Label-Retaining Cells in the Rat Pancreas

Location and Differentiation Potential in Vitro

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Islets of Langerhans are micro-organs scattered throughout the pancreas that contain insulin-producing cells, called β-cells. Although new light has been recently shed on β-cell development, information on the phenotype and location of β–stem cells remains scarce. Here, we provide evidence that β–stem cells are slow-cycling cells located within and around the islets of Langerhans. First, using a bromodeoxyuridine (BrdU) pulse/chase approach, we detected BrdU-retaining cells in vivo in the islet area of rat pancreata. These cells were negative for endocrine markers but expressed Pdx1, a marker for pancreatic stem cells. Next, using an in vitro model that mimicked endocrine cell development, we found that BrdU-retaining cells were capable of differentiating into β-cells. Taken together, these observations demonstrate that BrdU retention is a property of β–stem cells. *Diabetes* 52:2035–2042, 2003

Recent data indicate that stem cells exist in many tissues, including the hematopoietic tissue, central and peripheral nervous system, epidermis, prostate, liver, and retina (1–7). Specific markers for stem cells in hematopoietic tissue and the central and peripheral nervous system have been identified and used to prospectively monitor the fate of these cells (1,2,4). For other tissues, such as the epidermis or prostate, specific markers are not available and alternative strategies are used to characterize stem cells. One strategy is based on the slow rate of stem cell proliferation. Briefly, cells are labeled by a pulse of DNA precursor, such as tritiated thymidine or bromodeoxyuridine (BrdU), which is incorporated into the DNA. This results in labeling most of the cells. Next, a prolonged chase period results in dilution of the DNA precursor in the cells that actively proliferate. Label-retaining cells are then identified. This method has been used to determine the location of follicular and epidermal stem cells in mammals (5) and has provided evidence that prostate epithelial stem cells may be located in the proximal prostatic duct area (6).

Several studies suggest that stem/progenitor cells may be present in the pancreas throughout the lifespan, since new insulin-producing cells (β-cells) can develop in the pancreas in adulthood under specific conditions. The exact location of these stem cells has been investigated in various pancreatic tissues, including the pancreatic ducts (8,9), the acini (10), and the islets themselves (11–13). Pdx1, a specific transcription factor necessary for pancreas development, has been identified as a potential marker for β–stem cells (13–17). However, the exact nature of these cells remains poorly understood and additional cell markers and properties need to be defined.

The objective of the present work was to improve the characterization of β–stem cells in the pancreas. To this end, we investigated whether BrdU-retaining cells were present in vivo in the rat pancreas. These cells were indeed found, and we sought to determine whether they were β–stem cells. Using an in vitro model that mimics endocrine cell development from embryonic pancreatic epithelium, we found that β-cells differentiated from the BrdU-retaining cells. Thus, BrdU retention is a specific property of pancreatic β–stem cells.

RESEARCH DESIGN AND METHODS

Animals. Pregnant Wistar rats were purchased from the Janvier breeding center (CERJ, Le Genet, France). The animals had free access to food pellets and water. The first day postcoitum was designated as embryonic day 0.5 (E0.5). The rats were killed with CO_2.

BrdU labeling and in vivo chase. To label slow-cycling cells, we injected BrdU (50 μg/g body wt) (Sigma-Aldrich, Saint Quentin Fallavier, France) into neonatal (3-day-old) rats twice daily for 3 days. The rats were killed either 2 h or 4 weeks after the last injection. The pancreata were dissected and fixed in formalin for immunohistochemistry studies.

BrdU labeling and in vitro chase. To label embryonic pancreatic cells, we injected BrdU (100 μg/g body wt) into pregnant rats, twice daily on E11.5 and E12.5 and once at 10:00 A.M. on E13.5. The embryos were harvested 1 h later and dissected. The dorsal pancreatic buds were dissected as described previously (18). Briefly, the stomach, pancreas, and a small portion of the intestine were dissected together and incubated with 0.5 mg/ml collagenase A (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 30 min. They were then washed several times with Hank's balanced salt solution (HBSS; Gibco, Cergy-Pontoise, France) at 4°C. The epithelium was then mechanically removed from the surrounding mesenchyme using needles on a 0.25% Agar, 25% HBSS, 75% RPMI (Gibco) gel in a Petri dish.

Pancreatic epithelia were then embedded in 500 μl of collagen gel (10% RPMI 10× [Sigma-Aldrich], 80% type I rat-tail collagen [2.5 mg/ml, Sigma-Aldrich], and 10% sodium bicarbonate in 0.1 mol/l NaOH) in four-well plates, as previously described (18,19). Once the gel had polymerized, 500 μl of RPMI 1640 (Gibco) containing penicillin (100 units/ml), streptomycin (100 μg/ml), Hepes (10 mmol/l), L-glutamine (2 mmol/l), nonessential amino acids (1×, Gibco), and 1% heat-inactivated calf serum (Hyclone) were added. Cultures were maintained at 37°C in a humidified atmosphere of 95% O_2/5% CO_2. Human recombinant fibroblast growth factor 7 (FGF7) (50 ng/ml) (R&D System) diluted in PBS was added once a day.

