Colony-forming cells in the adult mouse pancreas are expandable in Matrigel and form endocrine/acinar colonies in laminin hydrogel

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The study of hematopoietic colony-forming units using semisolid culture media has greatly advanced the knowledge of hematopoiesis. Here we report that similar methods can be used to study pancreatic colony-forming units. We have developed two pancreatic colony assays that enable quantitative and functional analyses of progenitor-like cells isolated from dissociated adult (2–4 mo old) murine pancreas. We find that a methylcellulose-based semisolid medium containing Matrigel allows growth of duct-like “Ring/Dense” colonies from a rare (~1%) population of total pancreatic single cells. With the addition of roof plate-specific spodin 1, a wingless-int agonist, Ring/Dense colony-forming cells can be expanded more than 100,000-fold when serially dissociated and replated in the presence of Matrigel. When cells grown in Matrigel are then transferred to a Matrigel-free semisolid medium with a unique laminin-based hydrogel, some cells grow and differentiate into another type of colony, which we name “Endocrine/Acinar.” These Endocrine/Acinar colonies are comprised mostly of endocrine- and acinar-like cells, as ascertained by RNA expression analysis, immunohistochemistry, and electron microscopy. Most Endocrine/Acinar colonies contain beta-like cells that secrete insulin/C-peptide in response to D-glucose and theophylline. These results demonstrate robust self-renewal and differentiation of adult Ring/Dense colony-forming units in vitro and suggest an approach to producing beta-like cells for cell replacement of type 1 diabetes. The methods described, which include microfluidic expression analysis of single cells and colonies, should also advance study of pancreas development and pancreatic progenitor cells.

extracellular matrix proteins  |  Sry-related HMG box (Sox) 9  |  Prominin 1 (CD133)  |  Neurogenin 3  |  Dickkopf1 (Dkk1)

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dult pancreatic stem/progenitor cells are a potential unlimited source of insulin-secreting beta-like cells, but their existence is controversial. Candidate progenitor cells include adult duct (1–3), centroacinar (4), acinar (5), and insulin-low (6) cells. However, tissue culture strategies so far have been only marginally effective for expanding and differentiating putative progenitor cells into beta-like cells. In addition, the starting materials used for culture often contain a mixture of cells, complicating the interpretation of results (1). This lack of progress has been in part due to the absence of analytical tools that can effectively measure self-renewal and differentiation at the single-cell level. Delineation of lineage potential of a progenitor requires single-cell analysis. Otherwise, a population of cells under study may contain several monopotent progenitors that have various lineage potentials, and the cells will appear collectively as multipotent. In addition, if the cells of interest are scarce, the majority may mask the rare cell’s activities. One example of a population study is the in vivo genetic lineage tracing method using the crelization recombine (Cre) and locus of crossing over (Lox) system (7). In these studies, both positive (8, 9) and negative (10–15) results have been reported with respect to the question of whether exocrine pancreas in the adult gives rise to beta-like cells in injury models. These controversial data should be interpreted with care because of the possible failure of labeling rare cell populations (16). A different methodology capable of addressing this issue is needed. The single cell “pancreasphere” assay in liquid culture addresses lineage potential using limiting dilution (4, 6, 17). However, this method is labor-intensive, does not allow incorporation of the extracellular matrix (ECM) proteins important for cell function, and becomes highly cumbersome when attempting to manipulate individual colonies for replating to address self-renewal capacity.

To address these issues, we have investigated the use of the hematopoietic colony assay, which employs semisolid culture media. For this assay, single cells are mixed in viscous media containing methylcellulose, a biologically inert material purified from wood fibers. The methylcellulose medium restricts the movement of single cells, yet is soft enough to allow colony formation. A cell capable of forming a colony is termed a “colony-forming unit” (CFU). To test whether the adult pancreas contains CFUs, we replaced hematopoietic growth factors with factors that we thought would be helpful for growing pancreatic cells, including various growth factors (18–21) and ECM proteins, such as the commercially available Matrigel or an artificial ECM protein (22) containing an α1 laminin sequence (termed “laminin hydrogel”).

