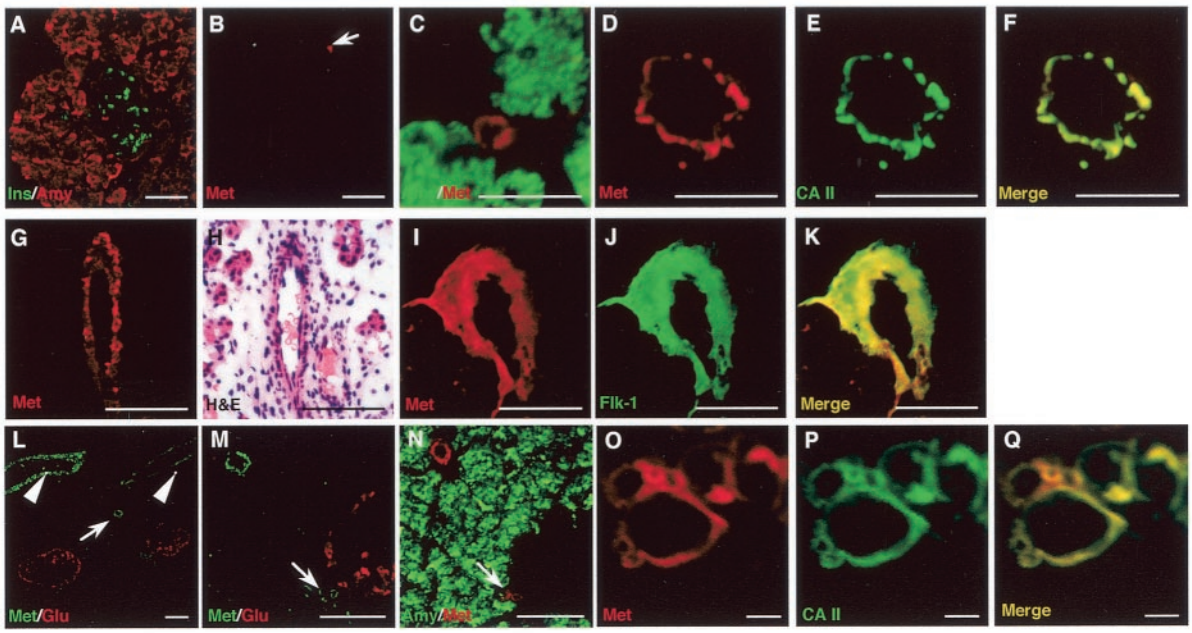

FIG. 3. Localization of c-Met–positive cells in the mouse pancreas. *A–C*: In the neonatal pancreas, a few c-Met–positive cells resided in acinar tissues (*B*, arrow), but not in islets. Note that c-Met was detected in cells expressing no endocrine or acinar cell markers. *A*: Insulin (green) and amylase (red). *B*: A serial section of *A* was stained by anti–c-Met antibody (red, shown as arrow). *C*: A serial section of *A* was stained by antibodies against amylase (green) and c-Met (red). *D–F*: c-Met–positive cells were found in some ducts. *D*: c-Met (red). *E*: CAII (green). *F*: Merge. *G–K*: Cells residing in several vascular endothelia also expressed c-Met. *G* and *I*: c-Met (red). *H*: Hematoxylin and eosin stain. *J*: Flk-1 (green). *K*: Merge. *L–N*: In the adult pancreas, c-Met is expressed in ductal cells (*L*, arrow), vascular endothelial cells (*L*, arrowheads), and cells residing around acinar tissues (*M* and *N*, arrow). Note that these immunoreactive regions for c-Met are maintained from the neonatal through the adult stage. *O–Q*: c-Met–positive ductal cells in the adult pancreas also expressed the ductal cell marker CAII. *O*: c-Met (red). *P*: CAII (green). *Q*: Merge. Scale bar: 100 μm (*A*, *B*, *G*, *H*, and *L–N*), 50 μm (*D–F* and *I–K*), 25 μm (*C*), and 10 μm (*O–Q*).

