Int. J. Dev. Biol. 46: 201-207 (2002)

Original Article

Pancreatic lineage analysis using a retroviral vector in embryonic mice demonstrates a common progenitor for endocrine and exocrine cells

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ABSTRACT The origin of pancreatic endocrine cells is unknown. Some studies have suggested that there is a common pancreatic progenitor which gives rise to both endocrine and exocrine cells, while others have suggested separate endocrine and exocrine lineages. Previous conclusions have been based on indirect data, such as the co-expression of molecular markers. We directly assessed the relationship between endocrine and exocrine cells during development using a lineage tracer. A replication-incompetent retrovirus was used to introduce the reporter gene alkaline phosphatase into single cells in explants of mouse embryonic pancreas. After a week in culture, the subsequent fate of the infected cells could then be determined. The results show that a common pancreatic progenitor cell exists, which gives rise to both endocrine and exocrine cells.

KEY WORDS: *pancreas, lineage, islet, exocrine, retrovirus*

Introduction

The mammalian pancreas develops as a bud from the early embryonic gastrointestinal tract. A striking feature of the mature pancreas is that it is composed of two functionally separate tissues, endocrine and exocrine. The endocrine cells secrete hormones such as insulin and glucagon into the blood, and are organized into 'islet' tissue; the exocrine cells secrete digestive enzymes such as carboxypeptidase and amylase into the gut, and are organized into a branching epithelial tissue. These two tissues are physically separate in many lower animals. For example, insulin-secreting endocrine cells are located in the brain in insects. The endocrine and exocrine tissues exist as completely separate organs along the gastrointestinal tract in different species of fish (Falkmer, 1985). In higher vertebrates the endocrine and exocrine tissues are located together within the pancreas. The developmental relationship between these two tissues is debated. The question is whether endocrine and exocrine cells derive from a common pancreatic progenitor cell, or alternatively whether they derive from separate endocrine and exocrine progenitors (Andrew *et al.*, 1998; Gittes *et al.*, 1996; Pictet, 1972; Pictet *et al.*, 1976; Slack, 1995).

Numerous observations support the idea that endocrine and exocrine cells derive from separate precursor populations. Analysis of gene expression patterns in pancreatic endocrine and exocrine cells reveals the expression of distinct sets of genes from the time they first differentiate (Pictet, 1972; Rutter *et al.*, 1964; Slack, 1995). In fact, endocrine cells express many genes also expressed in neurons, a finding which led to the proposal that endocrine cells derive from neuroectoderm, rather than endoderm (Alpert *et al.*, 1988). The interpretation of such data is limited, however, since it is based on the questionable assumption that the lineage relationship between two cells can be deduced from their common expression of a molecular marker (Edlund, 1998; Guz *et al.*, 1995; Le Douarin, 1988; Teitelman and Lee, 1987; Teitelman *et al.*, 1987). Indeed, Herrera has shown the inherent danger in drawing conclusions about lineage from the expression of common markers. While several authors have proposed that embryonic cells expressing both insulin and glucagon are the progenitors of differentiated alpha (glucagon) and beta (insulin) cells, Herrera's genetic tests show this not to be the case (Herrera, 2000).

Genetic data consistent with separate endocrine and exocrine lineages includes the finding that targeted disruption of different pancreatic transcription factors affects predominantly one or the other lineages, rather than both (Edlund, 1998). For example, mice bearing null mutations of *pax4*, *pax6*, or *Neuro-D* have abnormal endocrine, but not exocrine development (Naya *et al.*, 1997; Sosa-Pineda *et al.*, 1997; St-Onge *et al.*, 1997). Conversely, targeted disruption of either the *p48* or Notch pathway genes prevents exocrine, but not endocrine development (Apelqvist *et al.*, 1999;

Abbreviations used in this paper: AP, alkaline phosphatase.

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0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

Krapp *et al.*, 1998). Several mutations in zebrafish perturb exocrine, but not endocrine development (Pack *et al.*, 1996).

Although it is remains unclear whether endocrine and exocrine cells derive from separate precursor cells within the endoderm, there is strong evidence against a neuroectodermal or mesodermal origin. Despite the earlier suggestions that endocrine cells derive from the neural crest (Alpert *et al.,* 1988), recombination experiments between chick and quail embryos showed that endocrine cells do not originate from the neural crest or from early neuroectoderm (Fontaine, 1980; Le Douarin, 1988; Pictet *et al.*, 1976). Mesenchyme is the other candidate source for endocrine cells. However, recombination experiments in E 11.5 pancreatic explants between mesenchyme and epithelium from wild type and ROSA 26 mice, respectively, did not demonstrate any migration of endocrine cells from the mesenchyme into the epithelium, at least after this relatively late stage of development (Percival and Slack, 1999).