Pancreatic development is controlled by sequential activation of key transcription factors (23). Around embryonic day 8.5 (E8.5), commitment to the early pancreas is promoted by factors such as pancreatic and duodenal homeobox 1 (Pdx-1) (24) and NK6 homeobox 1 (NK6.1) (25). The early Pdx-1+ cells are tripotent for duct, acinar and endocrine lineages (26, 27), and their subsequent commitment to endocrine cells is dependent on the activation of neurogenin (Ngn) 3 (28–32), a helix-loop-helix transcription factor. The wingless-int (Wnt) proteins are a family of secreted, lipid-modified proteins critical for organ development (33) and for self-renewal and differentiation of several classes of adult stem cells (34). Wnt signaling exerts stage-specific effects during pancreatic development. Although Wnt signaling activation in early (E9) Pdx-1+ cells results in agenesis of the pancreas (35, 36), it enhances the growth and differentiation of more developed (around E11.5) Pdx-1+ progenitors (36). The roof plate-specific spondin 1 (Rspo1) has recently been identified as a Wnt signaling ligand (37). Rspo1 binds with high affinity to Wnt receptor low-density lipoprotein receptor-related protein


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(Lrp) 6 (38), which can be blocked by the Lrp6 inhibitor dickkopf1 (Dkk1) (37). Whether RSPO1 may affect activity of adult pancreatic progenitor cells has not been tested.

Here we report that in the adult mouse pancreas there are cells that form colonies in semisolid media. We have named these cells Pancreatic CFUs (PCFUs). These PCFUs make up only ~1% of the total cells of the adult pancreas, but can give rise to “Ring” or “Dense” ductal-like colonies as well as Endocrine/Acinar colonies in which some cells express endocrine markers and others express acinar markers. Our results demonstrate extensive in vitro progenitor cell activities displayed by adult PCFUs and show that RSPO1 stimulates PCFUs to self-renew and differentiate in vitro.

Results

**“Ring” Colonies Can Form from Dissociated Adult Murine Pancreatic Cells.** Dissociated pancreatic cells from adult mice (2–4 mo old) were suspended in colony assay medium containing 5% (vol/vol) Matrigel (referred to as the “Matrigel colony assay”) (Fig. S1). Three weeks later, morphologically distinct pancreatic colonies were observed, which we have named “Ring” (Fig. L4). Ring colonies are hollow spheres or cysts when viewed under the stereomicroscope. To understand how individual colonies develop, the locations of young colonies on the plate were noted and followed over time. Starting at day 2 postplating, Ring colonies begin as a small cluster of cells that are highly light reflective when viewed under phase-contrast illumination. These young colonies are named “Small Bright” (Fig. L4). Colony formation is not strain-specific; cells from both CD1 outbred and C57BL/6 (B6) inbred mice form colonies. It will be shown in a later section that a single cell can form a colony. A PCFU that gives rise to a “Ring” colony is termed a PCFU-Ring.

**Enrichment for PCFUs-Ring.** PCFUs-Ring comprise ~1% of plated, dissociated adult pancreatic cells (1.18 ± 0.70%; range, 0.56–2.32%). Therefore, enrichment for these scarce cells is highly desirable. Because cystic cell clusters, similar to Ring colonies, were described in cultures initiated with enriched ducts (1, 39–41), we tested known ductal markers, CD133 (42–46) and Sry-related HMG box (Sox) 9 (15, 47, 48), to see if they could be used as markers for fluorescence-activated cell sorting. CD1 mice transgenic for a Sox9 promoter-driven EGFP reporter (49) were used for subsequent studies. CD133 Sox9/EGFP cells (R3 window in Fig. 1B), which accounted for 3.78 ± 1.54% (range, 2.00–6.10%) of total pancreatic cells (Fig. 1B and Fig. S2), had the highest frequency of PCFUs-Ring compared with the other subpopulations (Fig. 1C). CD133 Sox9/EGFP cells did not give rise to Ring colonies even when plated up to 2.5 × 10^4 cells/well. Microfluidic quantitative (q) RT-PCR analysis demonstrated that single micromanipulated CD133 Sox9/EGFP cells (n = 21) expressed ductal (cytokeratin [CK]19 and CK7) but not acinar (Elastase1) or endocrine (Insulin2) markers (Fig. S3), supporting the ductal identity of CD133 Sox9/EGFP cells. Microfluidic qRT-PCR is a relatively new technology that allows reaction volumes to be in the nanoliter range, thus enabling detection of gene expression from as little as a single cell (50).