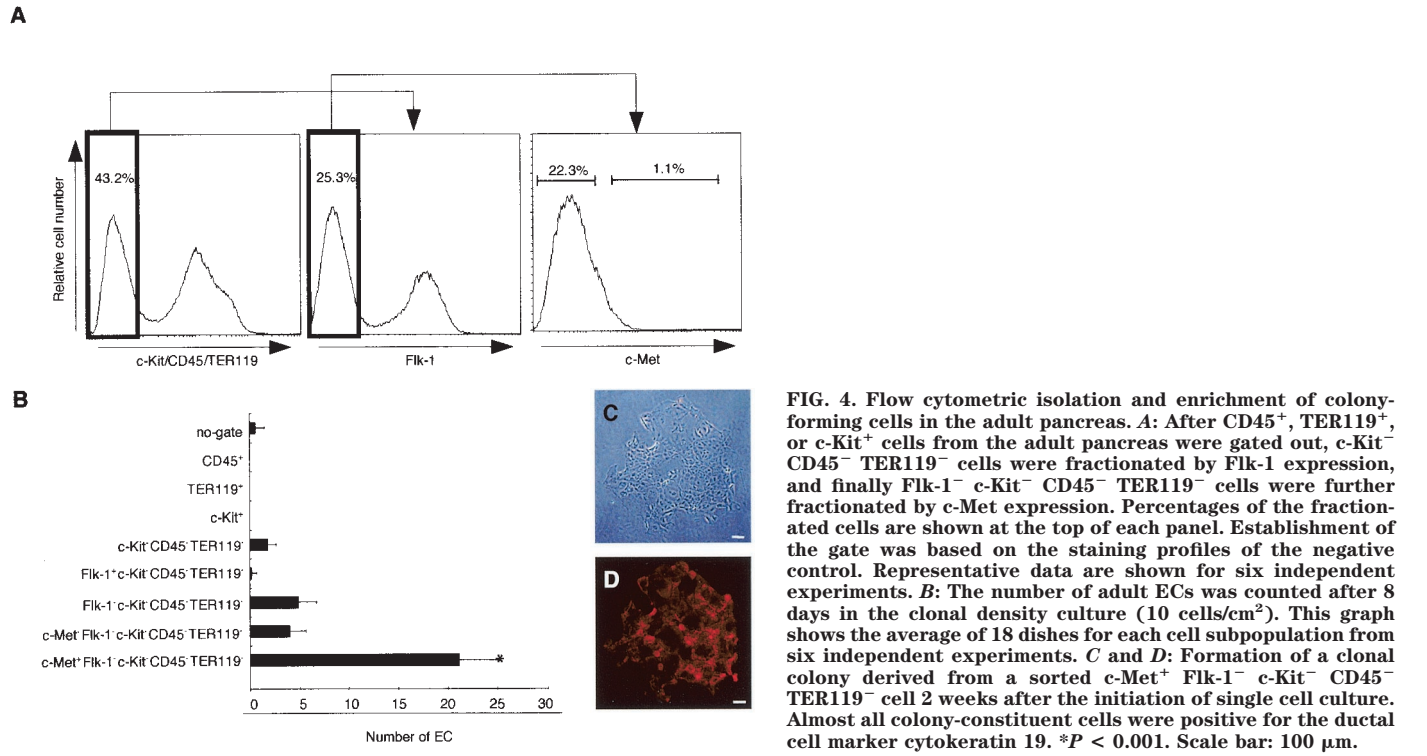

FIG. 4. Flow cytometric isolation and enrichment of colony-forming cells in the adult pancreas. *A*: After CD45$^+$, TER119$^+$, or c-Kit$^+$ cells from the adult pancreas were gated out, c-Kit$^-$ CD45$^-$ TER119$^-$ cells were fractionated by Flk-1 expression, and finally Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells were further fractionated by c-Met expression. Percentages of the fractionated cells are shown at the top of each panel. Establishment of the gate was based on the staining profiles of the negative control. Representative data are shown for six independent experiments. *B*: The number of adult ECs was counted after 8 days in the clonal density culture (10 cells/cm^2). This graph shows the average of 18 dishes for each cell subpopulation from six independent experiments. *C* and *D*: Formation of a clonal colony derived from a sorted c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell 2 weeks after the initiation of single cell culture. Almost all colony-constituent cells were positive for the ductal cell marker cytokeratin 19. *P < 0.001. Scale bar: 100 μm.

expression of several marker genes, including insulin genes, emerged in cells derived from some ECFCs (Table 2). These data suggest that c-Met is a convenient cell surface marker of cells with high proliferative capacity and multiple differentiation potential in adult pancreas, and it is useful to isolate such cell populations by using FACS to examine their characteristics clonally. Since our culture environment, however, is still not sufficient to induce complete differentiation of adult ECFCs into endocrine and acinar cells, further investigation of their potential for differentiation is required.

Cell expansion from sorted neonatal c-Met–positive cells. When we cultured no-gated cells derived from neonatal pancreas at high density (1,000 cells/cm^2), several epithelial-like cells proliferated, reaching semiconfluency after 1 month in culture. They also produced cell aggregates that mimicked pancreatic islet structures. We