Immunohistochemistry. At the end of the 7-day culture period, the pancreatic rudiments were photographed, fixed in 3.7% formalin, preembedded in agarose gel (4% of type VII low-gelling-temperature agarose [Sigma] in Tris-buffered saline), and embedded in paraffin. Sections 4 μm in thickness

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BrdU, bromodeoxyuridine; FGF7, fibroblast growth factor 7; HBSS, Hank's balanced salt solution.

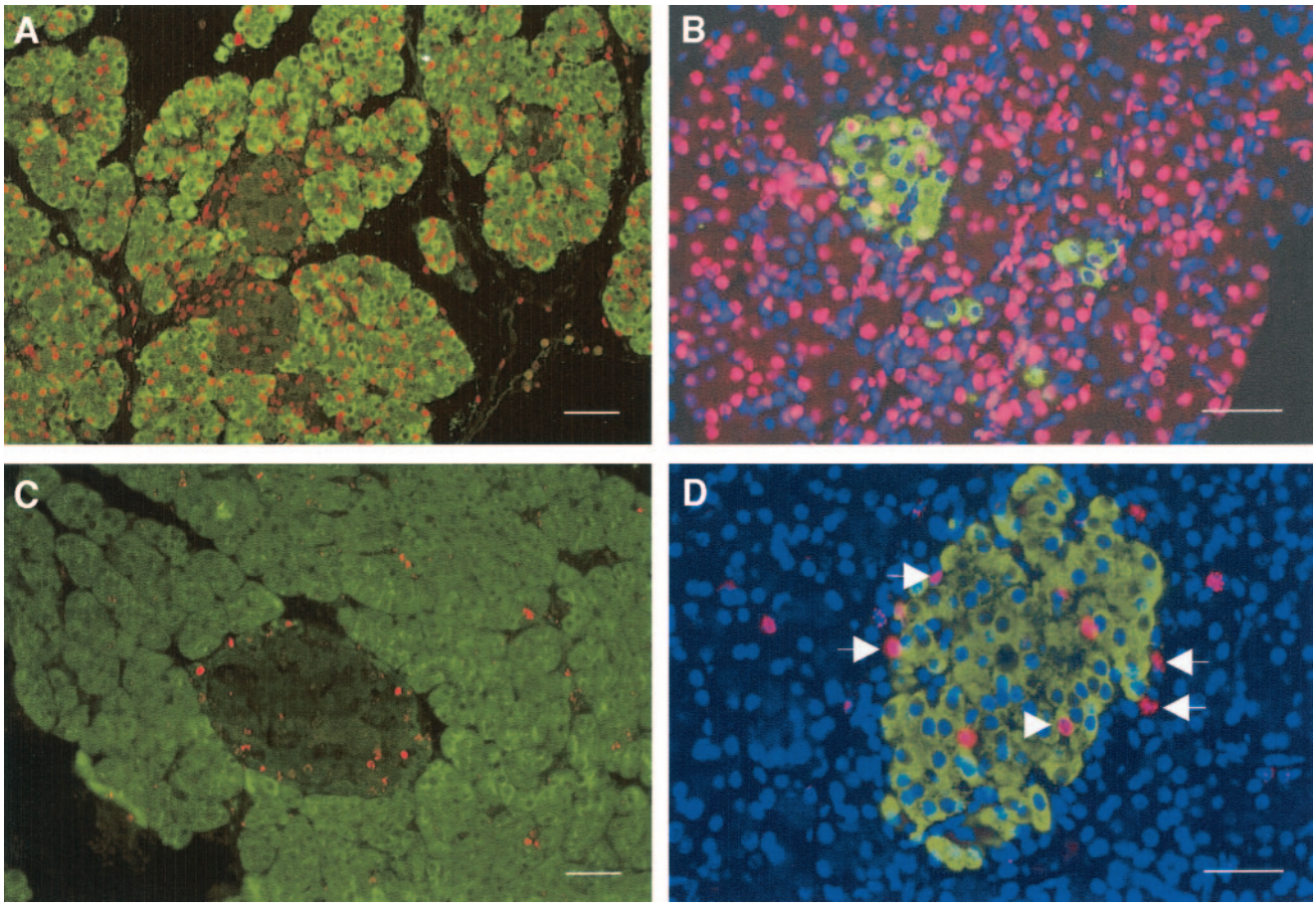

FIG. 1. BrdU labeling and dilution in vivo. Neonatal rats were injected with BrdU between days 3 and 5. They were killed on either day 5 (*A* and *B*) or 4 weeks later (*C* and *D*). In *A–D*, BrdU was revealed in red. In *A* and *C*, carboxypeptidase-A was revealed in green. In *B* and *D*, insulin was revealed in green and nuclei were stained with Hoescht. Arrow in *D*: BrdU-positive cells located around the islets and negative for insulin. Arrowhead in *D*: BrdU-positive cells located around the islets and positive for insulin. Scale bar: 50 μm.

were collected and processed for immunohistochemistry, as described previously (18,20). The antibodies were used at the following dilutions: mouse anti–human insulin (Sigma, 1:2,000), rabbit anti-insulin (Diasorin, 1:2,000), guinea pig anti-insulin (Dako, 1:1,000), rabbit anti-glucagon (Sigma, 1:2,000), rabbit anti-somatostatin (Dako, 1:500), rabbit anti-PP (Eurodiagnostica, 1:500), rabbit anti-carboxypeptidase A (Biogenesis, 1:600), and mouse anti-BrdU (Amersham, 1:2). The rabbit anti-Pdx1 antibody was raised against the synthetic peptide (C) SPQPSSIAPLRPQEPR conjugated to *Concholepas concholepas* hemocyanin. The fluorescent secondary antibodies were fluorescein isothiocyanate anti-rabbit antibody (Jackson Immunoresearch, Baltimore, MD) 1:200, aminomethyl coumarin acetate anti–guinea pig antibody (Jackson Immunoresearch) 1:200, and Texas red anti-mouse antibody (Jackson Immunoresearch) 1:200. Photographs were taken using a fluorescent microscope (Leica; Leitz, Rockleigh, NJ) and digitized using a cooled three-chip charge-coupled–device camera (Hamamatsu C5810; Hamamatsu, Middlesex, NJ). Confocal microscopy was performed using a Zeiss Axiovert 200 confocal microscope.