Gene expression analysis by conventional qRT-PCR of sorted subpopulations from B6 pancreas confirmed that markers for acinar (Amylase 2A) and endocrine cells (Glucagon and Insulin

![Fig. 1. “Ring” colonies are formed in Matrigel-containing culture from CD133 Sox9/EGFP cells isolated from dissociated adult murine pancreata.](image-url)
genes) were enriched in the CD133− fraction, whereas CD133+ cells were enriched for ductal markers. Only CD133+ but not CD133− cells were able to give rise to Ring colonies (Fig. S4). Thus, we speculate that Ring colonies may not come from acinar differentiation, as suggested by a previous study (51).

Ring colony-forming frequency of dissociated pancreatic cells is the same with or without passing through a sorter, so heterogeneity in colony-forming ability is not due to the physical stress of sorting (Fig. S5). Ring colonies might have been derived from hematopoietic cells present in the pancreas at the time of procurement. However, we found no colony formation from femur-derived bone marrow cells even after plating up to 2.5 × 10^6 cells/well. This negative result was not due to compromised bone marrow cells, as control experiments showed that the marrow cells formed hematopoietic colonies in the presence of hematopoietic factors (Fig. S6).

**Single Cells Can Form Ring Colonies.** To test whether a single cell can form a colony, freshly sorted CD133+ Sox9/EGFP+ cells were individually handpicked and plated (1 cell/well, 96-well plate) to observe colony formation. We found that 23 out of 120 (~19%) wells developed Ring colonies. This result indicates that (i) a single CD133+ Sox9/EGFP+ cell is sufficient to form a colony and (ii) not all CD133+ Sox9/EGFP+ cells can form colonies. To test the lineage composition of single handpicked colonies, we examined the expression of a gene panel by microfluidic qRT-PCR analysis (Fig. 1D). We found that all Ring colonies expressed high levels of housekeeping and ductal genes (Fig. 1D, green box) and low levels of endocrine and acinar genes (Fig. 1D, red box). To confirm protein expression, 3-wk-old Ring colonies were handpicked, pooled, fixed, and analyzed by whole-mount or frozen-section immunofluorescent staining and confocal imaging. Consistent with the microfluidic qRT-PCR analysis, ductal protein marker expression was present in all colonies examined, and each colony was positive for ductal proteins, Sox9/EGFP, Mucin1, or Osteopontin1 (Spp1) (52) (Fig. 1E). Three-wk-old Ring colonies were further examined by transmission electron microscopy. Cells in a Ring colony had multilobed nuclei (Fig. S7A), and microvilli and tight junctions were located at the apical surfaces of the cells facing a lumen (Fig. S7B), indicating a ductal cell identity and proper polarity. Cell polarity is important for pancreas development (53). We conclude that most cells in Ring colonies are duct-like.

However, a few cells in many of the colonies express amylase or C-peptide (a surrogate marker for de novo synthesized insulin) (Fig. 1E). About 70% (19/28) of colonies derived from single cells expressed some endocrine and acinar lineage markers (Fig. 1D, red box). This is evidence that some PCFUs—Ring are tripotent.

**“Dense” Colonies Are Induced from PCFUs—Ring by the Wnt Agonist RSPO1 but Not the Wnt Antagonist Dkk1.** To determine if Wnt signaling affects colony formation, adult CD133+ Sox9/EGFP+ cells were plated in the Matrigel colony assay with exogenous RSPO1 or Dkk1. A different type of colony (Fig. 2A) was formed from 2,500 input cells, was not changed by RSPO1 or Dkk1. (Fig. S5). This colony was enriched for ductal markers. Only CD133+ Sox9/EGFP+ cells were able to give rise to this type of colony (Fig. S4). Thus, we speculate that Ring colonies may not come from acinar differentiation, as suggested by a previous study (51).

