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Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors

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SUMMARY

The location and lineage of cells that give rise to endocrine islets during embryogenesis has not been established nor has the origin or identity of adult islet stem cells. We have employed an inducible Cre-ER^{TM}-LoxP system to indelibly mark the progeny of cells expressing either *Ngn3* or *Pdx1* at different stages of development. The results provide direct evidence that NGN3+ cells are islet progenitors during embryogenesis and in adult mice. In addition, we find that cells expressing *Pdx1* give rise to all three types of pancreatic tissue: exocrine, endocrine and duct. Furthermore, exocrine and endocrine cells are derived from *Pdx1*-expressing progenitors throughout embryogenesis. By contrast, the pancreatic duct arises from PDX1+ progenitors that are set aside around embryonic day 10.5 (E9.5-E11.5). These findings suggest that lineages for exocrine, endocrine islet and duct progenitors are committed at mid-gestation.

Key words: Mouse, Pancreas, NGN3, PDX1

INTRODUCTION

There is considerable interest in the possibility of growing pancreatic islets for the treatment of diabetes (Bonner-Weir et al., 2000; Ramiya et al., 2000; Soria et al., 2000). To this end, it is important to understand the normal ontogeny of islets so that precursors or islet stem cells can be unequivocally identified and purified. Two general approaches have been used to address this issue. In the first instance, the common expression of molecular markers in two cells was taken as evidence that the cells have a common origin (Teitleman and Lee, 1987; Le Douarin, 1988; Teitelman et al., 1993). This led to the widely held belief that progenitors that transiently expressed two or more hormones gave rise to mature hormone-secreting cells (Alpert et al., 1988; Teitelman et al., 1993; Upchurch et al., 1994; Upchurch et al., 1996). For example, cells that express both glucagon and insulin were thought to give rise to two types of fully differentiated cells, the glucagon-secreting (α) and insulin-secreting (β) cells. However, by tagging cells that expressed specific hormones with the Cre-LoxP system, Herrera (Herrera, 2000) demonstrated that this is not the case; the double positive 'progenitors' do not give rise to differentiated islet cells (Herrera, 2000).

A second line of experiments used to draw conclusions about pancreatic and islet progenitors is the phenotype of knockout mice (Nielsen et al., 1999; Dohrmann et al., 2000; Edlund, 1999; Kim and Hebrok, 2001). These studies have focused attention on two transcription factors, *Pdx1* (Ohlsson et al., 1993; Guz et al., 1995) and *Ngn3* (also known as *Math4B* or *Atoh5*) (Sommer et al., 1996). The pancreas duodenal homeobox gene, *Pdx1*, is one of the earliest genes expressed in the developing pancreas. *Pdx1* expression is first detected in three endodermal domains on the dorsal and the two ventrolateral sides of the fore-midgut junction at E8.5 (Guz et al., 1995; Offield et al., 1996). As organogenesis continues, PDX1 is detected throughout the region that forms the pancreas and in part of the presumptive stomach and duodenum. After birth, *Pdx1* expression is restricted to β cells within the endocrine islets in the pancreas, as well as a few epithelial cells in the dorsal side of the stomach and all the mucosa cells in the duodenum (Offield et al., 1996). *Pdx1* mutants have no pancreas, promoting the idea that PDX1+ cells are general pancreatic progenitors (Jonsson et al., 1994; Offield et al., 1996). However, *Pdx1* mutants do generate some glucagon- and insulin-expressing cells in early embryogenesis and a pancreatic bud forms and later regresses. This raises the question of whether a *Pdx1*-independent pathway exists for pancreas generation. Herrera (Herrera, 2000) addressed some of these issues by tagging *Pdx1*-expressing cells with a Cre-LoxP system. His experiments convincingly demonstrate that PDX1+ progenitors give rise to mature α and β islet cells, though the origin of the exocrine and duct cells were not addressed.

The *Ngn3* knockout phenotype is particularly interesting in that exocrine tissue and pancreatic ducts are nearly normal, but differentiated endocrine cells (islets) are absent (Gradwohl et al., 2000). This phenotype, and the fact that *Ngn3* is co-expressed with *Pax6*, *Nkx6*.1 and *Neurod* (*Neurod1*) in some

cells (three genes that appear to be expressed in immature endocrine cells) has led to the conclusion that embryonic NGN3+ cells are endocrine progenitors (Jensen et al., 2000; Schwitzgebel et al., 2000). However, this conclusion is not entirely justified because *Ngn3* may function non-autonomously for islet development, e.g. NGN3+ cells may be required to induce adjacent (NGN3–) cells to form islet cells. Results from ectopic *Ngn3* expression demonstrated that *Ngn3* is sufficient to induce endocrine cell development, suggesting that *Ngn3* functions in a cell-autonomous fashion. However, the lack of insulin-expressing cells in these ectopic islets raises the question of whether these NGN3+ progenitors give rise to mature β cells (Apelqvist et al., 1999; Schwitzgebel et al., 2000; Graphin-Botton et al., 2001). The fact that PAX6, NKX6.1 and NEUROD are detected in cells other than those expressing NGN3+ (Jensen et al., 2000; Schwitzgebel et al., 2000) raises the possibility that other NGN3– cells also contribute to endocrine cell populations.

We have used a direct lineage tracer to determine whether the PDX1+ cells give rise to all pancreatic tissue and whether NGN3+ cells are indeed endocrine progenitors. The *Pdx1* or *Ngn3* promoters were used to drive the expression of the phage recombinase CRE or an inducible form of CRE, CRE-ER^{TM} (Metzger et al., 1995; Danielian et al., 1998; Sauer, 1998; Rossant and McMahon, 1999; Nagy and Mar, 2001). This procedure irreversibly tags the progeny of PDX1+ or NGN3+ cells in reporter mice that express the marker human placental alkaline phosphatase (HPAP) only after CRE-dependent excision of a transcription stop sequence.

The advantage of using the CRE-ER^{TM} version for lineage analysis is that this protein remains in the cytoplasm and is inactive until a ligand, tamoxifen (TM), is provided (Metzger et al., 1995; Danielian et al., 1998). Once TM is added, TM-bound CRE-ER^{TM} is transported to the nucleus and catalyzes recombination, thereby allowing the expression of the HPAP reporter gene (Lobe et al., 1999). Thus, cells can be marked at any time during development, not just at the time when *Pdx1* or *Ngn3* is first expressed. An added feature of the reporter line, the Z/AP mice (Lobe et al., 1999), is that cells that fail to undergo recombination maintain *lacZ* expression. Thus, cells that have never expressed *Pdx1* or *Ngn3* can be identified by β-galactosidase staining or antibodies against β-galactosidase.

By controlling the time at which tamoxifen is given, we are able to characterize the progeny of specific sets of PDX1+ or NGN3+ cells born at different stages of development. This improved lineage marking approach provides direct evidence that all three types of pancreatic tissues are derived from PDX1+ progenitors and NGN3+ cells are endocrine progenitors that gave rise to all four types of islet cells. We further demonstrate that the competence of a large majority of the early pancreatic progenitors is restricted to either duct or an endocrine/exocrine fate before embryonic day 12.5.

MATERIALS AND METHODS

Animal care and maintenance

For normal mating, outbred strain ICR (Taconic, Germantown, NY) or CD-1 (Charles River, Hartford, CN) mice were used. The hybrid B6CBAF1 strain was used to generate transgenic animals. Noon of the day when vaginal plugs first appear is regarded as 0.5 day post conception (dpc). Tamoxifen and BrdU administration were performed by intraperitoneal injections.