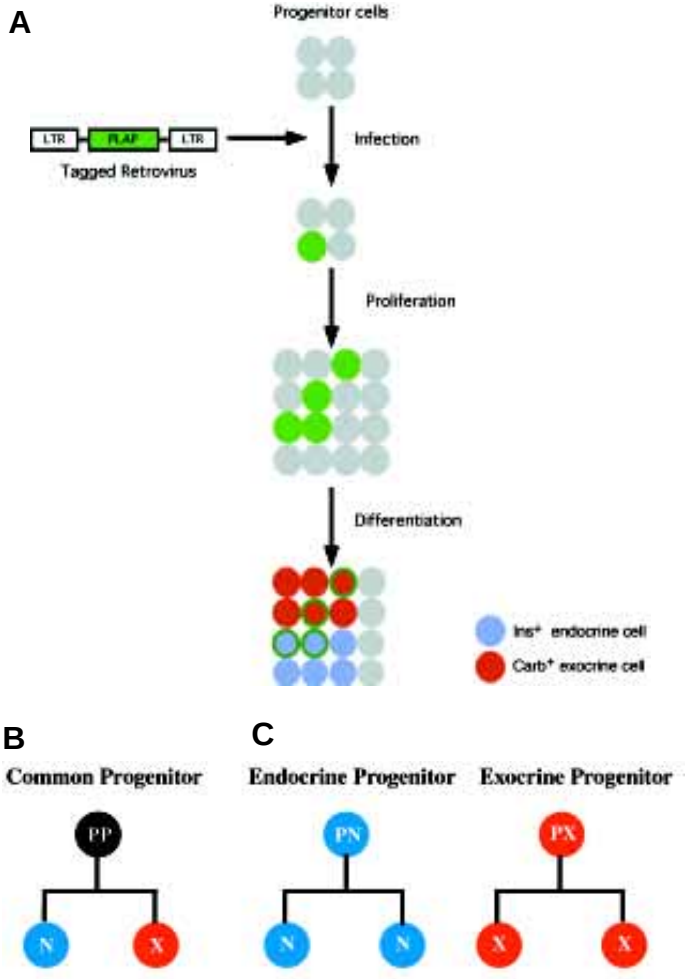

Fig. 1. Experiment to determine the lineage relationship between pancreatic endocrine and exocrine cells. (A) *The pancreatic bud from an embryonic day E 11.5 mouse is dissected into culture, then infected with a limiting dilution of replication-incompetent retrovirus encoding the* alkaline phosphatase *gene in order to label a single cell. The labeled cell within the explant divides and differentiates in culture for one week, at which time its progeny are analyzed by immunohistochemistry to determine endocrine or exocrine cell type. The particular example shown assumes a common endocrine-exocrine progenitor. (B,C) Possible outcomes of the lineage experiment.* **(B)** *If a common progenitor cell exists, then it can give rise to clones containing both endocrine and exocrine cells.* **(C)** *If separate endocrine and exocrine progenitors exist, then the resultant clones will be restricted to either endocrine or exocrine cell type. (PP, common pancreatic progenitor; PN, endocrine progenitor; PX, exocrine progenitor; N, endocrine; X, exocrine).*

Some evidence supporting a common progenitor derives from a transgenic mouse model of islet regeneration. In this model, the interferon gene is expressed under the control of the insulin promoter, resulting in massive islet destruction and regeneration. Although during normal development endocrine and exocrine genes are not co-expressed within the same cell, in this transgenic mouse model a few cells within the regenerating islets expressed both insulin and amylase (Gu *et al.*, 1994).

The best evidence for a common pancreatic precursor is based on the expression of *PDX-1*, an insulin transcription factor that marks a population of early pancreatic progenitor cells, which subsequently appear to form both endocrine and exocrine pancreas. However, detailed analysis of the expression pattern and role of *PDX-1* in the pancreas shows the situation is complex. Early in development *PDX-1* is expressed in endodermal cells in the region where the pancreas will form, but also in non-pancreatic regions of the duodenum and stomach. Within the early pancreatic bud *PDX-1* is expressed in most cells. As the pancreas undergoes further differentiation and branching, *PDX-1* expression becomes localized to clusters of cells in the pancreatic duct and surrounding epithelium. In the adult, *PDX-1* expression is limited to endocrine (mostly beta) cells (Ahlgren *et al.*, 1996; Guz *et al.*, 1995; Offield *et al.*, 1996). Mice with null mutations of *PDX-1* initially form pancreatic buds containing endocrine cells, but further morphological development does not occur. The glucagon and insulin cells subsequently disappear, and exocrine cells never form. At birth *PDX-1* deficient mice completely lack pancreatic tissue (Ahlgren *et al.*, 1996; Offield *et al.*, 1996; Jonsson *et al.*, 1994). Taken together, these data support a crucial role for *PDX-1* in pancreas development, but do not clearly define the fate of the early *PDX-1* expressing cells.