provisionally designated cells that produce such aggregates as CAPCs. Ramiya et al. (6) reported that these islet-like structures were generated from stem cells derived from ductal epithelia as culture proceeded. Although the culture conditions were different, similar cells also appeared in our culture condition. To determine which cell subpopulation contained these CAPCs in vivo, we cultured directly sorted cells from neonatal pancreas using antibodies for c-Met, Flk-1, c-Kit, CD45, and TER119. When we cultured cells at low density (<200 cells/cm^2), it was difficult to find cells 1 month later. However, in middle- to high-density culture conditions (500–1,000 cells/cm^2), cells sorted into no-gated, CD45$^-$ TER119$^-$, c-Met$^+$ c-Kit$^-$ CD45$^-$ TER119$^-$, and c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cell subpopulations proliferated and gen-

TABLE 2
Expression of various marker genes in independent adult epithelial colonies in single cell culture

	HGF + EGF										HGF + EGF + GLP-1 (1–37)					HGF + EGF + GLP-1 (7–36)				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Preproglucagon (α-cell)	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	+	+
Preproinsulin I (β-cell)	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	+	+	−
Preproinsulin II (β-cell)	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	+	−
PP (γ-cell)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Preprosomatostatin (δ-cell)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Amylase 2 (exocrine cell)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
HS lipase (exocrine cell)	−	−	−	+	−	+	−	−	−	−	−	+	+	−	+	+	−	+	+	−
Cytokeratin 19 (ductal cell)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbonic anhydrase II (ductal cell)	+	−	−	+	−	+	−	+	−	−	−	−	+	+	+	+	−	+	+	+
Pdx1	+	−	−	−	+	+	−	−	−	−	−	+	+	+	−	+	−	+	+	−
c-Met	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HPRT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lineages included in a colony	1	1	1	2	1	2	1	1	1	1	1	2	4	2	3	4	1	3	4	2

HPRT, hypoxanthine phosphoribosyltransferase.