Transgene constructs and Cre recombination

To generate the *Pdx1-Cre* construct, a 5.5 kb *Sal*I-*Sma*I fragment (blunt-ended) containing the *Pdx1* promoter from pKSpdx-1*Sal*I (#571, a kind gift from C. Wright, Fig. 1) was ligated to the *Xba*I-digested (blunt-ended) pMC-Cre (a kind gift from A. McMahon). The insert was released by *Sal*I and *Not*I digestion. For *Pdx-1-Cre-ER^{TM}*, the coding region of the *Cre-ER^{TM}* cDNA was directly fused to the starting ATG of the PDX1 protein by PCR. Three primers (p5, 5′-ttgaaacaagtgcaggtgttcg-3′; p75, 5′-gttgcatcgaccggtaatgcaggcaaattttg-gtgtacggtcagtaaattggacatggtggcagccggcact-3′; and p71, 5′-gttgc-atcgaccggtaatgca-3′) were used. First, p5 and p75 were used to amplify a 400 base pair fragment from the *Pdx1* genomic DNA. Then this fragment was used together with p5 and p71 and the *Cre-ER^{TM}* plasmid (A. McMahon) to obtain a fragment that has the 5′ end coding region directly fused to the *Pdx1* promoter. This fragment was digested with *Age*I and ligated to the *Xho*I (blunt-ended)-*Age*I double-digested pBSCre-ER^{TM} that contains the full-length *Cre-ER^{TM}*-coding region (courtesy of A. McMahon) to give pGD19. Then a 2.2 kb insert was released from pGD19 by *Sma*I-*Spe*I digestion and ligated to *Sma*I-*Not*I digested pKSpdx-1*Sal*I, and a *Spe*I-*Not*I digested PCR fragment that contains the SV40 polyadenylation signal to give pGD35. The 8 kb insert was released by *Sal*I-*Not*I digestion and was used for pronucleus injection.

To generate the *Ngn3* constructs (Fig. 1), a 7.6 kb fragment containing *Ngn3* (pGD15, contains the 6.5 kb upstream region of *Ngn3*) was isolated using *Ngn3* cDNA to screen a mouse RPI-22 BAC library (Genetics Institute, Buffalo, NY). Three primers were used to fuse the Cre-ER^{TM}-coding region to the *Ngn3* ATG to generate a fragment that had part of the *Ngn3* promoter and the 5′ end of the recombinase. The primers used are p73 (5′-acacttgactccttgatcgctg-3′), p71 (5′-gttgcatcgaccggtaatgca-3′) and p74 (5′-ttttcccaaccgcaggatg-tccaatttactgaccgtacac-3′). This fragment was subsequently digested with *Apa*I and *Age*I and ligated to the *Kpn*I (blunt-ended)-*Apa*I (partial digestion)-digested pGD15, and a 2.4 kb *Age*I-*Sac*II (partial digestion, blunt-ended) fragment of pGD35. The insert from the final construct (pGD29) was released by *Not*I digestion.

The *Ngn3-Cre* construct was obtained by directly ligating a *Xba*I-*Bss*HII (partial digestion) blunt-ended fragment from pGD15 to the pMC-Cre plasmid to generate pGD 84. The insert was released by *Not*I-*Sal*I digestion and used for transgenic animal production. To eliminate the possible expression variance between transgenic lines, at least three independent lines were generated for each construct.

For lineage analysis, *Cre-ER^{TM}* or *Cre* transgenic animals were crossed with the reporter line, Z/AP (a kind gift from C. Lobe). Females received an intraperitoneal injection of 1 mg (or 0.7 mg for low dose) of TM at E8.5, E9.5, E10.5, E11.5, E12.5, E14.5 or E16.5 (Fig. 1). Double transgenic progeny were identified by PCR (primers used for the reporter line: p108, 5′-atcgctgatttgtgtagtcggt-3′; and p116, 5′-caacagttgcgcagcctgaatg-3′) (primers for the Cre-ER^{TM} line: p111, 5′-tgccacgaccaagtgacagc-3′; and p112, 5′-ccaggttacggatatagttcatg-3′) and the excision event by HPAP staining. To assay the excision event in postnatal animals, 3- to 8-week-old double transgenic animals received one or three 2 mg doses of TM on consecutive days and reporter gene expression was scored 3 or 7 days after the last injection. The AP activity was detected using the method of Lobe et al. (Lobe et al., 1999). After color development, the tissue was washed in PBS and counterstained with Hematoxylin.

Statistical analysis

Fifty random fields from stained sections were digitally photographed using an Optronics camera and printed at 125× magnification to count the number of HPAP+ or HPAP– islet cells and the area of HPAP-positive and -negative clones in exocrine and duct tissues. The frequency of all HPAP+ cells in exocrine tissue at various stages of

development is set at 1 (see Fig. 4). This eliminates differences in the efficiency of TM-induced recombination when comparing different stages of development. The frequency of HPAP+ islet cells is calculated as the percentage of HPAP+ islet cells/the percentage of HPAP+ exocrine tissue. If this number remains the same at different stages of development, it would indicate that PDX1+ cells from different stages of development contain a comparable ratio of endocrine and exocrine progenitors. The frequency of HPAP+ duct cells is calculated as the percentage of HPAP+ duct cells/ the percentage of HPAP+ exocrine tissue.

Islet isolation

Mouse islets were isolated by perfusing a pancreas with collagenase, shaking to disrupt cell contacts, and followed by centrifugation in Ficoll as described elsewhere (Warnock, 1990). Individual islets were handpicked under a dissection microscope and washed in PBS before RNA was extracted for analysis by RT-PCR.

Immunohistochemistry and in situ hybridization

The primary antibodies used were mouse anti-human placental alkaline phosphatase (Accurate Chemicals, Westbury, NY), guinea pig anti-human C-peptide, guinea pig anti-glucagon, rabbit anti-pancreatic polypeptide, rabbit anti-somatostatin (Jackson Immunoreserarch, West Grove, PA), mouse anti β galactosidase (Promega, Madison, WI), guinea pig anti-vimentin and rat anti PECAM (Research Diagnostics, Flanders, NJ). The secondary antibodies used were Cy3-conjugated donkey anti-guinea pig IgG, Cy3-conjugated donkey anti-rabbit IgG, Cy3 or FITC-conjugated sheep anti-mouse IgG, biotin-conjugated donkey anti-rat IgG, biotin-conjugated donkey anti-guinea pig IgG, streptavadin-HRP, streptavadin-FITC and streptoavadin-Cy3 (Jackson Immunoresearch, West Grove, PA). For the BrdU incorporation assay, a cell proliferation kit (Pharmacia, Piscataway, NJ) was used. Biotin labeled *Wisteria floribunda* agglutinin is from Sigma-Aldrich (St Louis, MO).

RESULTS

Pancreatic tissues are exclusively derived from PDX1+ progenitors

We generated transgenic mice using the *Pdx1* promoter (Wu et al., 1997) to drive expression of *Cre* recombinase (Fig. 1A). In situ hybridization on adjacent pancreas sections demonstrated that the *Cre* transgene expression pattern recapitulated the endogenous *Pdx1* pattern in all three independent transgenic lines (data not shown). One of the three lines was used for lineage analysis by crossing the animals with a reporter line, Z/AP (Lobe et al., 1999), that expresses the marker human placental alkaline phosphatase (HPAP) after *Cre* recombinase removes a transcription stop sequence.