RESULTS

Identification of slow-cycling cells in the adult rat pancreas. To label slow-cycling cells in the pancreas, we took advantage of the high rate of pancreatic cell proliferation in the neonatal period as compared with adulthood (21). Newborn Wistar rats received two subcutaneous BrdU injections per day from day 3 to day 5 after birth. Figure 1 shows that this resulted in labeling the vast majority of acinar cells (carboxypeptidase-positive cells) (Fig. 1*A*) and some β-cells (insulin-positive cells) (Fig. 1*B*). After a 4-week chase period, we looked for cells that had retained the BrdU. As shown in Fig. 1*C*, nearly all the acinar cells were negative for BrdU and the few BrdU-positive cells were mainly present within or around the pancreatic islets. The BrdU-retaining cells within the islets were often insulin-positive, whereas those around the islets were insulin-negative (Fig. 1*D*).

To determine the nature of the BrdU-retaining cells, we performed consecutive immunostainings for BrdU, Pdx1, insulin, somatostatin, glucagon, and pancreatic polypeptide on the same sections. As shown in Fig. 2*A–C*, BrdU-retaining cells expressing Pdx1 were detected within and around the islets. Pdx1 is a transcription factor expressed in precursor cells and in differentiated β- and δ-cells, which express insulin and somatostatin, respectively (22). Some of the BrdU- and Pdx1-positive cells stained positive and others negative for insulin (Fig. 2*D–F*). The BrdU-positive, Pdx1-positive, and insulin-negative cells stained negative for somatostatin (Fig. 2*F*), glucagon, and pancreatic polypeptide (data not shown), strongly suggesting that they were undifferentiated cells. The presence of these cells was confirmed by confocal microscopy. As shown in Fig. 3, BrdU-retaining cells that stained positive for Pdx1 but negative for endocrine markers were detected after the 4-week chase period. Taken together, these results indicate that BrdU-retaining cells expressing Pdx1 but negative for the markers found in differentiated cells are present in the pancreas.

BrdU dilution and retention in vitro. To determine

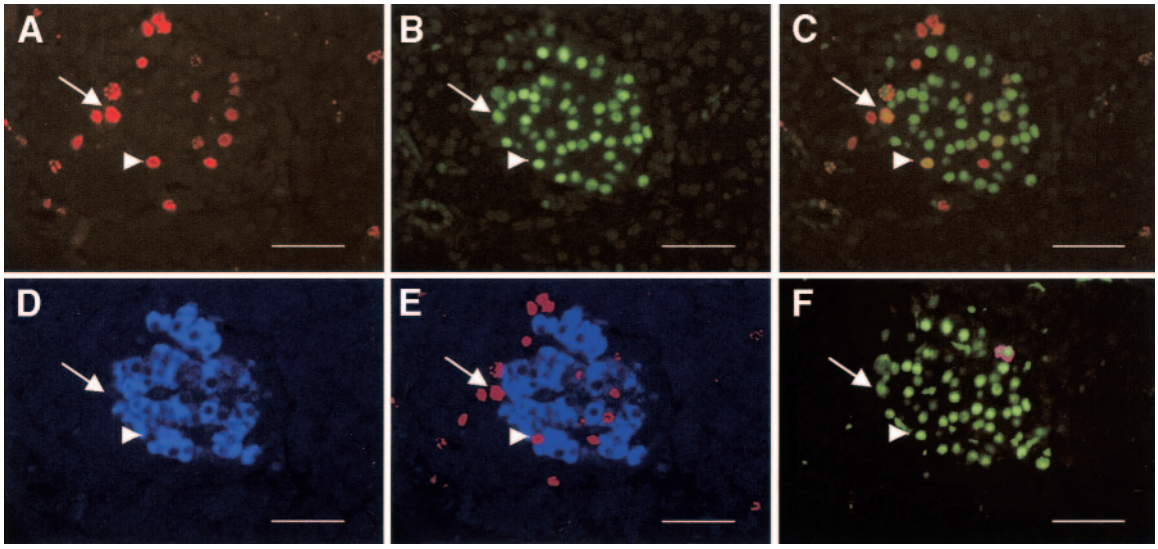

FIG. 2. Phenotypic characterization of BrdU-retaining cells. Neonatal rats were injected with BrdU between days 3 and 5 and killed 4 weeks later. Immunostaining was performed to detect BrdU-retaining cells (*A*) and Pdx1-expressing cells (*B*). *C*: Fusion of *A* and *B*. *D*: Detection of insulin-expressing cells. *E*: Fusion of *A* and *D*. *F*: Detection of somatostatin-expressing cells (pink) and Pdx1 (green). Arrow: a BrdU-positive cell positive for Pdx1 and negative for insulin and somatostatin. Arrowhead: BrdU-positive cells positive for both Pdx1 and insulin. Scale bar: 50 μm.