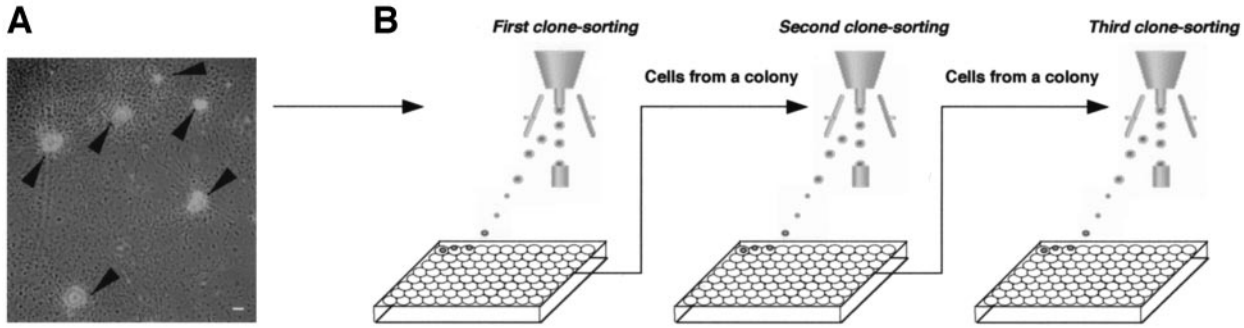

FIG. 5. Clonal analysis of CAPCs derived from c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ sorted cells. *A*: Islet-like cell aggregates that budded from the monolayer cell sheet were observed in long-term cultures of neonate-derived c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells (arrowheads). *B*: The CAPCs were then clone sorted by FACS and individually cultured in 96-well tissue culture plates. Second and third clone sortings were also conducted by using cells included in a clonal colony. Scale bar: 50 μm.

erated cell aggregates (Fig. 5*A*). No cells were found when we cultured cells from other cell subpopulations, even at much higher-density conditions (>10,000 cells/cm^2). These results show that CAPCs express c-Met but not Flk-1, c-Kit, CD45, or TER119 in the neonatal pancreas.

To characterize these cells clonally, we conducted single-pass sorting by FACS and cultured them in individual wells of 96-well plates (Fig. 5*B*, first clone sorting). Colonies were formed by 3.13 ± 1.04% of sorted CAPCs (average of six plates [576 wells], three independent experiments). Among the progeny of a single cell, the expression of genes characteristic of not only pancreatic endocrine and acinar lineages, but also for progenitor cells (*Pdx-1*, *ngn3*, and *nestin*), was detectable (Table 3).

TABLE 3
Expression of various marker genes in independent colonies after serial clone sorting of CAPCs

	First clone sorting					Second clone sorting					Third clone sorting				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pancreas-related genes															
Preproglucagon	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Preproinsulin I	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Preproinsulin II	+	−	−	+	+	−	+	+	+	+	+	+	−	+	+
IAPP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
glut-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Preprosomatostatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Amylase 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HS lipase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cytokeratin 19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbonic anhydrase II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nestin	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+
c-Met	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hepatocyte-related genes															
Albumin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-*Tetoprotein*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose-6-phosphatase	+	+	+	−	+	+	−	−	+	+	+	+	−	+	+
Intestine/stomach-related genes															
Fabp-2	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
GIP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CCK	+	+	−	+	+	+	+	+	−	+	+	−	+	+	+
TPH	−	+	−	−	+	+	−	+	−	+	+	+	+	+	−
Pepsinogen F	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
Gastrin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Transcription factor genes															
Pdx1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ngn3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HNF-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*HNF-3*α	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*HNF-3*β	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*HNF-3*γ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HNF-6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HPRT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

CCK, cholecystokinin; GIP, gastric inhibitory peptide; HPRT, hypoxanthine phosphoribosyltransferase; IAPP, islet amyloid polypeptide; TPH, tryptophan hydroxylase.