The first control, shown in Fig. 2A, demonstrates that pancreatic cells in these transgenic mice do not express HPAP without the presence of CRE recombinase. A second control, Fig. 2B, shows that when *Cre* is expressed under the control of a general promoter, PGK (Lallemand et al., 1998), to induce recombination in all cells, every cell expresses HPAP. In the double transgenic *Pdx1-Cre;Z/AP* pancreas, acini, islets and ducts all express HPAP (Fig. 2C). In every section examined, the acini, islets and ducts were labeled by HPAP stain. Histological staining and the organization of the cells identified the various tissues. For example, ducts were identified by the alignment of cells and nuclei (see Fig. 2A,C) and by staining with *Wisteria fluoribunda* agglutinin (*WFA*, which stains ducts but not blood vessels) (Lammert et al., 2001). The only

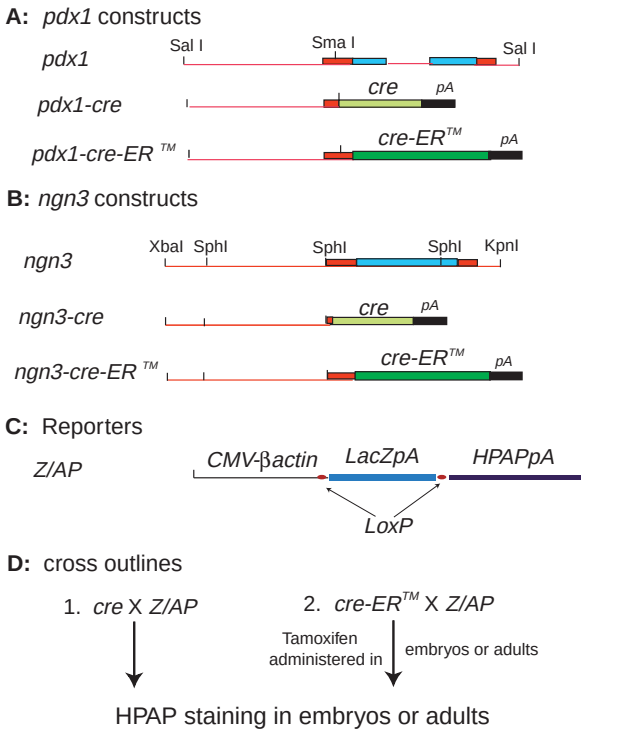

Fig. 1. The structure of transgenes (A-C) and the mating scheme (D). The *Pdx1Cre* transgene has the *Cre*-coding region directly fused to the *Sal*I-*Sma*I region of the 5′ promoter region of *Pdx1*. The *Pdx1-Cre-ER*^{TM} has the *Cre-ER*^{TM} ATG fused with the *Pdx1* ATG (A). The *Ngn3-Cre* construct directly fuses the *Cre*-coding region to the 6.5 kb upstream sequence of *Ngn3*. The *Ngn3-Cre-ER*^{TM} has the *Cre-ER*^{TM} ATG fused with the *Ngn3* ATG directly (B). Promoters and introns are represented by red lines, untranslated regions by red boxes and coding regions by blue boxes. The transgene polyadenylation signal, which is from the large T antigen polyA region, is noted by black boxes. The yellow and green boxes represent the coding regions of *Cre* or *Cre-ER*^{TM}. The reporter used is the *Z/AP* transgenic line (C) (Lobe et al., 1999). When *Pdx1-Cre* or *Ngn3-Cre* strain is crossed with reporters, no further manipulation is needed (D1). When *Pdx1-Cre-ER*^{TM} or *Ngn3-Cre-ER*^{TM} is used, tamoxifen (TM) is injected at different embryonic or postnatal days as indicated (D2). HPAP staining was examined 3-8 weeks after birth.

pancreatic cells not expressing HPAP were mesenchymal cells: endothelial cells (recognized by PECAM antibodies, Fig. 2C) and mesenchyme-derived smooth muscle cells. Further evidence for this point is shown in Fig. 2D where we took advantage of the fact that the reporter mouse (Z/AP) expresses β-galactosidase in all cells unless *Cre* recombinase is present (in which case the *lacZ* gene is removed; see Fig. 1C). We used PECAM and vimentin antibodies that label blood vessels and mesenchymal cells, respectively, to determine whether these are the only cells that maintain β-galactosidase expression. Indeed, as shown in Fig. 2D, PECAM or vimentin antibodies also label all cells that maintain *lacZ* expression. These data also show, as expected, that PDX1+ cells do not give rise to mesodermal tissues (blood vessels and mesenchyme).

Taken together, these data (Fig. 2A-D) show that all pancreatic cells (islets, exocrine and ductal cells) are derived from progenitors that express *Pdx1*. These data extend previous results and interpretations of PDX1 expression patterns and the

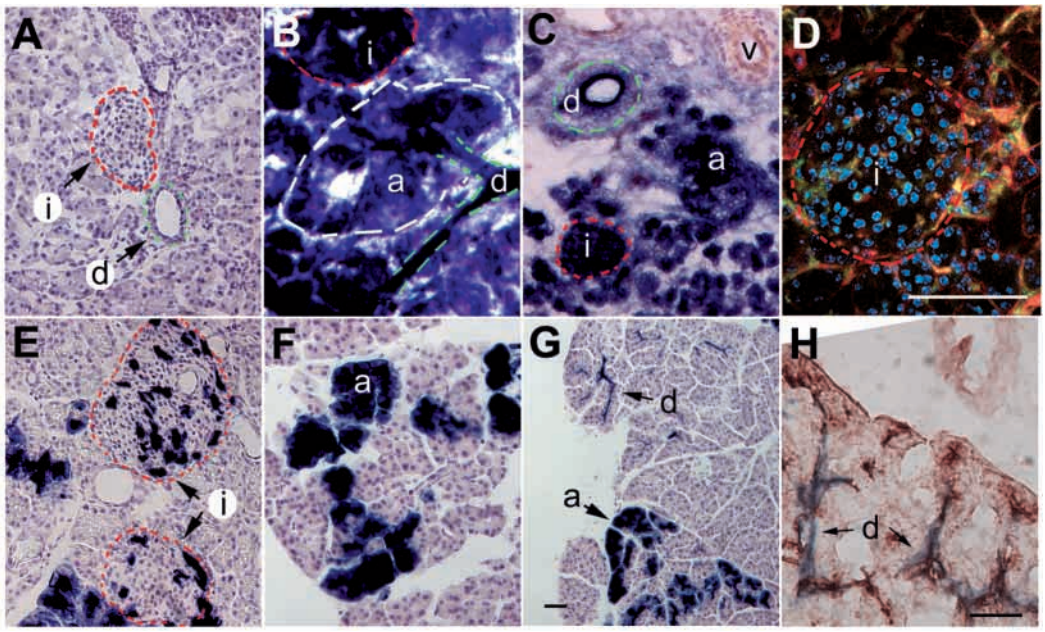

Fig. 2. PDX1+ progenitors give rise to three types of pancreatic tissues in adult mice. Reporter *Z/AP* mice were crossed with *pGK-Cre* (B), *Pdx1-Cre* (C,D) or *Pdx1-Cre-ER*^{TM} (E-H, tamoxifen was given at E9.5). The pancreata of double transgenic mice were stained for HPAP activity 4 or 8 weeks after birth. (A) HPAP staining is not observed in controls in the absence of recombination. (B) Positive control shows that all pancreatic cells stain for HPAP when a general deletor line, *pGK-Cre* was mated with the reporter line. (C,D) In pancreata of *Pdx1-Cre*;*Z/AP* mice (4 weeks old), all exocrine, endocrine, and duct cells express HPAP, whereas mesenchyme and blood vessels do not. (C) Blood vessels are stained light brown with an anti-mouse PECAM antibody. (D) A confocal image of a *Pdx1-Cre;Z/AP* pancreas section which is double labeled by β-galactosidase antibodies (stained red with Cy3) and antibodies against PECAM + vimentin (antigens present in endothelial or mesenchymal cells only, stained green with FITC). Yellow and orange stain (resulting from the combination of red and green) are observed, but not green or red only, demonstrating that only the blood vessel or mesenchymal cells maintain *lacZ* expression. Note that this section was not stained for HPAP, i.e. the dark areas are acini, islets (i) and ducts. (E-H) The HPAP staining pattern (8 weeks old) when *Pdx1-Cre-ER*^{TM} animals are crossed with Z/AP and TM is administered at E9.5. Islet (E), acini (F) and ducts (G-H) are labeled in a mosaic fashion. In H, the small ducts are stained with *Wisteria floribunda* agglutinin as brown and HPAP as blue. Note the blue staining in the small duct but not the adjacent acinar cells. (a, acini, broken white lines; d, duct, broken green lines; i, islets, broken red lines; v, blood vessel). Scale bars: in D, 20 μm; in G, 20 μm; in H, 20 μm for A, B, C, E, F and H.

genetic targeting of the PDX1 locus (Jonsson et al., 1994; Offield et al., 1996).