whether pancreatic cells that retain BrdU can differentiate into β-cells, we used an in vitro system of cultured epithelium from rat embryonic pancreata. This in vitro system mimics endocrine cell development: endocrine cells differentiate from E13.5 pancreatic epithelial cells and associate to form islets of Langerhans containing mature β-cells surrounded by glucagon-expressing cells (18,23,24). First, to determine whether this in vitro system mimics in vivo BrdU dilution and retention, we labeled embryonic pancreatic cells by injecting BrdU into pregnant rats between E11.5 and E13.5. On E13.5, the pancreata were dissected and depleted of their surrounding mesenchyme. At the end of the pulse period, the vast majority of the cells stained positive for BrdU (Fig. 4*A* and *C*). We then cultured the epithelia for 7 days and found that few cells were still BrdU-positive at that point (Fig. 4*B* and *D*). Most of the acinar cells were BrdU negative (Fig. 5*A*–*C*). On the other hand, β-cells positive for BrdU were observed (Fig. 5*D*–*G*). Taken together, the data indicate that this in vitro model of pancreas development mimics in vivo BrdU

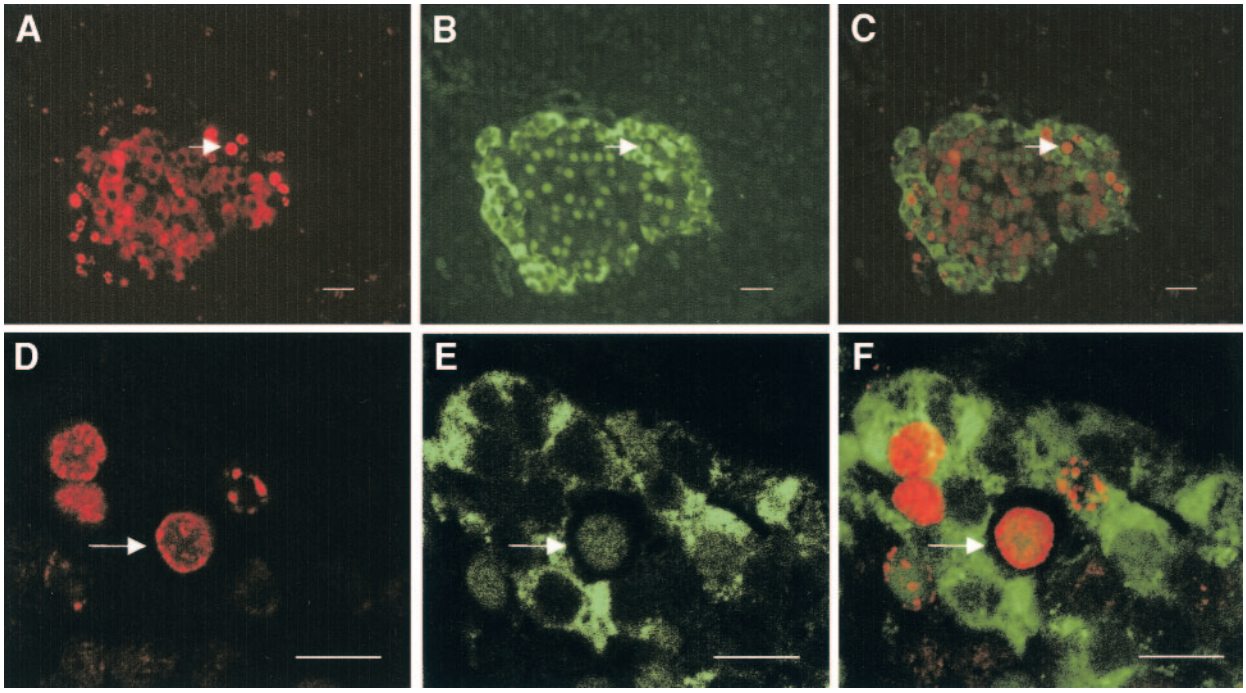

FIG. 3. Analysis of BrdU-retaining cells by confocal microscopy. Neonatal rats were injected with BrdU between days 3 and 5 and killed 4 weeks later. Pancreatic sections were labeled with a cocktail of anti-BrdU and anti-insulin antibodies raised in mice and revealed in red (*A*). The same section was labeled with a cocktail of anti-Pdx1, -glucagon, -somatostatin, and –pancreatic polypeptide antibodies raised in rabbits and revealed in green (*B*). *C*: Fusion of *A* and *B*. Arrow: a BrdU-positive cell positive for Pdx1 and negative for endocrine markers. *D*–*F*: Confocal analysis of the section shown in *A*–*C*. *D*: BrdU-retaining cell negative for insulin. *E*: The same cell positive for Pdx1 but negative for glucagon, somatostatin, and pancreatic polypeptide. *F*: Fusion of *D* and *E*. Scale bar: 10 μm.