Since previous arguments about the lineage relationship between endocrine and exocrine cells are inconclusive, i.e. based on co-expression of molecular markers or on phenotypic analysis of mice with disrupted development, we decided to test directly for the presence of a common endocrine-exocrine progenitor cell by a lineage marking experiment.

Replication-incompetent retroviruses enable the delivery of a lineage label into single cells not easily accessible to intracellular injection. Because the marker gene encoded by the retrovirus integrates directly into the genome of the infected cell, it reliably labels all progeny without dilution through subsequent generations. Both beta –galactosidase and alkaline phosphatase have been introduced as markers into embryonic cells in numerous studies, and shown not to disrupt normal development (Cepko, 1998; Price, 1987; Sanes *et al.*, 1986; Turner and Cepko, 1987; Cepko, 1988). We used a retrovirus encoding alkaline phosphatase (Fields-Berry *et al.*, 1992) to label a precursor cell in the E 11.5 pancreatic bud, and then determine whether it gave rise to endocrine cells, exocrine cells, or both.

Results

A lineage tracing experiment was used to determine directly whether the early pancreatic rudiment contains distinct populations of progenitor cells for endocrine and exocrine lineages, or alterna-

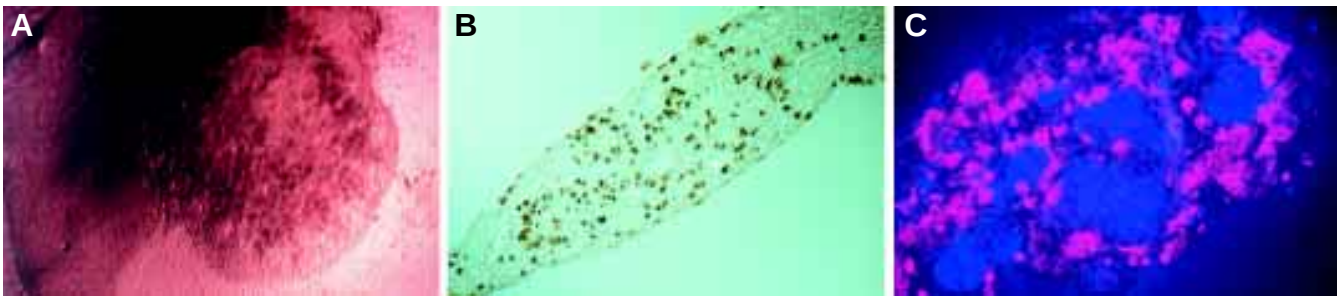

Fig. 2. Development of pancreas explants in culture. (A) *E 11.5 pancreas rudiment cultured on a filter for one week. The rudiment is approximately one millimeter in diameter.* **(B)** *BrdU incorporation (brown) after one day in culture, at the time of infection.* **(C)** *Immunofluorescent staining for insulin (blue) and carboxypeptidase (red/pink) after one week in culture. Unstained cells appear black.*

tively whether there is a common progenitor cell that gives rise to both lineages.

We used a replication-incompetent retroviral vector to deliver the marker gene alkaline phosphatase (AP) into a single precursor cell in a cultured E 11.5 pancreatic rudiment (see Fig. 1A). Since the retroviral AP integrates directly into the genome of the cell it infects, all subsequent progeny of that cell will express AP and thus can be identified. By using a dilution of retrovirus calculated to infect a single cell per explant and then allowing that cell to divide and differentiate in culture, we produced clones of AP labeled progeny. The fate of cells within each clone could then be identified by the expression of cell-type specific markers. There are two possible outcomes for such an experiment as shown in Fig. 1 B,C. If a common pancreatic progenitor cell (PP) exists in the E 11.5 rudiment, then when infected it will produce a clone of both endocrine (N) and exocrine (X) progeny. If there are different progenitors for endocrine and exocrine cells (PN and PX), then each when infected will produce a clone restricted either to pure endocrine or to pure exocrine progeny.