**Dense Colonies Have Enhanced Expression of Neurogenin 3 (an Endocrine Progenitor Marker) Compared with Ring Colonies.** Individual 3-wk-old Ring and Dense colonies both expressed the ductal markers Sox9 and Mucin1 at similar levels (Fig. 2 D, 1).

**“Endocrine/Acinar” Colonies Form from Dissociated Dense or Ring Colonies in a Laminin Hydrogel.** ECM proteins, especially laminin, are important for adult murine β-cell function (54). The inefficient endocrine commitment in the Matrigel colony assay (Figs. 1 and 2) prompted us to test whether other ECM proteins affected β-cell
survival or differentiation. An artificial ECM protein (22) containing an 18-amino-acid sequence from α1 laminin (designated laminin hydrogel) was produced and tested. The laminin hydrogel-containing colony assay will be referred to as the “laminin colony assay.” Importantly, when total colonies from 3-wk-old culture, grown from adult CD133<sup>+</sup> Sox9/EGFP<sup>+</sup> cells (n = 120 in 96-well plate) were implanted in Matrigel and RSPO1, and the resulting 3-wk-old Dense colonies (n = 4) were individually handpicked and dissociated into single-cell suspension. Half of the dissociated cells were analyzed for ductal gene expression, while the other half were replated into the laminin colony assay. The resulting 2-wk-old Endocrine/Acinar colonies were then analyzed for endocrine and acinar gene expression. We found that all of the single Dense colonies and their derivatives expressed trilineage markers, suggesting that all PCFUs–Dense are tripotent (Fig. S8). In contrast, half of the control single Ring colonies (6 and 8) did not have endocrine potential.

Wnt Signaling Promotes PCFUs–Ring/Dense Expansion. Adult CD133<sup>+</sup> Sox9/EGFP<sup>+</sup> cells were plated in the Matrigel and RSPO1 colony assay for 3 wk. The resulting colonies were dissociated and serially replated in the Matrigel colony assay over an additional four generations in the presence of RSPO1, vehicle, or Dkk1. Continuous exposure of PCFUs–Ring to exogenous RSPO1 induced exponential growth of PCFUs–Ring/Dense (Fig. 4) and led to a ~5 × 10<sup>3</sup>-fold net expansion over 11 wk. Thus, PCFUs–Ring/Dense are expandable in culture.

Fig. 3. “Endocrine/Acinar” colonies are formed from dissociated and replated Ring and Dense colonies in laminin hydrogel-containing culture. (A) Representative photomicrographs of 2-wk-old Endocrine/Acinar colonies. (B, Upper) Total 3-wk-old colonies grown in Matrigel-containing culture (stimulated without or with 750 nM RSPO1) were dissociated and replated into laminin hydrogel colony assay for 2 wk. Colony-forming efficiency was calculated as the number of Endocrine/Acinar colonies generated divided by total number of input cells. (Lower) A total of 20 3-wk-old Ring (grown in Matrigel) or Dense colonies (grown in Matrigel and RSPO1) were picked, pooled, and dissociated into single-cell suspension. Total cell number was determined and the cells subsequently were replated into laminin hydrogel colony assay in quadrupled wells for 2 wk. The conversion efficiency was calculated as percentage Endocrine/Acinar colony-forming efficiency times total cell number and then divided by 20. (C) Whole-mount immunostaining of Endocrine/Acinar colonies. (D) Transmission electronmicroscopy of Endocrine/Acinar colonies showing cells with insulin- (Upper) or acinar-like (Lower) granules. (E) Microfluidic qRT-PCR analysis of individually handpicked colonies. Each bar is from a single colony. (F) In vitro glucose change assay on pooled Endocrine/Acinar colonies. Concentrations of theophylline are 10 mM.
Discussion

We report here the use of single-cell methylcellulose colony assays to delineate the ex vivo differentiation and expansion properties of adult pancreatic cells. Structures similar to Ring colonies have been described previously using semipurified pancreatic ducts grown in culture containing high (>33% (vol/vol)) concentrations of Matrigel (1, 39–41, 55). However, in our culture system, low [5% (vol/vol)] concentrations of Matrigel, when mixed with methylcellulose, are sufficient to grow adult pancreatic cells. Advantages of semisolid, methylcellulose-based assays are the ability to vary all active components and easily quantify and manipulate the resulting colonies. Colonies are evenly distributed across the well and can be counted accurately, and a single colony can be easily handpicked for subsequent analysis. The culture system we describe is efficient and easy to maintain. Up to 2.5 × 10^6 cells can be plated in 500 μL of semisolid media in one well of a 24-well plate. No media change is required during the 3-wk culture period.