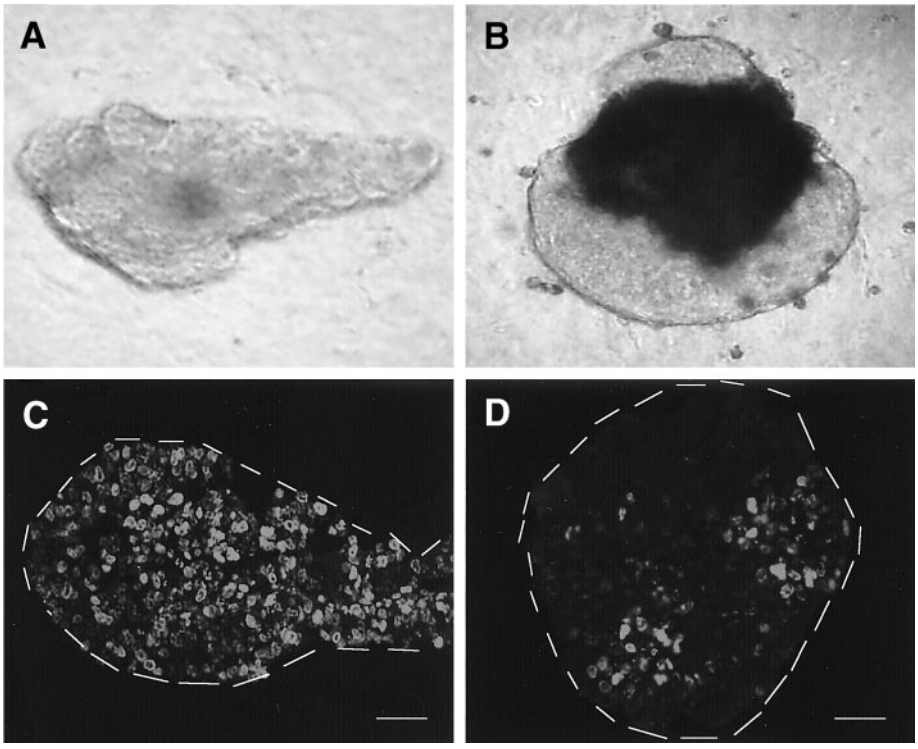

FIG. 4. BrdU labeling and in vitro dilution. Pregnant rats were injected with BrdU between E11.5 and E13.5. The embryonic pancreatic epithelia were dissected on E13.5 and grown in culture for 7 days. *A*: Dissected epithelium on E13.5. *B*: Dissected epithelium after 7 days of culture. *C*: BrdU labeling on E13.5. *D*: BrdU labeling after 7 days of culture. Scale bar: 50 μm.

dilution and retention. These results suggest that acinar cells differentiate and subsequently proliferate, thus losing their BrdU label. β-Cells differentiate from cells labeled by BrdU during the pulse period but do not subsequently proliferate. Results after short-term in vitro BrdU pulses further support such a hypothesis: when epithelia were cultured for 4 days and pulsed in vitro for 2 h with BrdU, acinar cells incorporated BrdU, whereas β-cells did not (Fig. 6).

Potential of BrdU-retaining cells to differentiate into β-cells. We used the above-described in vitro system to determine whether BrdU-retaining cells were able to differentiate into β-cells. Embryonic epithelia derived from BrdU-injected pregnant rats were cultured in the presence of FGF7. As previously shown under such conditions (19), epithelial cell proliferation occurred but endocrine cell differentiation was repressed. Few cells remained positive for BrdU (Fig. 7, compare *A* and *B*). Some of the BrdU-