Embryonic Pancreas Explants In Vitro *can be Successfully Infected with a Retroviral Vector to Produce Labeled Clones of Progeny*

A whole organ explant culture system provides easy access for retroviral infection while maintaining the cells in their normal microenvironment. The embryonic pancreas rudiment was dissected from the duodenum and placed on a tissue culture filter, as shown in Fig. 2A. Since the retrovirus infects only dividing cells (Cepko, 1998), we assessed BrdU incorporation to determine the optimal time and conditions for retroviral infection. Cell division rates were much higher in cultures grown at the air-media interface on a filter than in cultures grown submerged in media embedded in collagen (data not shown). Because we found cell division to be extremely low in the period immediately following dissection, we allowed the explants to recover in culture overnight prior to infection. On day one of culture, at the time of infection, cell division had risen to robust levels. Fig. 2B shows that in a two-hour pulse exposure, approximately ten percent of the cells in the cultured explant incorporate BrdU. This division rate is comparable to that seen in embryonic pancreas *in vivo* at the same stage. The rate of cell division was maintained at the same level throughout the seven-day culture period (data not shown).

We found the optimal embryonic stage for retroviral labeling to be E 11.5, and the optimal culture period following infection to be one week. At this stage most cells at the time of infection had not yet differentiated to express either insulin or carboxypeptidase. During the ensuing week in culture, many cells fully differentiated and expressed either insulin or carboxypeptidase (Fig. 2C). Rudiments taken from earlier stages (e.g. E10.5) did not differentiate as extensively in culture.

We infected each of several hundred E11.5 explants with a limiting dilution of a replication-incompetent retrovirus encoding the placental alkaline phosphatase gene. After a week in culture the infected cell produces a clone of progeny expressing the retroviral alkaline phosphatase gene, which can be detected histochemically by reaction with BCIP/NBT to produce a purple product. Serial sections through the explant reveal a spatially localized clone of cells marked by retroviral AP expression, surrounded on either side by unstained cells (Fig. 3).

Retrovirally-Tagged Progenitor Cells Produce Both Endocrine and Exocrine Progeny

To determine the differentiated cell types within each clone, we used fluorescence immunohistochemistry to detect co-expression of retroviral AP and a cell-type specific marker. We found that many AP-labeled clones contained both endocrine (insulin-expressing) and exocrine (carboxypeptidase- expressing) cells. An example of one such clone is shown in Fig. 4. The top three panels show adjacent serial sections through a portion of a pancreatic explant in which cells expressing retroviral AP are identified by FITC-

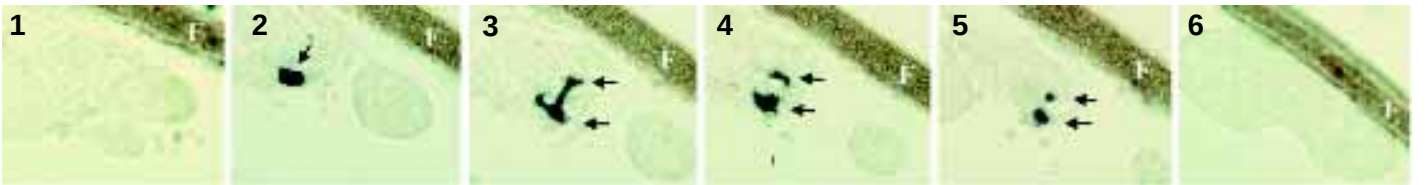

Fig. 3. Serial sections through an infected embryonic pancreas after a week in culture show the cells comprising a clone of retrovirally labeled cells. *Sequential (6 micron) sections are numbered one through six. Cells expressing the retroviral gene are stained for alkaline phosphatase (dark purple; arrows). The gold line (F) is the tissue culture filter.*