Exocrine/endocrine cells and duct cells arise from different lineages

Four transgenic mouse lines driving Cre-ER^{TM} expression under the control of the *Pdx1* promoter were also generated. In situ hybridization and immunohistochemical analyses demonstrated that three lines recapitulate endogenous *Pdx1* expression (the other line had a low copy number of the transgene and did not express the transgene; data not shown). Two lines (p35.6 and p35.10) were chosen to cross with the Z/AP reporter line (Fig. 1D) to determine whether cells that express *Pdx1* at different stages of development give rise to a different spectrum of mature pancreatic cells.

The CRE-ER^{TM} protein requires tamoxifen (TM) to catalyze LoxP site-mediated excision. We determined the time window during which TM is effective by injecting 1 mg of TM into E5.5, E6.5 and E7.5 plugged females and testing for HPAP labeling in *Pdx1-Cre-ER*^{TM}*;Z/AP* pancreata of newborn mice. We failed to find any HPAP+ cells when TM was injected at E5.5 (n=5). When TM was administered at E6.5 (n=4), less than 0.1% pancreatic cells were labeled in the double transgenic animals. We observed HPAP staining in more than 3% of pancreatic cells in all double transgenic animals (only non-duct tissue) when TM was injected at E7.5 (n=5). Because *Pdx1* expression is first detected at E8.5 (Guz et al., 1995; Offield et al., 1996), these results suggest that TM is active for less than 48 hours in injected mice. Therefore, this system allows one to mark the progeny of PDX1+ cells indelibly within a 48 hour window at any stage of development by activating CRE recombinase with tamoxifen injections.

TM was administered at E9.5 and the HPAP staining pattern was tested in pancreata of 8-week-old double transgenic animals. As shown in Fig. 2E-G, HPAP labeling is found in all three types of pancreatic tissue: endocrine (Fig. 2E), exocrine (Fig. 2F) and ducts (Fig. 2G,H). In all cases, only part of the pancreatic tissue is labeled, resulting in a mosaic HPAP staining pattern. We examined six 8-week-old adults and found that 3-15% of exocrine, endocrine and duct cells were labeled. Both transgenic lines, p35.6 and p35.10, give similar HPAP staining patterns.

One possible reason for incomplete labeling is that PDX1+ cells are born continuously from PDX1– cells during embryogenesis and the TM marks cells expressing PDX1 within a 48 hour period. Another possibility is that the TM-induced recombination is not 100% efficient. We could not apply TM at more than a 1 mg dose/animal without toxic side effects and this concentration might be insufficient to induce all the Cre-ER^{TM}-positive cells to undergo excision (Danielian et al., 1998). When a higher TM dose (1.5 mg/animal) is injected, we observed an increased portion (as high as 25-30%) of the pancreatic tissue being labeled, though the relative amount of each labeled tissue remains constant (data not shown). Thus, the efficiency of TM induced recombination does not effect the conclusion as to the eventual fate of the progeny of PDX1+ cells.

The mosaic pattern of the HPAP staining displays three

features. First, HPAP+ cells within islets are scattered randomly (Fig. 2E), consistent with a previous report concluding that islets are not clonal (Deltour et al., 1991). The second feature is that the HPAP+ cells within acini or ducts are most often found in contiguous groups as if they are derived from a single cell (Fig. 2F-H). This is consistent with the idea that tight junctions between developing exocrine and duct-like cells restrict their migration during development (Pictet et al., 1972). The third feature is that the HPAP+ acinar clones and ductal clones are not adjacent (Fig. 2G,H). This result indicates, but does not prove, that a single PDX1+ progenitor does not give rise to both mature acinar and ductal tissue.

Pancreatic duct progenitors express *Pdx1* between E10.5 and 12.5

We performed additional lineage analyses of PDX1+ cells by injecting TM at several other embryonic stages and postnatal mice, in each case scoring for HPAP+ cells 8 weeks after birth. If TM is administered at E9.5, E10.5 or E11.5, exocrine, endocrine and duct cells are labeled (Fig. 3). However, when TM is given at E8.5 or E12.5 and anytime thereafter, we only observed islet and acini cells being labeled (Fig. 3B-E). Stated otherwise, HPAP label is observed in duct cells only if TM is given between E9.5, E10.5 and 11.5, not before and not after. This result is not dependent on the time at which the labeled progeny are assayed. We have examined additional animals at 3, 6 or 12 weeks after birth and in every case the duct cells are labeled if and only if the TM was give between E9.5 and E11.5.

We quantitated the frequency with which PDX1+ cells adopt exocrine, endocrine or duct cell fates by injecting TM at several developmental stages. Fifty fields of view were randomly chosen from sections of adult (8-week-old) pancreata and the HPAP+ exocrine, endocrine and duct cells (see Materials and Methods) were counted. As shown in Fig. 4, PDX1+ cells at all stages of development give rise to islet and exocrine cells with similar ratios. When we normalize the variation of TM efficiency to the number of HPAP+ exocrine cells (the most abundant pancreatic tissue), HPAP+ islet cells show less than a twofold difference from stage to stage. In marked contrast, animals receiving TM at E10.5 have 35 times more HPAP+ duct cells than those receiving TM at E8.5. In other words, PDX1+ cells at E10.5 are 35 times more likely to adopt an adult duct fate than the PDX1+ cells from E8.5. Because TM remains active for 24-48 hours, we believe that the vast majority of the duct progenitors (>95%) express high level of *Pdx1* between embryonic days 9.5 and 12.5. At the same time, the results show that exocrine and endocrine precursors (also *Pdx1*-expressing cells) are found at all stages of embryonic development.

When postnatal mice receive TM, HPAP+ cells are found in exocrine and endocrine tissues. As we do not find any duct cells labeled when TM is injected into postnatal mice, we conclude that *Pdx1*-expressing duct progenitors are very rare, if not absent, in postnatal mice.

NGN3+ cells give rise to islets

We isolated a 6.7 kb *Ngn3* genomic fragment and generated four transgenic lines that express Cre-ER^{TM} under the control of the *Ngn3* promoter (Fig. 1B). In situ hybridization using *Ngn3* and *Cre-ER^{TM}* probes on adjacent pancreas sections showed that the transgene is correctly expressed, i.e. the transgene is expressed in cells that transcribe the endogenous NGN3 gene (Fig. 5A,B). Two of the four transgenic lines (p29.4 and p29.10) were crossed with the reporter line, Z/AP. Lineage analysis was performed by giving TM at several stages of development and assessing HPAP+ cells in the pancreas 3-8 weeks after birth.

All animals had HPAP+ cells within the islets, regardless of the stage at which TM was administered (Fig. 5C,D). In addition, we note that in every case only some of the cells within an islet were labeled. By randomly counting sections from about one-fifth of each pancreas, we found that 3-18% of the islet cells were labeled, with similar variation between littermates or between the p29.4 and p29.10 transgenic lines.