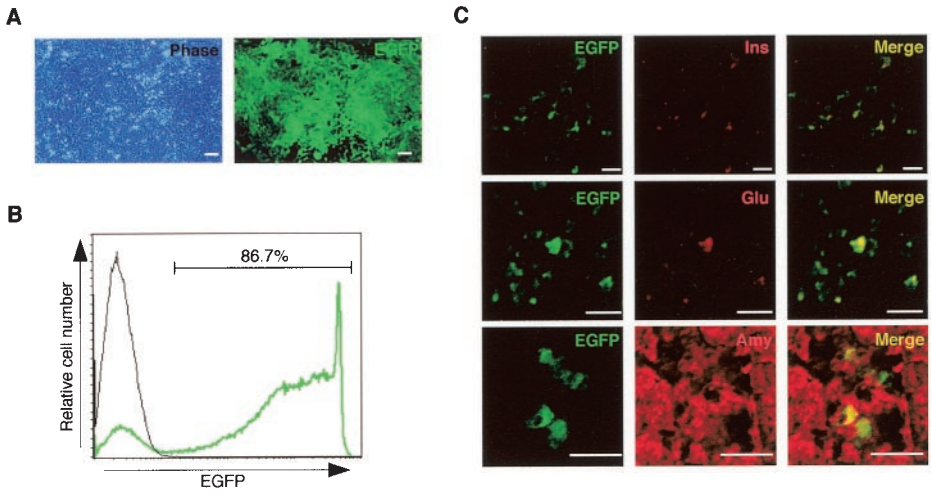

FIG. 6. Clonally expanding CAPCs in culture can differentiate into pancreatic endocrine and acinar cells in vivo. *A*: For use as donor cells, an expanding CAPC clone was marked genetically with EGFP by retrovirus infection. *B*: After a single round of infection, FACS analysis revealed that 85.4 ± 4.2% (mean ± SD) of cells highly expressed EGFP (n = 3). *C*: One month after transplantation of cells, the pancreata of recipient mice were fixed and sectioned. In islets, several EGFP-positive cells expressing the β-cell marker insulin or the α-cell marker glucagon were observed. An acinar cell marker amylase was also detected in EGFP-positive cells residing in acinar tissues, showing that donor cells had given rise to both endocrine and acinar cells in vivo and reconstituted these tissues with host cells. Scale bar: 100 μm (*A*) and 40 μm (*C*).

Furthermore, the expression of hepatocyte- and intestinal/stomach-related genes was also detected (Table 3). To examine whether CAPCs expanded with self-renewing cell divisions, we conducted subcloning experiments for cells in a CAPC-derived colony (Fig. 5*B*, *second and third clone sorting*). In Table 3, secondary formed colonies (colony number 6–10) and tertiary formed colonies (colony number 11–15) were derived from cells in the colonies numbered 1 and 6, respectively. RT-PCR analysis showed that various endoderm marker genes were expressed in colonies formed by second and third single-cell sorting, similar to primary formed colonies (Table 3). These data indicate that long-term cultured c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells can differentiate into multiple cell types in endodermal organs, while they maintain multipotent cells by self-renewing cell divisions.

In vivo differentiation of clonally expanding CAPCs. In order to determine whether a clonally expanding CAPC in culture could differentiate into both pancreatic endocrine and acinar cells in vivo after transplantation, we injected them through the common bile duct into a recipient pancreas. Cells derived from colony 1 (Table 3) were used for this in vivo analysis. Before cell transplantation, donor cells were marked genetically with EGFP by retrovirus infection to distinguish donor cells from recipient cells (Fig. 6*A*). The concentrated vesicular stomatitis virus pseudotyped retrovirus allowed high transduction frequencies, and >85% of cells highly expressed EGFP after a single round of infection (Fig. 6*B*).

At 1 month posttransplantation, all five recipient mice exhibited donor cell integration into pancreatic tissues. Staining for the β-cell marker insulin, α-cell marker glucagon, and acinar cell marker amylase showed that donor cells had given rise to both endocrine and acinar cells in vivo (Fig. 6*C*). However, engrafted cells were widely scattered in the pancreas, and few of them existed with recipient α- and β-cells in islets. Integration and engraftment of donor cells in islets might require an adequate tissue injury for islet neogenesis. Because the engraftment of donor cells was slightly found, it is also required to improve the protocol of cell transplantation and to prepare a number of transplantable functional cells to realize the repopulation of islet and acinar tissues. This result, however, is the first report to show that derivatives of a single cell can survive in the pancreas for at least 1 month and differentiate into endocrine and acinar lineage cells in vivo as well as in vitro.