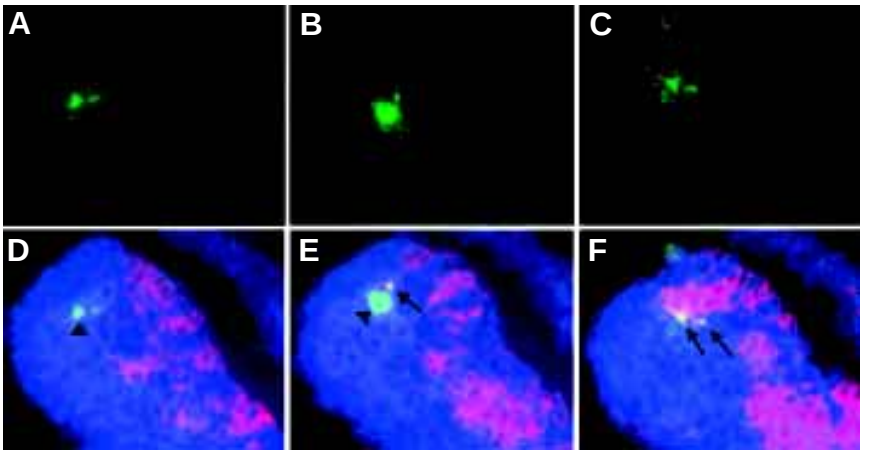

Fig. 4. Immunofluorescent analysis of a retrovirally labeled clone to determine differentiated cell types. *The top panels* **(A,B,C)** *are sequential (6 micron) sections through an infected explant in which each cell expressing retroviral AP is FITC –labeled (green). The bottom panels* **(D,E,F)** *are the same three sections photographed to show the expression of insulin (AMCA-labeled; blue) and carboxypeptidase (CY-3 labeled, red/pink) in addition to retroviral AP. In the explant shown, the majority of the cells stain blue for insulin, as is evident on the left hand side of the sections. Fewer cells, scattered on the right side, stain red/pink for carboxypeptidase. Cells co-expressing retroviral AP (green) and insulin (blue) appear teal (arrowheads); cells co-expressing retroviral AP (green) and carboxypeptidase (red/pink) appear yellow/white (arrows). This is a typical example of a clone that contains both endocrine and exocrine cells.*

staining (green). The bottom three panels show the same sections additionally stained for insulin (AMCA; blue) and carboxypeptidase (CY-3; red) to identify the cell type of each AP-labeled cell. Two of the cells within the clone are endocrine (co-staining for insulin and retroviral AP produces teal color; arrowheads) and three of the cells are exocrine (co-staining for carboxypeptidase and retroviral AP produces yellow color; arrows).

Confocal microscopy was used to confirm that the co-expression of markers occurred within a single cell rather than in overlapping cells. This more detailed analysis seems important especially given the discordant results about co-expression of markers in developing pancreas (Ahlgren *et al.*, 1996; Guz *et al.*, 1995). Figure 5 shows a typical optical section (one micron). The co-expression of retroviral AP (FITC-labeled, green) and carboxypeptidase (CY-3 labeled, red) produces a yellow fluorescence that can be seen within the cytoplasm of three adjacent cells of one clone. The fluorochrome used for insulin staining (AMCA) is out of the detectable wavelength range for confocal microscopy, and therefore is not shown.

We quantitatively analyzed the composition of clones arising from labeled E11.5 pancreatic cells in a series of fifty explants (Table 1). All cells within each clone by definition expressed the retroviral AP gene. Forty eight percent of the clones contained both insulin-expressing and carboxypeptidase-expressing cells ('both endocrine and exocrine'). Thirty two percent of the clones contained insulin-expressing cells, but not carboxypeptidase-expressing cells ('endocrine only'). Eight percent of the clones contained carboxypeptidase-expressing cells, but not insulin expressing cells ('exocrine only'). Twelve percent of the clones contained no insulin or carboxypeptidase expressing cells (termed'undifferentiated' although some may express other markers, such as glucagon, that were not assayed). These results show that a majority of the labeled precursor cells produced both endocrine and exocrine progeny. A minority of the labeled precursor cells produced progeny of a single (endocrine or exocrine) cell type. The clones consisting of a single cell type presumably arose from a population of cells infected when already committed to either an endocrine or exocrine fate. The relative paucity of exocrine compared to endocrine clones may reflect the fact that exocrine differentiation occurs later than endocrine differentiation, such that at E 11.5 more differentiated endocrine than exocrine cells were infected.

A more detailed statistical analysis of these data is presented as an appendix. That mathematical analysis demonstrates that the large fraction of cultures expressing both endocrine and exocrine cells could not have arisen from a simultaneous infection of separate progenitor lines.