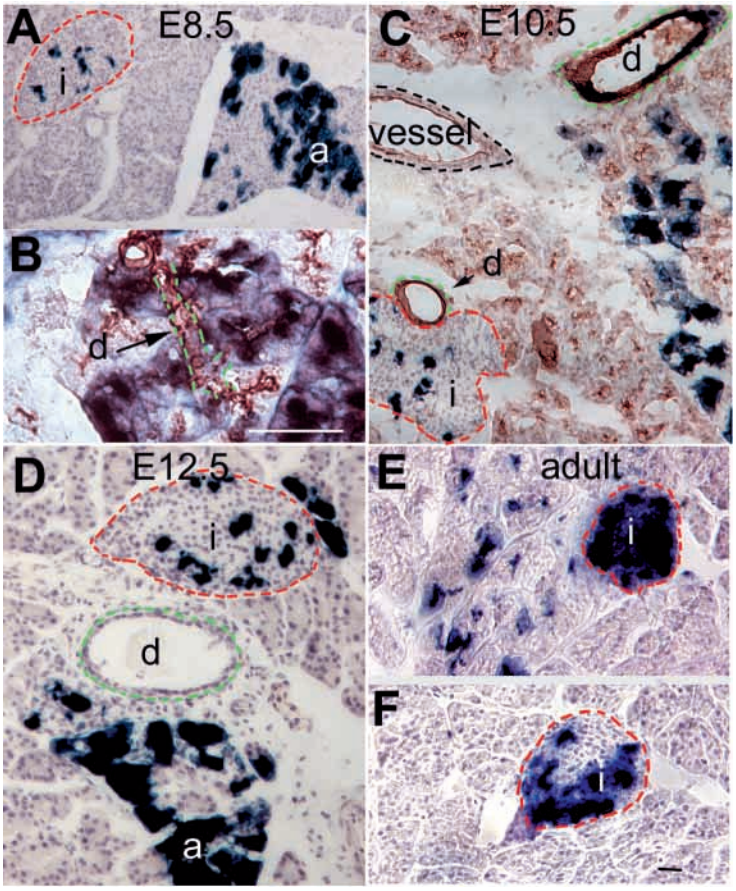

Fig. 3. Duct progenitors express *Pdx1* in the period between E9.5-E12.5. Tamoxifen is administered at different stages of development and the HPAP expression in 8-week-old double transgenic animals is determined (when TM is administered to 8-week-old animals, the HPAP staining is performed 3 days later). (A,B) Acini and islet staining when TM was injected at E8.5. In B, the duct is stained brown with HRP-conjugated *Wisteria floribunda* agglutinin. Note that the brown duct is not blue, whereas acinar cells surrounding the duct are HPAP+ and stain blue. (C) HPAP+ cells within ducts (d), islets (i) and acinar structures when TM was injected at E10.5. (D-F) Acini and islet staining when TM was administered at E12.5 (D), 3 weeks (E) or 8 weeks (F) after birth. (a, acini; d, duct, broken green lines; i, islets, broken red lines; blood vessel, broken black lines.) Scale bars: in B, 20 μm; in F, 20 μm for A, C, D, E and F.

A:

TM Injection	E8.5	E9.5	E10.5	E11.5	E12.5
HPAP+cells/ total islet cells	238/7425	267/6218	385/4942	318/5483	417/6421
HPAP+ cells/ total acini area	281/5740	279/4987	344/5472	224/4312	232/4551
HPAP+duct area/ HPAP+acini area	41/8946	536/7982	1432/8740	546/8670	94/9430

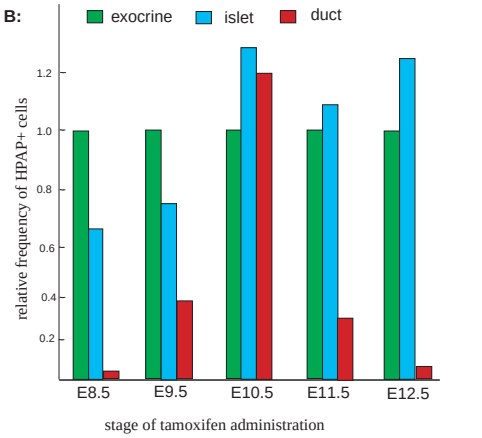

Fig. 4. Relative frequency of duct cell progenitors. Eight-week-old pancreata that received tamoxifen at different stages of development were stained for HPAP and counted (see Materials and Methods). Raw numbers are shown in A. The histogram (B) shows the relative frequency of HPAP+ cells in exocrine (green), islet (blue) or duct (red) tissue. Variations in HPAP labeling efficiency induced by TM at different stages were normalized to the exocrine tissue.

This slight variation could have several causes, including different levels of *Cre-ER*^{TM} expression in different genetic background or their different accessibility to TM in the uterus. This variation makes it difficult to give a reasonable estimate for the number of islet progenitors at different stages of development. Nonetheless, the main conclusion is that pancreatic cells expressing NGN3+ during embryogenesis give rise to progeny that populate the islets.

A systematic test of one-fifth of 15 sectioned pancreata from a single transgenic line was performed to look for labeled cells that were not in islets. A consistent finding of non-islet cells would suggest that NGN3+ cells give rise to more than just islets. However, we found a total of only nine HPAP+ cells that were not in islets compared with over 2000 HPAP+ cells present in islets (one cell in each of four pancreata that received TM at E8.5; four cells in five pancreata that received TM at E10.5, one cell found in three pancreata that received TM at E12.5 and three cells found in three pancreata that received TM at E14.5). These nine cells reside in pancreatic ducts (data not shown) and may result from rare mis-expression of the transgene or rare and transient expression in ducts. In any case, the vast majority of the NGN3+ cells in developing embryos divided and formed endocrine islets.

NGN3+ cells produce all four types of islet endocrine cells

Double immunostaining was performed using antibodies against HPAP and either insulin, glucagon, PP or somatostatin. All four types of HPAP/hormone double positive cells are

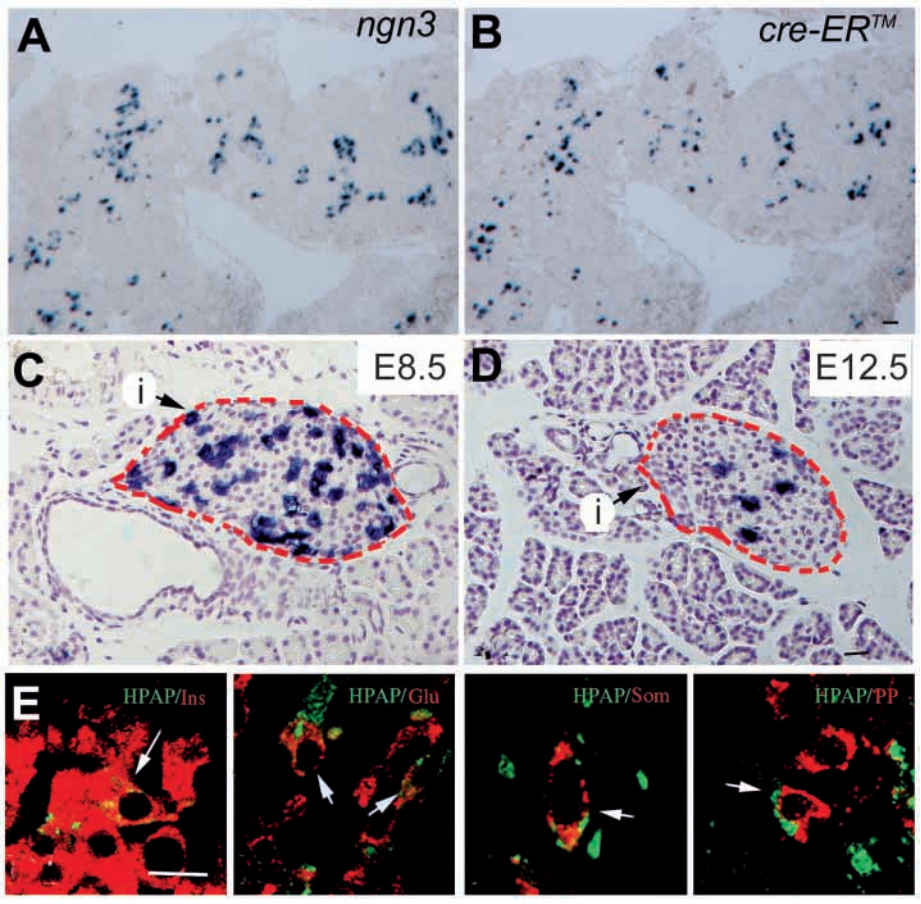

Fig. 5. NGN3+ cells form islets. Correct expression of the *Ngn3* promoter-driven transgene is assessed by in situ hybridization on adjacent sections. (A,B) Adjacent sections of an E13.5 transgenic pancreas probed with *Ngn3* (A) or *Cre-ER*^{TM} (B) cRNA. To study the lineage of the *Ngn3*-expressing cells, TM is given to embryos at different stages and the pancreata of double transgenic animals are examined for HPAP expression or co-expression of HPAP with various hormones at 3-8 weeks of age. (C,D) HPAP staining in islet cells when TM was injected at E8.5 or E12.5, respectively (8-week-old pancreata are shown). (E) A confocal image that shows hormone (Ins, Glu, Som, PP) and HPAP double-positive cells (arrows) when TM was injected at E8.5. Each picture is a single scan. Because HPAP is a membrane-bound protein while the hormones are cytoplasmic proteins, we do not always see colocalization of HPAP and hormone, as in HPAP/PP staining (i, islets, broken red lines). Scale bars: in B, 25 μm for A,B; in D, 25 μm for C,D; in E, 5 μm.

detected in animals that received TM at either E8.5 (Fig. 5E) or E12.5 (data not shown). We conclude that NGN3+ cells from different stages of embryogenesis give rise to all four types of endocrine islet cells. This result suggests that although high levels of hormones, such as somatostatin and pancreatic polypeptide, are not detected until very late in embryogenesis (after E17.5) (Gittes and Rutter, 1992), the progenitors for these cells are determined and are set aside before E9.5. Because the HPAP protein is membrane bound, whereas the four hormones are largely cytoplasmic, it is difficult to accurately count the number of each type of endocrine cell generated from a specific set of NGN3+ progenitors. Therefore, these data do not show whether NGN3+ progenitors at different stages of development give rise to the same ratios of all four endocrine cell types.