DISCUSSION

A previous study (6) has shown that cells derived from ductal epithelia generated islet-like cell aggregates and expressed c-Met. Because such ductal cell fractions, however, still contained many other kinds of cells, c-Met has not been defined as a possible marker to characterize stem/progenitor cells in the pancreas. Our present results indicate that c-Met is a useful marker to prospectively identify and isolate cells that fulfill the criteria of pancreatic stem/progenitor cells. By sorting c-Met–positive cells using FACS in this study, we achieved prospective isolation of a rare c-Met–positive cell population by completely excluding other lineage cells and revealed their high proliferative capacity and multiple differentiation potential. This stem/progenitor cell activity is restricted to the c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells, which constitute scarcely ~1% of both the neonatal and adult pancreas. The remaining 99% of the cells, in contrast, have much less activity of stem/progenitor cells. Furthermore, when neonatal c-Met$^+$ Flk-1$^-$ c-Kit$^-$ CD45$^-$ TER119$^-$ cells were cultured for longer periods (>1 month), a semiconfluent monolayer of cells appeared, from which islet-like cell aggregates budded out, and they gave rise to cells expressing multiple lineage markers for endodermal digestive organs in vitro. These findings suggest that c-Met–positive cells residing in the pancreas possess potential for wider cell lineage plasticity in organs of endoderm origin or that they are more primitive endodermal stem cells.

The c-Met/HGF interaction mediated by signaling between mesenchymal and epithelial cells plays important roles in pancreatic development (27,28). Whether this essential signaling is directly responsible for stem cell activity, however, remains to be determined. In this study, we showed that some ductal and acinar cells in the neonatal pancreas are immunoreactive for c-Met and that they could be specifically separated from other differentiated cells by using FACS. These sorted c-Met–positive cells proliferated exclusively in response to HGF and exhibited multipotent differentiation. These findings permit us to speculate that the c-Met/HGF interaction is critically responsible for stem cell growth and differentiation in the developing pancreas. Interestingly, our studies

showed that nestin, a recently reported (42) marker for stem-like cells that reside in ductal epithelia and islets, was expressed in both CAPCs (Table 3) and EC constituent cells (data not shown). Nestin, however, was also detected in the c-Met–negative cell fraction, suggesting that this antigen is expressed in a much broader cell population, including differentiated cells.

In the adult pancreas, immunohistochemical analysis showed expression of c-Met in several ductal cells. Also, most adult ECs derived from sorted c-Met–positive cells possessed ductal cell characteristics, such as expression of CK19, and they received a growth signal from HGF to grow in culture. These results indicate that adult ECFCs reside in some pancreatic ducts, express c-Met, and are capable of forming colonies in response to HGF after sorting. Therefore, the c-Met/HGF interaction, in addition to its role in pancreatic development, would be crucial for homeostatic cell turnover and regeneration from stem/progenitor cells residing in the adult pancreas. Our data also demonstrate that the multilineage differentiation capacity of ECFCs is much more strictly regulated in adults than in neonates. For this reason, neonatal "activating" stem cells, rather than those isolated from the adult pancreas, would be more suitable for clinical applications, such as cell transplantation, that require the production of multiple cell type lineages and self-renewing cell proliferation. Although most adult ECs possess a ductal phenotype, several colonies included cells expressing *Pdx-1* or the acinar cell marker HS lipase, suggesting that the original ECFC could potentially differentiate into both endocrine and acinar lineage cells. In fact, GLP-1 can induce expression of several marker genes in cells derived from adult ECFCs. However, this effect is not sufficient to produce many functional cells for therapeutic application. Differences of differentiation potential between c-Met–positive cells in the neonatal and adult pancreas may indicate that adult "silencing" stem cells residing in ducts cannot respond immediately to severe β-cell loss. Instead of these cells, intraislet β-cell progenitors, which emerge in islets following depletion of the resident cell population by a β-cell toxin (e.g., streptozotocin), may differentiate to recover insulin content in β-cell neogenesis (43). Screening for differentiation-inducing factors using our clonal cell culture system for stem/progenitor cells should identify a critical substrate that activates differentiation of endogenous PSCs and efficient reproduction of functional β-cells.