Discussion

Retroviruses have provided a powerful technique for lineage analysis, since they enable delivery of a stably inherited marker gene into embryonic cells (Cepko, 1998). This technique is particularly useful for organisms in which the small size of embryonic cells limits the practicality of direct intracellular injection of the lineage tracer. Retroviral lineage labeling has previously been carried out *in vivo* in embryonic organs accessible for percutaneous delivery of the vector, including the central nervous system, heart, skeletal muscle, and retina (Galileo *et al.*, 1990; Gourdie *et al.*, 1995; Hughes and Blau, 1990; Price, 1987; Walsh and Cepko, 1993). To obtain access to developing pancreas we found it more practical to use an *in vitro* organ culture system. Embryonic tissue explants

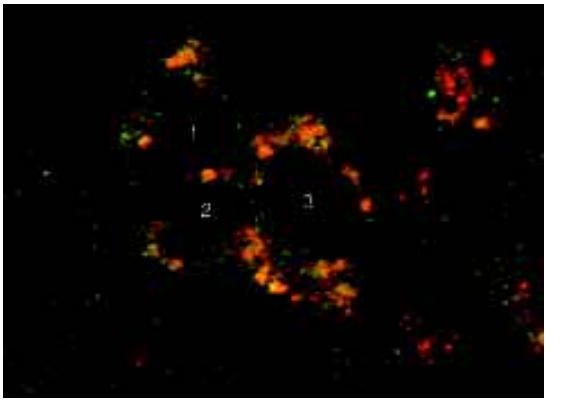

Fig. 5. Confocal image (1 micron section) of a clone containing both endocrine and exocrine cells. *Fluorescent immunohistochemistry shows that a single cell expresses the retroviral and cell-type specific markers. A cluster of three cells (marked 1, 2, and 3) expressing both membrane-associated retroviral AP (green) and carboxypeptidase (red) is shown; co-expression appears yellow.*

TABLE 1

COMPOSITION OF CLONES ACCORDING TO ENDOCRINE OR EXOCRINE CELL TYPE

CLONE COMPOSITION	#	%
both endocrine and exocrine (insulin and carboxypeptidase)	24	48
endocrine only (insulin)	16	32
exocrine only (carboxypeptidase)	4	8
undifferentiated (no insulin or carboxypeptidase)	6	12
total clones tabulated	50	100

A clone was defined as a spatially clustered group of cells within an explant marked by the retroviral gene. Fifty sequential clones were categorized according to whether they contained: (1) both insulin and carboxypeptidase expressing cells ('both endocrine and exocrine'); (2) insulin expressing cells, but no carboxypeptidase expressing cells ('endocrine only'); (3) carboxypeptidase expressing cells, but no insulin expressing cells ('exocrine only'); or (4) neither insulin nor carboxypeptidase expressing cells ('undifferentiated').

faithfully recapitulate *in vivo* organ development (Ahlgren *et al.*, 1996; Gittes, 1993; Golosow and Grobstein, 1962; Pictet, 1972), preserving normal tissue interactions, while allowing direct access to the developing tissue.

Retroviral lineage labeling *in vitro* has not been previously reported. We were able successfully to carry out retroviral lineage labeling in our *in vitro* system by using conditions that optimized cell division at the time of infection and maximized subsequent differentiation into mature endocrine and exocrine cells. Single cell infection was achieved by dilution of the virus. Although it is formally possible that some explants contained more than one infected progenitor cell, previous studies have confirmed that at limiting dilutions, infection with more than one retrovirus in the same region is an exceedingly rare event (Fields-Berry *et al.*, 1992; Turner and Cepko, 1987).

We found that cells labeled in the E 11.5 rudiment frequently produced both endocrine and exocrine progeny, demonstrating that both endocrine and exocrine cells arise from a common progenitor cell. Furthermore, the high percent of clones containing both cell types indicates that at E 11.5 many precursor cells are present which retain the potential to develop along both endocrine and exocrine lineages. This finding suggests a model for pancreas development in which gut endodermal cells are first signaled to form pancreas, and as a later step to choose between endocrine exocrine fates.

There are two limitations to the conclusions that can be drawn from our studies. First, we have concentrated our analysis on one stage of embryonic development. Thus, we cannot draw conclusions about the nature or lineage of adult pancreatic progenitors. If there is an adult pancreatic stem cell population, these studies do not show whether those cells are likely to form both endocrine and exocrine cells or instead are composed of two separate pools of progenitors. Secondly, the labeling in our experiments was performed *in vitro*. While the morphological and molecular differentiation observed *in vitro* mimics *in vivo* development, this aspect of the experimental design must be taken into account.

It will be of interest to determine the factors that signal the choice between an endocrine or exocrine pathway of development. Several factors have already been identified which influence the relative proportion of endocrine and exocrine cells, including follistatin and TGF-beta (Miralles *et al.*, 1998; Sanvito *et al.*, 1994). Different extracellular matrix environments can also influence pancreatic cell fate (Gittes *et al.*, 1996). In other organs, more specific instructive signals have been demonstrated. In the neural crest, for example, multipotent precursor cells can be induced to produce either neural or glial cells (Shah *et al.*, 1996).