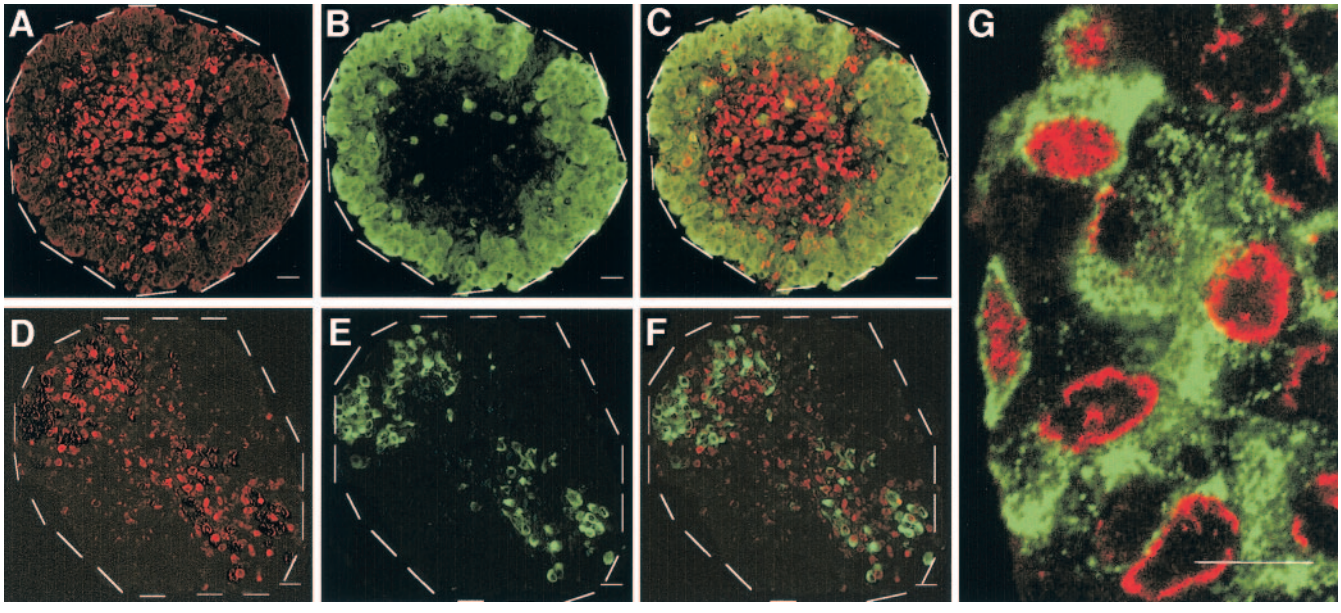

FIG. 5. Phenotypic characterization of BrdU-retaining cells after 7 days of culture. Pregnant rats were injected with BrdU between E11.5 and E13.5. The embryonic pancreatic epithelia were dissected on E13.5, grown in culture for 7 days, and sectioned. *A*: BrdU staining. *B*: Carboxypeptidase-A staining. *C*: Fusion of *A* and *B*, indicating that the vast majority of carboxypeptidase-A–positive cells had diluted BrdU. *D*: BrdU staining. *E*: Insulin staining. *F*: Fusion of *D* and *E*, showing insulin-expressing cells positive for BrdU. *G*: Confocal microscopy showing insulin-expressing cells positive for BrdU. Scale bar: 10 μm.

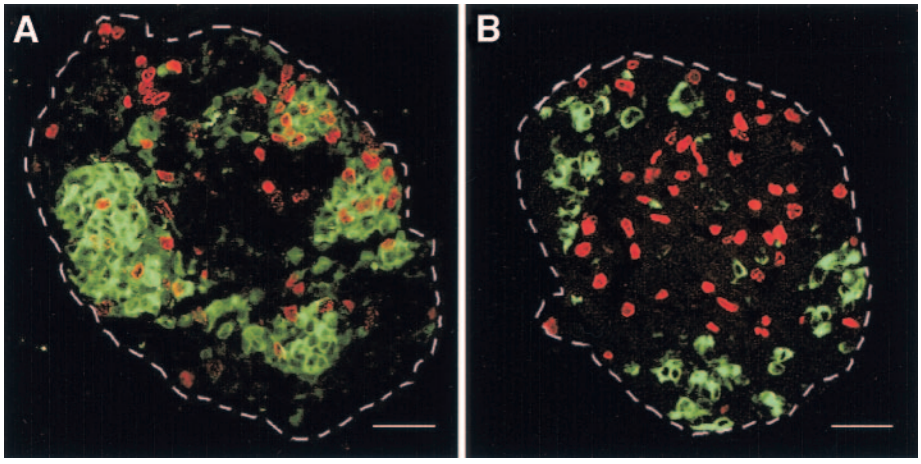

FIG. 6. In vitro cell proliferation after a short-term BrdU pulse. Embryonic pancreatic epithelia were dissected on E13.5 from pregnant rats that had not been injected with BrdU. They were grown for 4 days, pulsed for 2 h with BrdU, and sectioned. *A*: Staining for BrdU (red) and carboxypeptidase-A (green). *B*: Staining for BrdU (red) and insulin (green). Scale bar: 50 μm.

retaining cells stained positive for Pdx1 and negative for insulin, suggesting that they were β-cell progenitors (Fig. 7*C–F*). To test this hypothesis, we investigated the ability of these cells to differentiate into β-cells. Epithelial rudiments derived from BrdU-injected pregnant rats were cultured for 7 days with FGF7 and then for 3 or 7 days without FGF7. As previously shown (19), β-cell development occurred en masse upon removal of FGF7. Some of the insulin-positive cells that developed during the second week of culture stained positive for BrdU (Fig. 8), indicating that they differentiated from precursor cells labeled by BrdU during the first week.

DISCUSSION

In this study, we demonstrate that β-cells develop from undifferentiated BrdU-retaining cells. BrdU retention can therefore be used as a property of pancreatic progenitor cells.

A large body of data indicates that progenitor cells are present in the pancreas throughout the lifespan. Under specific conditions, for example when regeneration is needed, these progenitors differentiate into mature pancreatic cells (8–11,13,25,26). However, the phenotype and location of the progenitor cells have not been completely elucidated. The crucial role of Pdx1 during pancreatic