A candidate mouse hepatic stem cell, hepatic colony-forming unit in culture (H-CFU-C), which has been reported previously (24) by our group, has a similar phenotype to a candidate PSC described here, including the expression of c-Met but not c-Kit, CD45, and TER119. The expression of CD49f (α6 integrin subunit), which is weakly expressed in H-CFU-Cs, is also detected in most c-Met–positive pancreatic cells. Furthermore, both cell types have the potential to differentiate into hepatic, pancreatic, gastric, and intestinal lineage cells in vitro. These findings suggest that pluripotent cells, like H-CFU-C in the fetal liver, are also present in the pancreas. However, these putative hepatic and pancreatic stem cells are not completely equivalent, as they possess several striking differences. For example, several extracellular matrices that exert strong growth induction of H-CFU-C do not have an effect on the ability of a candidate PSC to proliferate in culture (23). For this reason, both cell types should not be characterized as the same cell population. Our present data and several previous reports (42,44–48), however, suggest that hepatic or endodermal stem cells may persist in postnatal pancreatic tissue. In particular, it was reported (44,45,47) that pancreatic hepatocytes could develop from pancreatic ductal and/or acinar cells. These regions were defined in this study as being immunoreactive for c-Met and contained candidate PSCs in both neonates and adults. Alternatively, Wang et al. (49) showed that pancreatic cells capable of significant liver reconstitution are not derived from the ductal pancreas. Although further intensive study is required to identify the basis for pluripotency in these cells, it is possible to speculate that pluripotent stem cells for endodermal digestive organs have common phenotypic and physiological characteristics and are uniformly present in the liver, pancreas, stomach, intestine, and other endoderm-derived organs.

As a result of cell transplantation, clonally cultured CAPCs were capable of engrafting in the recipient pancreas and differentiating into endocrine and acinar cells. We infected donor cells with a retrovirus and marked them for EGFP expression in vitro to trace them after transplantation. Single-virus infection reproducibly achieved a high tagging efficiency, suggesting that gene-modified cells are theoretically useful for clinical cell-replacement therapy for the treatment of diabetes. Although insulin-positive donor-derived cells were also found when we transplanted CAPCs derived from EGFP transgenic mice (A.S., unpublished data), the possibility of cell fusion, uptake of DNA from dead cells, or reactivation of the retrovirus after cell transplantation cannot be completely ruled out. Further examination is required to address this issue and to achieve efficient differentiation and replacement of recipient pancreatic tissues by CAPCs.

Purification and characterization of PSCs will allow us to determine their full developmental potential by examining sorted stem cells directly. For this experimental goal, further enrichment of PSCs must be conducted by combining several antibodies with the anti–c-Met antibody, and culture conditions should be improved for clonogenic expansion of directly sorted cells. Manipulation of such cells may accelerate their proliferation and differentiation, make them able to generate functional β-cells more efficiently, and enhance hormone secretion from other endocrine lineage cells. Prospective identification, direct sorting, selective expansion, and precise differentiation control of pluripotent stem cells could provide a means of successful islet cell transplantation in patients with diabetes due to absolute or relative loss of β-cells.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan (14207046, 14081201, and 14657279) and by JSPS Research Fellowships for Young Scientists. This work was also supported by grants from The Mitsubishi Foundation and the Nissan Science Foundation and by grants for the leading project from the Ministry of Education, Science and Culture of Japan.

We thank N. Ukawa and Y. Jinzenji for technical support and Y. Morita for FACS operation.

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