Using these semisolid media, 3D assays, we have found the following (Fig. 5): (i) In the presence of Matrigel and exogenous RSPO1, two types of colonies form, ~50% are “Dense” and the rest are “Ring” colonies. Matrigel is known to contain many growth factors. Whether other factors are required for RSPO1 to induce Dense colony formation remains to be determined. Because PCFUs–Dense constitute a subset of the total PCFUs–Ring (Fig. 2B and C) and there is no marker to distinguish the two populations, we refer to these progenitor-like cells collectively as PCFUs–Ring/Dense. (ii) PCFUs–Ring/Dense are rare (~1%) in the adult mouse pancreas but are enriched in the CD133^Sox9/EGFP^ cell fraction (Fig. 1C). It should be noted that although CD133^Sox9/EGFP^ cells are enriched for ductal cells as well as PCFUs–Ring/Dense, this does not prove that ductal cells are the originator of the Ring, Dense, or Endocrine/Acinar colonies. Further investigation is required. (iii) When colonies grown in Matrigel are dissociated and replated in the laminin/1% (wt/vol) methylcellulose (Sinetsu Chemical), 5% (vol/vol) Matrigel or 100 μg/mL laminin hydrogel (see sequence in Fig. S9), 50% (vol/vol) conditioned media from murine embryonic-stem-cell–derived pancreatic-like cells, 5% (vol/vol) FCS, 10 mmol/L nicotinamide (Sigma), 10 ng/mL human recombinant activin–β, 0.1 nmoL/L, exendin-4, and 1 ng/mL vascular endothelial growth factor–A. When indicated, RSPO1 or Dkk1 (R&D Systems) was used at 750 ng/mL or 200 ng/mL, respectively. Cells were plated in 24-well ultralow protein-binding plates (Costar) and incubated in a humidified 5% (vol/vol) CO2 atmosphere. Primary colony numbers were scored after 3 wk in culture. For replating experiments, individual colonies were lifted from the methylcellulose medium by using a 10-μL Eppendorf pipette under direct microscopic visualization, collected in microcentrifuge tubes, and dissociated into single-cell suspension by using a 0.2% (wt/vol) trypsin-EDTA at 37 °C for 5 min. The single-cell suspension was then mixed in Matrigel or laminin hydrogel colony assay as described above.

In Vitro Colony Assays. Unless specified otherwise, cells were resuspended at a density of 2.5 × 10^5 cells/0.5 mL in methylcellulose-based colony culture medium as described previously (20, 21). In short, 1 mL culture mixture contained DMEM/F12 media, 1% (wt/vol) methylcellulose (Sinetsu Chemical), 5% (vol/vol) Matrigel or 100 μg/mL laminin hydrogel (see sequence in Fig. S5), 50% (vol/vol) conditioned media from murine embryonic-stem-cell–derived pancreatic-like cells, 5% (vol/vol) FCS, 10 mmol/L nicotinamide (Sigma), 10 ng/mL human recombinant activin-β, 0.1 nmoL/L, exendin-4, and 1 ng/mL vascular endothelial growth factor–A. When indicated, RSPO1 or Dkk1 (R&D Systems) was used at 750 ng/mL or 200 ng/mL, respectively. Cells were plated in 24-well ultralow protein-binding plates (Costar) and incubated in a humidified 5% (vol/vol) CO2 atmosphere. Primary colony numbers were scored after 3 wk in culture. For replating experiments, individual colonies were lifted from the methylcellulose medium by using a 10-μL Eppendorf pipette under direct microscopic visualization, collected in microcentrifuge tubes, and dissociated into single-cell suspension by incubation with 0.25% (wt/vol) trypsin-EDTA at 37 °C for 5 min. The single-cell suspension was then mixed in Matrigel or laminin hydrogel colony assay as described above.