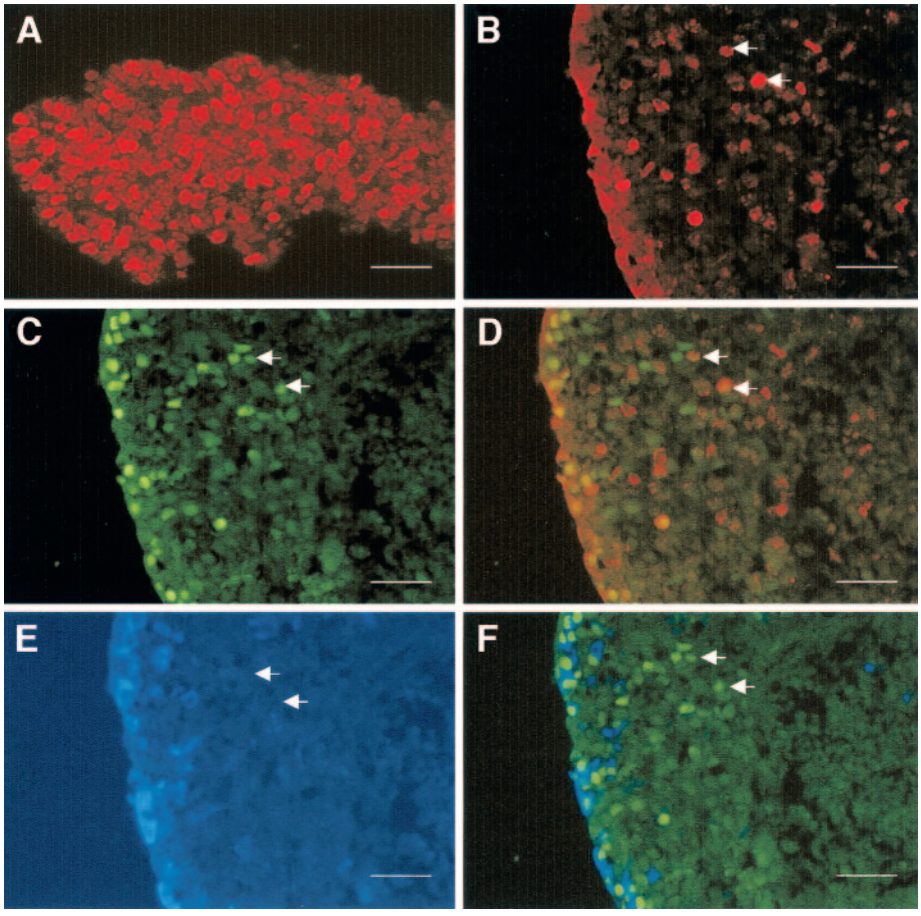

FIG. 7. BrdU dilution in vitro upon FGF7 treatment. Pregnant rats were injected with BrdU between E11.5 and E13.5. The embryonic pancreatic epithelia were dissected on E13.5, grown in culture for 7 days in the presence of FGF7, and sectioned. *A*: BrdU staining on E13.5 before culture. *B*: Staining for BrdU after a 7-day culture period in the presence of FGF7. *C*: Staining for Pdx1 after a 7-day culture period in the presence of FGF7. *D*: Fusion of *B* and *C*. *E*: Staining for insulin after a 7-day culture period in the presence of FGF7. *F*: Fusion of *D* and *E*. Arrows indicate cells positive for BrdU and Pdx1 and negative for insulin. Scale bar: 50 μm.

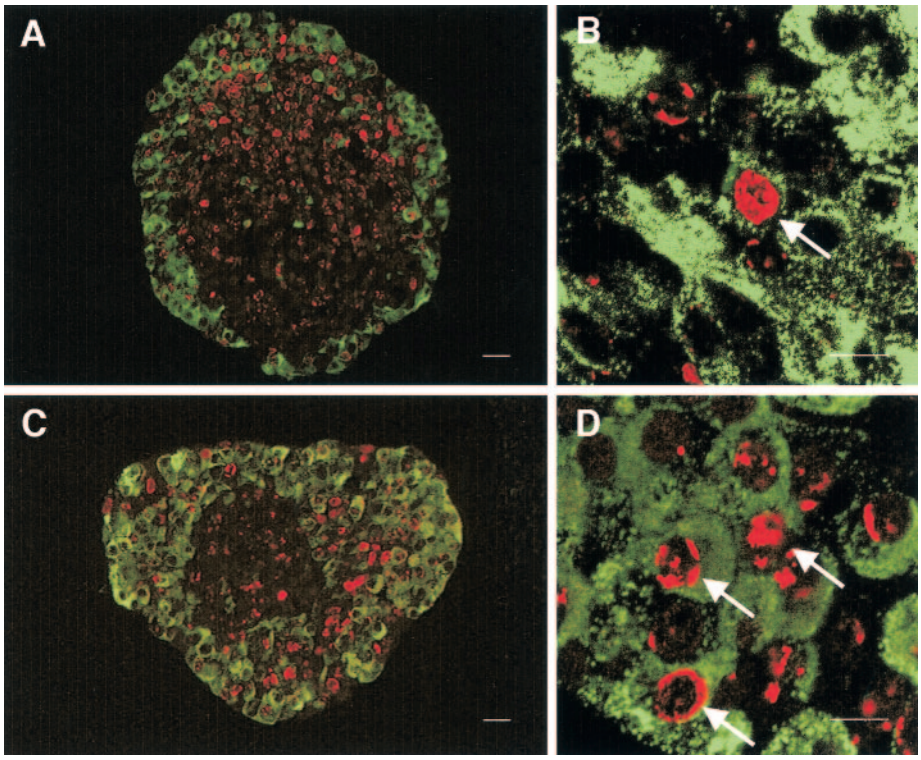

FIG. 8. β-cell differentiation and BrdU retention after FGF7 removal. Pregnant rats were injected with BrdU between E11.5 and E13.5. The embryonic pancreatic epithelia were dissected on E13.5, cultured for 7 days with FGF7, and then cultured without FGF7. They were sectioned and stained for BrdU (red) and insulin (green). *A* and *B*: The epithelia were sectioned 3 days after FGF7 removal. *C* and *D*: The epithelia were sectioned 7 days after FGF7 removal. *A* and *C*: Conventional microscopy. *B* and *D*: Confocal microscopy. Arrow: cells positive for both insulin and BrdU. Scale bar: 10 μm.