NGN3+ progenitors contribute to postnatal islet cell development

We employed this same experimental design to determine whether *Ngn3*-expressing cells contribute to islet maintenance or renewal in adult mice. Previous studies suggested that *Ngn3* expression is turned off in postnatal mice as judged by immunohistochemistry (Jensen et al., 2000; Schwitzgebel et al., 2000). We reasoned that a small number of NGN3+ cells, perhaps with transient or low *Ngn3* expression levels, could escape detection by immunohistochemistry. Indeed, *Ngn3* expression is detected by RT-PCR in handpicked islets from 8-week-old mice (data not shown).

We tested whether HPAP-labeled islet cells are observed when TM is administered in three or eight week old *Ngn3-Cre-ER^{TM};*Z/AP mice. One or three doses of 2 mg of TM were injected into 3-week- (n=15) and 8-week-old (n=10) double transgenic animals. The 25 pancreata were sectioned 1, 4 or 7 days after the last TM injection. HPAP+ islet cells were detected in 15 of 25 pancreata (nine out of the 15 in 3-week-old mice, and six out of the 10 in 8-week-old mice), with no significant difference between individual animals (Fig. 6). Without TM injection, no pancreatic cells expressing HPAP were found in the five double transgenic animals examined. The level of Cre-ER^{TM} in the other 10 animals may have been too low to label a significant number of cells. We performed double labeling using antibodies against HPAP and insulin and found that many HPAP+ cells express insulin (data not shown), suggesting that they are mature β-cells. Scanning one-fifth of all sections (including three pancreata that were characterized 24 hours after TM injection) revealed no HPAP+ cells in non-islet tissues.

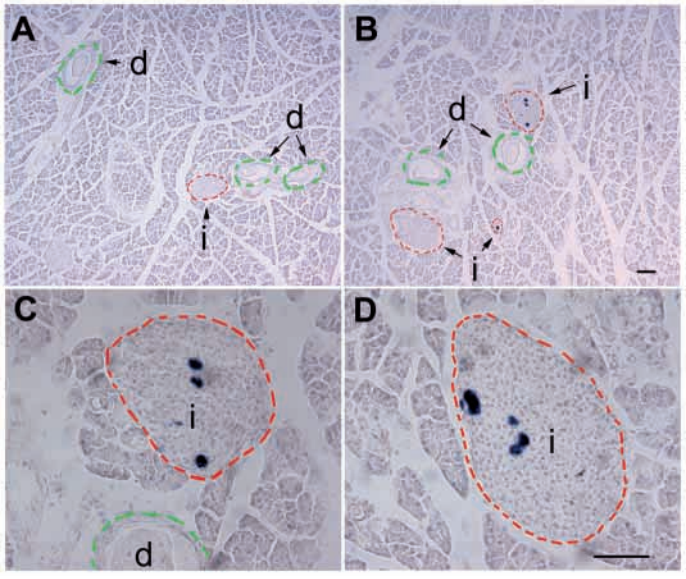

Fig. 6. NGN3+ cells contribute to the renewal or maintenance of islets in adult mice. Tamoxifen was injected into 3- or 8-week-old *Z/AP;Ngn3-Cre-ER^{TM}* animals and HPAP expression was examined 1, 3, or 7 days later. (A) A control showing that no HPAP+ cells are detected in an 8-week-old *Ngn3-Cre-ER^{TM}*; Z/AP pancreas without TM injection. (B) HPAP+ cells detected in islets 1 day after TM was injected into an 8-week-old mouse. (C) An enlarged area of B. (D) HPAP+ cells in islets 7 days after TM injection. In no case were HPAP+ cells observed in non-islet structures (d, duct, broken green lines; i, islets, broken red lines). Scale bars: in B, 25 μm for A,B; in D, 25 μm for C,D.

These data suggest that NGN3-expressing cells act as islet progenitors in adults, recapitulating the developmental mechanism used to make islets during embryogenesis. The data do not rule out the possibility that NGN3+ cells are born within mature pancreatic ducts and quickly migrate to existing islets. However, the results are most simply explained by the assumption that NGN3+ endocrine progenitors reside within islets (Fernandes et al., 1997). Furthermore, because such characterization is performed during normal development, we do not know whether this conclusion applies to the pancreatic regeneration.

Mature pancreatic duct cells derive from progenitors that do not express *Ngn3*

To test whether *Ngn3*-expressing progenitors also give rise to adult pancreatic ducts, we determined the position of HPAP+ cells in *Ngn3-Cre;Z/AP* double transgenic mice. In this case NGN3+ cells are labeled immediately after *Ngn3* expression is initiated, without the requirement for tamoxifen.

In two independent *Ngn3-Cre* transgenic lines, all islets cells, in both newborn and adult animals, express HPAP (Fig. 7A,B). Corresponding to this complete expression of HPAP, all the islet cells (except for endothelial cells within islets) lack β-galactosidase staining (data not shown). These data provide additional evidence that all islet cells derive from NGN3+ progenitors. By counting HPAP+ cells in 150 sections of four double transgenic pancreata (10-week-old, see Materials and Methods) we found less than one in a thousand exocrine or duct cells labeled (103/146,250 for acini and 16/20,270 for ducts). Thus, the vast majority of mature pancreatic duct cells derive from progenitors that have not expressed NGN3. However, as noted above, the data do not rule out the possibility that some endocrine progenitors reside in mature pancreatic ducts, transiently turn on *Ngn3* and then migrate to mature islets.

We also examined the location of the HPAP+ cells in embryonic stages of double transgenic animals. At E14.5, most HPAP+ cells are scattered, interspersed with mesenchymal cells, and reside in what appear to be duct-like structures (Fig. 7C). By E16.5, the HPAP+ cells are found in cords or islet-like aggregates that resemble immature islets, whereas other duct-like structures do not contain HPAP+ cells (Fig. 7D). Given that all of the HPAP+ cells end up in islets a short time later (Fig. 7A,B), these data suggest that some of the duct-like

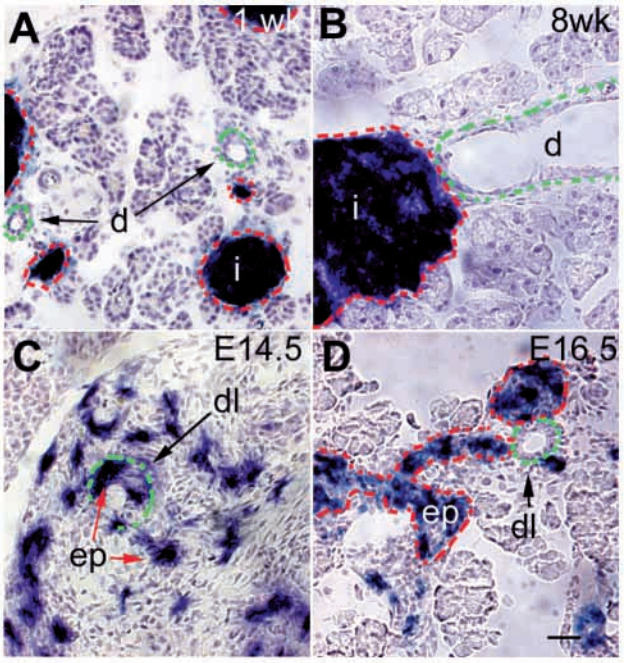

Fig. 7. Duct cell progenitors do not express *Ngn3*. *Ngn3-Cre* transgenic animals are crossed with the Z/AP reporter line and HPAP expression in pancreas is characterized in postnatal mice (A,B) or embryos (C,D). (A,B) In 1- or 8-week-old animals, only islet cells express HPAP (i, islet, broken red line), and no duct cells express HPAP (d, duct, broken green line). (C) In E14.5 pancreas, HPAP+ cells reside in duct-like structures (dl, duct-like structures, broken green line), as identified by the presence of a hole. Yet, these HPAP+ cells are endocrine progenitors (ep, red arrow). (D) At E16.5, the HPAP+ cells or endocrine progenitors (ep, broken red lines) form cord-like or islet-like structure and some duct-like structures that do not express HPAP (dl, broken green lines) are also observed. Scale bar: 25 μm.