Flow Cytometry and Cell Sorting. The cell suspension was first incubated with anti-mouse CD16/32 (10 μg/mL; BioLegend) for 5 min on ice to diminish nonspecific binding. Biotin-conjugated anti-mouse CD133 (clone 13A4; 5 μg/mL; eBioscience) or the control biotin-conjugated rat immunoglobulin (Ig)GT1 isotype (5 μg/mL; eBioscience) antibodies were added, and the cells incubated on ice for 20 min. After washing twice, cells were treated with streptavidin-labeled allophycocyanin (2 μg/mL; BioLegend) on ice for 10 min. Cells were washed twice and resuspended in PBS/BSA/DNase I containing DAPI (0.2 μg/mL). Cell sorting was performed on an Aria-special order research product (SORP) (Becton Dickinson). All analyses included an initial gating of forward and side scatters to exclude cell debris. Sorting further excluded doublets by gating on forward scatter width and side scatter width, and live cells were selected by DAPI negative staining (Fig. S2). The purity of the sorted population was routinely more than 95%.

In Vitro Colonies. Unless specified otherwise, cells were resuspended at a density of 2.5 × 10^5 cells/0.5 mL in methylcellulose-based colony culture medium as described previously (20, 21). In short, 1 mL culture mixture contained DMEM/F12 media, 1% (wt/vol) methylcellulose (Sinetsu Chemical), 5% (vol/vol) Matrigel or 100 μg/mL laminin hydrogel (see sequence in Fig. S9), 50% (vol/vol) conditioned media from murine embryonic-stem-cell–derived pancreatic-like cells, 5% (vol/vol) FCS, 10 mmol/L nicotinamide (Sigma), 10 ng/mL human recombinant activin–β, 0.1 nmoL/L, exendin-4, and 1 ng/mL vascular endothelial growth factor–A. When indicated, RSPO1 or Dkk1 (R&D Systems) was used at 750 ng/mL or 200 ng/mL, respectively. Cells were plated in 24-well ultralow protein-binding plates (Costar) and incubated in a humidified 5% (vol/vol) CO2 atmosphere. Primary colony numbers were scored after 3 wk in culture. For replating experiments, individual colonies were lifted from the methylcellulose medium by using a 10-μL Eppendorf pipette under direct microscopic visualization, collected in microcentrifuge tubes, and dissociated into single-cell suspension by incubation with 0.25% (wt/vol) trypsin-EDTA at 37 °C for 5 min. The single-cell suspension was then mixed in Matrigel or laminin hydrogel colony assay as described above.

Single-Cell Manipulation. Freshly sorted cells were mixed in 1% methylcellulose and 15% FCS at a density of 3,000 cells/mL and plated into a 35-mm Petri dish. Individual cells were visualized under a microscope and lifted one by one using a fine Pasteur pipet with a diameter of ~30 μm at the opening. The presence of a manipulated single cell is confirmed by visualization under a microscope.

qRT-PCR. Total RNA extraction and reverse transcription were as described (56). β-actin was used as an internal control for normalization. Duplicate samples were used in all analyses. Microfluidic qRT-PCR was performed using the BioMark 48.48 Dynamic Array system (Fluidigm). Single handpicked colonies or cells were collected in 10 μL reaction buffer, followed by preamplification (14 or 22 cycles for a colony or cell, respectively) according to the manufacturer’s instructions (Fluidigm). Amplified cDNA was loaded onto a 48.48 Dynamic Array using the NanoFlex integrated fluidic circuit (IFC) controller (Fluidigm). Threshold cycle (Ct), as a measurement of fluorescence intensity, was determined by the BioMark PCR analysis software (Fluidigm) for each sample as described (56).

**Fig. 5.** Model of PCFU–Ring/Dense expansion and differentiation.
and expressed as a heat map or relative expression (delta Ct) of the gene. All values are shown as mean ± SD. P values were calculated using student’s two-tailed t test; P < 0.05 was considered significant.

**Other Methods.** Please see SI Experimental Procedures. Antibodies used in immunostaining are listed in Table S2.

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