The persistence of stem cells relatively late in development, which retain the potential to differentiate along strikingly different lineages, has been increasingly documented in other systems. There are, for example, epidermal, intestinal epithelial, hematopoeitic and mesenchymal stem cells with multilineage potential, some of which persist even into adulthood (Fuchs, 2000; Johansson *et al.*, 1999; Pittenger *et al.*, 1999; Prockop, 1997; Stemple and Anderson, 1992; Weissman, 2000). The present study demonstrates the existence of multipotent precursor cells in the pancreas during development. This finding supports efforts to identify pluripotent stem cells in the adult pancreas. Such cells could be used to replace the pancreatic cell types that are absent or damaged in human diseases, once the factors necessary to induce their differentiation along specific pathways are elucidated (Smith, 1998; Vogel, 1999).

Materials and Methods

Embryonic Pancreas Explant Cultures

Embryos were obtained from CD-1 mice (Charles River Laboratories) on embryonic day 11.5 (E 11.5), the morning of the vaginal plug being defined as E 0.5. The pancreatic rudiment was dissected and cultured at the air-media interface on tissue culture filter inserts (Gittes, 1993) over M-199 (Gibco-BRL) supplemented with 7% fetal bovine serum (Gibco-BRL) and antibiotic-antimytotic premix (Gibco-BRL). Cultures were incubated in humidified air with 5% CO_2 at 37°C for one week following retroviral infection (see below), with the media changed every other day.

BrdU Incorporation

Explants were incubated with BrdU (Amersham Cell Proliferation Kit) at a dilution of 1:1000 in pre-warmed media for two hours, then washed in PBS prior to paraformaldehyde fixation, paraffin embedding and sectioning. BrdU was detected by the using the manufacturer's directions, except that the nickel intensifier was omitted from the DAB solution.

Retroviral Infection

The replication-incompetent retroviral vector encoding placental alkaline phosphatase (DAP virus) was generously provided by Constance Cepko. After one day in culture, dissected pancreatic rudiments were incubated individually in 2 μl of diluted retrovirus in Terasaki plate wells (Nunc) for thirty minutes in humidified 5% CO_2 at 37°C. With the goal of infecting each rudiment with a single virus, we determined a limiting retrovirus dilution where more than half of the virus-exposed rudiments were uninfected. The virus stock (10^8 pfu/ml) was diluted 1:8 in tissue culture media with polybrene [10 μg/ml]. We made several partial thickness nicks in the rudiment in order to allow retrovirus to contact the epithelium directly (rather than only the surrounding mesenchyme). The rudiment was then placed back onto tissue culture filters over fresh, pre-warmed media and returned to the incubator for a week in culture.

Immunohistochemisty and Histochemistry

We found detection of the retroviral placental alkaline phosphatase activity of the virus to be more sensitive in whole mount than in paraffin-embedded sections. Rudiments were fixed in 4% paraformaldehyde for one hour and then washed twice in PBS for thirty minutes. The rudiments were then pre-blocked for 6 hours in 5% donkey serum, 1% BSA, 1%Tween, incubated with anti-PLAP (Elcatech) at a dilution of 1:4000 overnight at 4 degrees, washed twice for two hours in PBS with 0.05% Tween, incubated with biotinylated anti-mouse (Jackson ImmunoResearch) at 1:200 for four hours, washed twice for two hours, incubated with FITC-conjugated Streptavidin (Jackson ImmunoResearch) for four hours, and washed twice

for two hours. To prepare the tissue for sectioning, it was dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at 6 microns. Insulin and caboxypeptidase immunoreactivities were detected by standard methodologies. Specifically, insulin-immunoreactivity was detected on the sections using guinea pig anti-insulin antibody at 1:400 (Linco) followed by biotinylated anti-guinea pig antibody at 1:200 (Jackson ImmunoResearch) and AMCA-conjugated streptavidin at 1:500 (Jackson ImmunoResearch). Carboxypeptidase-immunoreactivity was detected using rabbit anti-carboxypeptidase A at 1:200 (Biogenesis) followed by Cy3-conjugated anti-rabbit antibody at 1:500 (Jackson ImmunoResearch).

In some experiments alkaline phosphatase activity was detected histochemically using a BCIP/NBT substrate (Vector Laboratories Alkaline Phosphatase Substrate Kit). In these experiments the tissue was heated to 65 degrees prior to the reaction to abolish endogenous intestinal alkaline phosphatase activity.