development indicates that this transcription factor is a marker for pancreatic progenitor cells. Pdx1 is expressed during embryonic life in the gut region that gives rise to the pancreas, and Pdx1-deficient mice do not develop a pancreas (14,15,17,27,28). However, additional markers and properties of pancreatic progenitor cells need to be defined (29). A well-accepted criterion for stem cells is slow cycling, which is a particularly important attribute because it minimizes errors in DNA replication (30). Slow cycling has been used as a criterion for identifying stem cells in the epidermis, central nervous system, and prostatic epithelium (5,6,31). In these tissues, when cell labeling by a DNA precursor such as [^{3}H]thymidine or BrdU was followed by a chase period, the label was diluted from proliferating cells but not from slow-cycling cells. These slow-cycling cells that remained positive for the label were found to be stem cells. In the present study, we investigated whether BrdU-retaining cells were present in the rat pancreas and whether they represented putative endocrine progenitor cells, as identified by Pdx1 expression. We detected BrdU-retaining cells in the pancreata of 1-month-old rats pulsed in the neonatal period with BrdU; these BrdU-positive cells were also present after 2 months (data not shown). Some BrdU-retaining cells expressed Pdx1 and stained negative for insulin, glucagon, somatostatin, and pancreatic polypeptide, suggesting that they were pancreatic progenitor cells.

In addition to Pdx1, other molecules may be markers for pancreatic progenitor cells, such as nestin (32), an intermediate filament that has been identified as a marker for neural stem cells (33). Nestin-positive cells have been detected in the pancreas (32,34), and β-cells have been shown to differentiate in vitro from nestin-expressing cells. However, a role for nestin as a marker for pancreatic stem cells has been challenged by two studies (35,36) in which nestin was only detected in mesodermal-derived cells in the pancreas during both the prenatal and postnatal periods. In our experiments, none of the BrdU-retaining cells expressed nestin (data not shown).

To demonstrate that BrdU- and Pdx-positive cells are endocrine cell progenitors, these cells must be shown to differentiate into endocrine cells. Several in vivo models can be used to investigate the neogenesis of pancreatic endocrine cells, including partial pancreatectomy, duct ligation, and β-cell destruction with streptozotocin (11,12, 26,37). Therefore, to determine whether β-cells develop from BrdU-retaining cells, we needed a neogenesis model in which all preexisting β-cells were destroyed before neogenesis occurred because we had no means of differentiating newly formed β-cells from preexisting β-cells positive for BrdU at the end of the pulse period (Fig. 1). In the partial pancreatectomy and duct ligation models, β-cells are not destroyed before regeneration occurs (26,37). Theoretically, the model of β-cell regeneration after streptozotocin treatment would have been appropriate for our study; however, it has not been fully validated, and in the vast majority of published experiments, diabetes induced by streptozotocin in adult rodents did not lead to any noticeable β-cell regeneration (38). Therefore, we elected to use an in vitro model to determine whether BrdU-retaining cells were able to differentiate into β-cells. In this in vitro model, E13.5 pancreatic epithelia containing progenitor cells are grown in culture. During the culture period, the cells differentiate into mature endocrine cells (18,23,24). Here, pregnant females were pulsed with BrdU between E11.5 and E13.5. Epithelia were dissected, and a 7-day chase period was performed in vitro in the presence of FGF7, a growth factor previously shown to strongly activate pancreatic epithelial cell proliferation (39). BrdU was chased from the vast majority of the cells. The few insulin-positive cells detected at the end of the chase period stained negative for BrdU (data not shown). A

few BrdU-positive, Pdx-positive, and insulin-negative cells were detected. They represented <1% of the cells. Upon FGF7 removal, these cells differentiated into β-cells (19), indicating that these BrdU-retaining cells were β-cell progenitors. Whether this is also the case for BrdU-retaining cells present in vivo in the adult pancreas will have to be explored in the future.

We found that the BrdU-positive, Pdx-positive, and endocrine-negative cells were located within or around the islets. Various locations have been suggested for endocrine progenitor cells. A large body of data suggests that in adults, endocrine progenitor cells are located within the pancreatic duct tree. Furthermore, during embryonic life, endocrine cells bud from duct-like structures (40). In various models of pancreatic regeneration, such as partial pancreatectomy, duct ligation, or γ-interferon overexpression, β-cells seem to bud from ductal structures (8,26,37). In these regeneration models, but also in the normal human adult pancreas, intermediate cells coexpressing endocrine and duct markers have been detected, further suggesting that β-cells may derive from duct cells (25,37,41). Finally, in vitro, β-cell generation from duct cell preparations has been obtained (9). However, recent studies suggest that pancreatic progenitors may also be present within the islets themselves. In earlier studies, after streptozotocin-mediated destruction of pancreatic β-cells, a population of insulin-containing cells reappeared within the islets (11,12). Furthermore, low levels of neurogenin 3, a transcription factor transiently expressed in endocrine progenitors, were detected in mature islets (13). In the present study, BrdU-positive, Pdx1-positive, and endocrine-negative cells were detected within and around the islets, supporting the hypothesis that some endocrine progenitors are located within the islets.

In conclusion, BrdU retention is a property of pancreatic progenitor cells located within the islets of Langerhans. This new criterion will now be used to further characterize pancreatic progenitor cells, following the pattern used for other cell types (42).

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