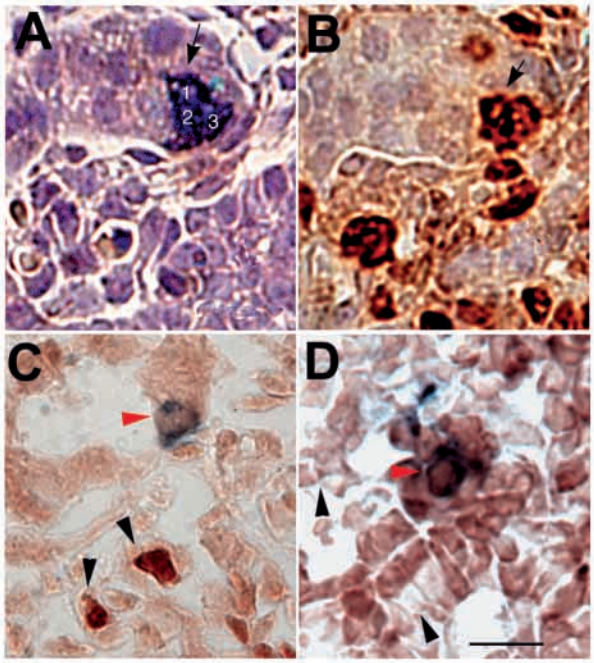

Fig. 8. The progeny of NGN3+ cells are mitotically active at E14, but they suppress *Ngn3* expression. *Ngn3-Cre-ER^{TM}; Z/AP* embryos were given TM at E8.5-E10.5 and the mitotic status of HPAP+ cells was assayed by BrdU incorporation. *Ngn3* expression and the differentiation of these HPAP+ cells was examined between E13.75 and E14 using NGN3 or NEUROD antibodies. (A) A clone composed of three HPAP+ cells (arrow), indicating that they are derived from a cell that expressed *Ngn3*. (B) An adjacent section to that in A shows these HPAP+ cells have incorporated BrdU. (C) HPAP staining (blue) and NGN3 protein staining (brown; with a polyclonal Ab). The cell indicated by the red arrowhead does not stain for NGN3 protein, whereas two other cells (black arrowheads) in this section are NGN3+. (D) An HPAP+ cell (blue stain, red arrowhead) that has differentiated and is expressing NEUROD protein (brown antibody stain). Other cells in this field are not HPAP+ but do stain for NEUROD. Scale bar: 8 μm.

structures and cords of epithelial cells observed in early embryos are clusters of endocrine, not duct, progenitors.

NGN3+ cells behave as expanding progenitors rather than stem cells

If NGN3+ cells are self-renewing stem cells, they should divide to form a clone within which at least one cell maintains *Ngn3* expression. In effect, the continued expression of NGN3 would be a molecular assay for self-renewal. The use of Cre-ER^{TM} allows us to label single NGN3+ cells by lowering the TM concentration so that the behavior of NGN-3+ clones derived from single cells can be followed.

Recombination by Cre-ER^{TM} was induced between E8.5-E10.5 using three low doses of TM injection. This produces a low frequency of HPAP+ labeling so that individual clones could be identified. We examined whether these HPAP+ cells still expressed *Ngn3* at E13.75-E14. At the same time, BrdU was administered 6 hours before sacrificing the animals to determine which cells were mitotically active. From 28 double transgenic embryos, we found 32 HPAP+ clones: 21 were composed of a single HPAP+ cell, five were composed of two-cell clones, and six were composed of three-cell clones (Fig. 8 and data not shown). We presume that these multi-cell clones derive from single NGN3+ progenitors because the frequency of the recombination was too low for individual HPAP+ cells to migrate to form clusters.

Immunostaining of adjacent sections shows that cells within the two- or three-cell HPAP+ clones incorporated BrdU (Fig. 8B), demonstrating that these NGN3+ derived cells were mitotically active. We performed in situ hybridization (data not shown) or immunostaining on HPAP+ cells and found that none of the tested HPAP+ cells maintain *Ngn3* expression (Fig. 8C and data not shown). We determined the differentiation status of some of these cells by staining with an antibody for NEUROD. All HPAP+ cells express NEUROD (Fig. 8D). To determine whether the low dose of TM administration only selectively labeled cells that expressed a high level of *Ngn3*, we used higher doses in more animals and found that no NGN3 was detected in HPAP+ cells, although more than 50 HPAP+ cells were found in some embryos (data not shown).

These data are consistent with the notion that most of NGN3+ cells are transient progenitors, committed to an endocrine fate. Furthermore, the disappearance of *Ngn3* expression suggests that NGN3+ cells are constantly born from NGN3– cells during development. This view is further

supported by the observation that only a small number of islet cells are labeled when one dose of TM is administered during embryogenesis.

DISCUSSION

The goal of these studies is to understand the normal development of the pancreas, particularly the lineage of pancreatic islets. Several methods have been used to mark cells and their progeny for lineage analysis. Although some success has been achieved using the Cre-LoxP genetic system (Herrera, 2000; Kimmel et al., 2000; Kisanuki et al., 2001; Rodriguez et al., 2000), one drawback of the system in its simplest form is that cells are marked from the time when the promoter driving *Cre* expression is first turned on in development. Marked progeny accumulate as development proceeds and new cells activate CRE. Thus, it is not possible to follow the progeny of cells born at defined developmental stages, such as embryonic development, postnatal growth or during regeneration. The inducible Cre-ER^{TM}-LoxP (Metzger et al., 1995; Danielian et al., 1998) improves on this system for lineage analysis. In conjunction with tamoxifen-independent CRE, we have employed it to analyze the progeny of cells expressing *Pdx1* and *Ngn3*. This direct cell lineage analysis eliminates many of the uncertainties connected with the use of gene co-expression as a lineage marker or the cell autonomy questions left open by the analysis of mutant phenotypes.

Pdx1 expressing progenitors give rise to all three types of pancreatic tissue

Previous lineage tracing showed that *Pdx1*-expressing cells give rise to mature α and β cells in adult mice (Herrera, 2000), but that study did not determine whether PDX1+ cells also gave rise to other pancreatic cell types, e.g. exocrine, duct and other endocrine cells. Gannon et al. (Gannon et al., 2000) examined other pancreatic cell types generated from *Pdx1*-expressing progenitors, but did not study whether all pancreatic cells are derived from PDX1+ precursors. We used the Z/AP (Lobe et al., 1999) reporter to monitor the recombination mediated by the *Pdx1* promoter driving CRE expression. Our results extend the findings of Herrera and demonstrate that all pancreatic tissue is derived from PDX1+ progenitors.