Acknowledgements

M.F. was supported by grants from the Juvenile Diabetes Foundation International, the National Institutes of Health (NRSA #T32HL07633), and a Janeway Child Health Center Award. D.M. is an investigator in the Howard Hughes Medical Institute. We thank Constance Cepko for providing the retrovirus and for generous scientific advice throughout the project; Jim Wells and Ming –Ko Chiang for many helpful discussions; Mark Fishman and John Potts for critical reading of the manuscript; and in particular Kimberly O'Donnell for scientific support and encouragement throughout this project.

Appendix

Statistical analysis by Henry A. Feldman. Our evidence for a common pancreatic progenitor cell (PP) is the large fraction of explant cultures that passed the retroviral vector from a single infected cell to both insulin-expressing (endocrine) and carboxypeptidase-expressing (exocrine) progeny. An alternative possibility is that the explants contained only endocrine progenitors (PN) and exocrine progenitors (PX) and that, despite our care to dilute the retrovirus, a substantial number of explants were infected twice or more, with the result that whenever both PN and PX cells received the vector, both insulin and carboxypeptidase were expressed in the culture.

To rule out this alternative, we constructed a mathematical model demonstrating that the hypothesis of dual progenitors and multiple infections is in conflict with our quantitative data. Suppose the explants consisted solely of PN, PX, and cells yielding undifferentiated progeny (U). Let the fractions of PN, PX, and U in the explant be ν, χ, and $1-\nu-\chi$ respectively. Let the dilution of retrovirus be such that the mean number of infected cells in an explant is λ. The number of infections in any given explant varies according to the Poisson law, with a fraction $e^{-\lambda}$ receiving no infection; $\lambda e^{-\lambda}$ receiving a single infection; and in general $\lambda^i e^{-\lambda}/i!$ receiving i infections.

Given those parameters, what would one expect for the data in Table 1? The fraction of explants receiving at least one infection, and thus showing the alkaline phosphatase label (AP), is $1-e^{-\lambda}$. Table 1 concerns 50 such cultures. In terms of ν, χ, and λ, we can predict the fraction of infected cultures that will express insulin only ($\Phi\nu$); the fraction that will express carboxypeptidase only ($\Phi\chi$,); and the fraction that will express both (Φ_2) or neither (Φ_0).

In order for a culture to express insulin only, the number of infections i in the explant must be at least 1; the number of infected PN cells n must be at least 1; and the remaining $i-n$ infections must occur in U cells. A set of i vectors can satisfy the above conditions in $i!/n!(i-n)!$ different ways, each combination occuring with probability $\nu^n(1-\nu-\chi)^{i-n}$. The probability of a culture expressing insulin only, given that it shows the AP label, is the sum of all such possibilities for $i \geq 1$ divided by the overall probability of at least one infection:

Similar arguments yield the fraction of cultures expressing carboxypeptidase only as; the fraction expressing both insulin and carboxypeptidase as; and the fraction expressing neither as.

For the hypothesis of dual progenitors and multiple infections to be viable, there must exist values of ν, χ, and λ that make $\Phi\nu$=32%, $\Phi\chi$=8%, Φ_2=48%, and Φ_0=12% as in Table 1. We used a numerical optimizing algorithm to seek such values and found the best fit at ν=9.04%; χ=4.61%; and λ=17.8 infections per explant. Even the best fit was not perfect; the estimated distribution of expression patterns was $\Phi\nu$=35.2%, $\Phi\chi$=11.2%, Φ_2=44.8%, and Φ_0=11.2%. The fitted value of λ was in flagrant conflict with our observation that only about 25% of explants showed the AP label, which would correspond to λ=0.29. When we fixed λ at the observed value of 0.29, the fitted value of Φ_2 fell to 5%, in contradiction of the observed 48% of cultures expressing both endocrine and exocrine function. Fixing λ at any value lower than 3.0 resulted in a poor match between the data reported in Table 1 and the fitted values of $\Phi\nu$, $\Phi\chi$, Φ_2, Φ_0, as indicated by a statistically significant lack of fit (chi-squared above 4.0, 1 df, $p<0.05$).

These results demonstrate quantitatively that the large fraction of cultures expressing both endocrine and exocrine function could not have resulted from simultaneous infection of separate progenitor lines, given the observed rate at which explants were infected, and that a common pancreatic progenitor cell must be responsible for our findings.

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Received: March 2001
Modified by Authors and Accepted for Publication: December 2001