Progenitor cells for endocrine/exocrine and duct tissue diverge during early embryogenesis

A significant finding of our lineage tracing is the early separation of the adult duct progenitors from the exocrine and endocrine cell lineage. Based on the observation that insulin- or glucagon-positive cells often appear close to duct-like structures during embryogenesis and during 'regeneration' after chemical treatment or surgical ablation (Teitleman and Lee, 1987; Dudek et al., 1991; Gu et al., 1994; Pang et al., 1994; Bonner-Weir et al., 1993), it is generally believed that the endocrine islets arise from the pancreatic duct (Edlund, 1999; Bonner-Weir et al., 2000). However, the lack of specific markers for early embryonic pancreatic ducts and a technique to follow individual cells in the pancreas made it difficult to determine the fate of cells within the embryonic duct-like structures, i.e. to determine whether those cells ever form adult ducts. Our results, based upon temporally controlled Cre recombination, demonstrate that exocrine and endocrine progenitors express *Pdx1* throughout early embryogenesis. By contrast, adult duct progenitors express *Pdx1* only between E9.5 and E12.5. These results suggest that the vast majority of progenitors for duct and exocrine/endocrine cells are separated before E12.5. This conclusion is consistent with the observation that islet cells form in the absence of ductal structures in vitro, i.e. endocrine cells can be generated without going through duct-like features (Gittes et al., 1996).

NGN3+ cells are endocrine progenitors in embryos and in adults

By irreversibly marking cells, we provide direct evidence that NGN3+ cells are endocrine progenitors during embryogenesis. Consistent with the finding that *Ngn3* expression is transient (Jensen et al., 2000; Schwitzgebel et al., 2000), our analysis of NGN3+ progenitors demonstrates that individual NGN3+ cells do not maintain *Ngn3* expression during embryogenesis from which we conclude that NGN3+ cells in early embryos behave as progenitors not stem cells.

Our data do not exclude the possibility that a population of endocrine stem cells exists that expresses a very low level of *Ngn3*. However, we and others find that endogenous *Ngn3* and the NGN3 transgene are uniformly expressed (Jenson et al., 2000; Schwitzgebel et al., 2000), i.e. there is no evidence for a population of cells that express a low level of *Ngn3*. We conclude that the majority, if not all, NGN3+ cells are endocrine progenitors that differentiate into endocrine cells without self-renewing.

Using the Cre-ER^{TM}-LoxP system, we demonstrate that NGN3+ cells exist in the adult pancreas and can therefore contribute to the maintenance of adult endocrine islets. Owing to the weak promoter activity of *Ngn3*, the levels of *Ngn3* and *Cre-ER* mRNAs in the transgenic animals is very low and we could not reliably detect these transcripts or the corresponding proteins. Thus, we could not directly determine the location of the *Ngn3-Cre-ER^{TM}*-expressing cells in situ. Nonetheless, our detection of *Ngn3* transcripts in purified islets and the fact that we never observe any HPAP+ cells in tissues other than islets, when TM is administered in adult animals, points to the possibility that islet progenitors reside in islets, consistent with the previous hypothesis by Fernandes et al. (Fernandes et al., 1997).

Many of the 'duct-like structures' in early embryonic pancreata are islet progenitors and do not give rise to adult ducts

By following the progeny of NGN3+ cells, we demonstrate that some of the structures previously described as 'pancreatic ducts' in embryos are derived from cells that expressed NGN3. Moreover, the results show that these cells within 'duct-like structures' later coalesce to form mature islets, i.e. they are not duct progenitors, but rather endocrine progenitors. Other duct-like structures, those that do not contain progeny of NGN3+ cells, presumably give rise to the adult duct system. These findings point to the heterogeneity in developmental potential among 'duct-like structures' in early embryo and further supports the notion that the endocrine/exocrine and ductal lineages are separated before E12.5. It also highlights the confusion in the literature surrounding the use of the term

'pancreatic duct' to refer to structures in the embryo that have not been demonstrated to be duct progenitors.

With respect to the relationship between adult pancreatic ducts and islet (NGN3+) progenitors, our results do not exclude the possibility that a minor population of mature pancreatic duct cells activate *Ngn3* expression and quickly exit the ducts to form islets. It is unlikely that the issue of ductal contribution to islets can be resolved until there are unequivocal markers for duct cells and their progenitors. In this regard, it should be noted that 'duct' markers such as cytokeratin 19, carbonic anhydrase II and various lectins also stain other pancreatic cell types or only stain subtypes of pancreatic duct in embryos and newborns (Githens, 1994).

Limitations to conclusions based on results with transgenic animals

As with other transgenic systems, our conclusion depends on the extent to which the transgene faithfully recapitulate the wild-type pattern of gene expression. The *Pdx1* promoter we used is well characterized and several studies showed that it drives the proper expression of the *Pdx1-Cre* or the *Pdx1-Cre-ER^{TM}* transgene (Wu et al., 1997). We confirmed this by in situ hybridization on multiple transgenic lines using *Pdx1* and the transgene as hybridization probes. Similarly, in situ hybridization on adjacent sections demonstrated that the *Ngn3* promoter used is sufficient to drive the transgene expression properly at the stages we tested (E13.5 and E15.5). Yet, we have not counted each and every cell nor tested whether *Pdx1* and *Ngn3* expression is subject to post-transcriptional regulation. Thus, the conclusions are limited to cells that had transcriptionally active *Pdx1* or *Ngn3* promoters, and do not speak to the possibility that some cells transcribe the *Pdx1* and *Ngn3* genes and regulate the function of their protein products. With our experimental design, it was not practical to label a single endocrine progenitor during embryogenesis and look for its progeny in the adult. Thus, our data do not show whether a single NGN3+ cell gives rise to all four islet cell types.

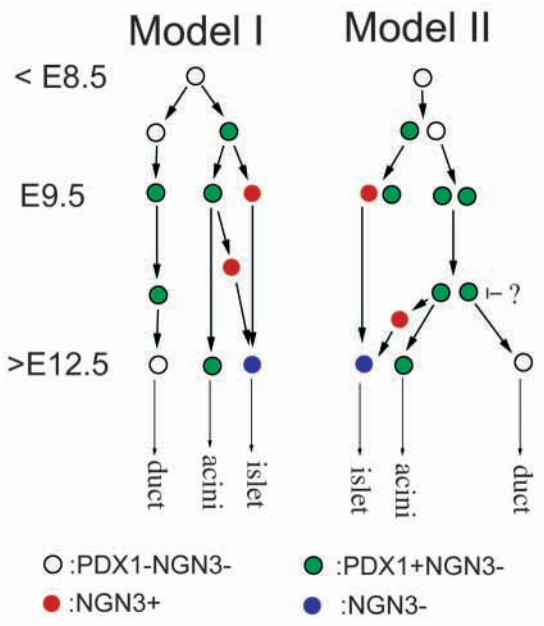

Fig. 9. Two models for the lineage of exocrine, endocrine and duct cells. In both models, the pancreatic duct progenitors only express *Pdx1* from E9.5 to E12.5. In Model 1, the duct lineage diverged from that of the endocrine/exocrine cells before E8.5. The endocrine and exocrine cells are distinguished by the presence of *Ngn3* expression. In Model 2, the duct and endocrine/exocrine lineage diverged between E9.5 and E12.5. In the later model, suppressive or inductive signals are needed to allow for the development of duct fate.

Time of pancreatic lineage commitment

Our data are consistent with at least two models for pancreatic cell commitment (Fig. 9). In model one, duct progenitors and endocrine/exocrine progenitors diverge before E8.5 (i.e. before the onset of *Pdx1* expression). At E8.5, an intracellular program or extracellular signals allows for *Pdx1* expression in endocrine/exocrine progenitors and this can continue throughout embryogenesis. By contrast, the pre-determined duct progenitors do not begin expressing *Pdx1* until E9.5 and then can only do so until E12.5. In the second model, the lineage of the duct and endocrine/exocrine progenitors does not separate until E12.5. In this scenario, cells within the prospective pancreatic region have the same potential before E8.5. When cells within the region start to express *Pdx1*, those that first express *Pdx1* give rise to endocrine or exocrine tissues in adults. When cells express *Pdx1* between E9.5 to E12.5, some will form pancreatic duct and others will form endocrine or exocrine cells. In both models, the duct and endocrine/exocrine progenitors diverge before E12.5. And in both scenarios, the initiation of *Ngn3* expression within some PDX1+ cells establishes them as endocrine progenitors.

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