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Defining the cell lineages of the islets of langerhans using transgenic mice

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ABSTRACT In this Special Issue of the *Int. J. Dev. Biol.,* we summarize our own studies on the development of the mouse endocrine pancreas, with special emphasis on the cell lineage relationships between the four islet cell types. Considerable knowledge concerning the ontogeny of the endocrine pancreas has been gained in recent years, mainly through the use of two complementary genetic approaches in mice: gene inactivation and genetic labelling of precursor cells. However, neither gene inactivation in KO mice nor co-localisation of hormones in single cells during development can be taken as evidence for cell lineage relationships among different cell types. The β-cell lineage analysis was started by selectively ablating specific islet cell types in transgenic mice. We used the diphtheria toxin A subunit coding region under the control of insulin, glucagon or pancreatic polypeptide (PP) promoters, in order to eliminate insulin-, glucagon- or PP-expressing cells, respectively. Contrary to the common view, we demonstrated that glucagon cells are not precursors of insulin-producing cells. These results were in addition the first evidence of a close ontogenetic relationship between insulin and somatostatin cells. We pursued these analyses using a novel, more subtle approach: progenitor cell labelling through the expression of Cre recombinase in doubly transgenic mice. We were able to unequivocally establish that 1) adult glucagon- and insulin-producing cells derive from precursors which have never transcribed insulin or glucagon, respectively; 2) insulin cell progenitors, but not glucagon cell progenitors transcribe the *PP* gene and 3) adult glucagon cells derive from progenitors which do express *pdx1*.

KEY WORDS: *pancreas, islet, transgenic, mouse, lineage*

Introduction

The mammalian pancreas is not a bilateral organ, yet it originates from two separate endodermal outgrowths (Fig. 1). The islets of Langerhans are well-organized micro organs that constitute the endocrine portion of the pancreas. They are scattered amidst the exocrine tissue (the secretory acini and excretory ducts) (Fig. 2A). Islet endocrine cells are of four different types, each producing one hormone: insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells), or pancreatic polypeptide (PP-cells) (Fig. 2B) (Orci, 1982; Orci and Unger, 1975). Contrary to what is observed in adult islets, cells containing simultaneously more than one hormone are frequent in the embryonic pancreas; this has suggested the existence of a common precursor cell for all four islet cell types (Alpert *et al.,* 1988; Rall *et al.,* 1973; Teitelman *et al.,* 1993).

The pancreas forms as a double evagination of the duodenal entoblast, through a complex pathway of cell differentiation and organogenesis (Fig. 2C) (Pictet *et al.,* 1972). Islet endocrine cells are derived from the epithelia of embryonic pancreatic ducts; accordingly, the first hormone-containing cells found in the early buds are located exclusively within the walls of these ducts (Fig. 2D) (Edlund, 1998; Herrera *et al.,* 1991; Larsson, 1998; Le Douarin, 1988; Pictet *et al.,* 1972; Sander and German, 1997; Slack, 1995; St-Onge *et al.,* 1999; Yamaoka and Itakura, 1999).

Interestingly, the potential to form pancreatic endocrine tissue from ducts is not restricted to the period of embryonic development. Under certain pathological or experimental conditions, such as after partial pancreatectomy (Bonner-Weir *et al.,* 1993; Bonner-Weir *et al.,* 1983; Brockenbrough *et al.,* 1988; Peters *et al.,* 2000; Sumi and Tamura, 2000; Wang *et al.,* 1995), duct ligation (Wang *et al.,* 1995) or treatment with streptozotocin or exendin-4 (a GLP-1 agonist) (Xu *et al.,* 1999), formation of new islets occurs with remarkable efficiency in adult pancreata (reviewed by Slack (Slack, 1995)).

Abbreviations used in this paper: KO, knock-out; PP, pancreatic polypeptide; TGF, Transforming Growth Factor;

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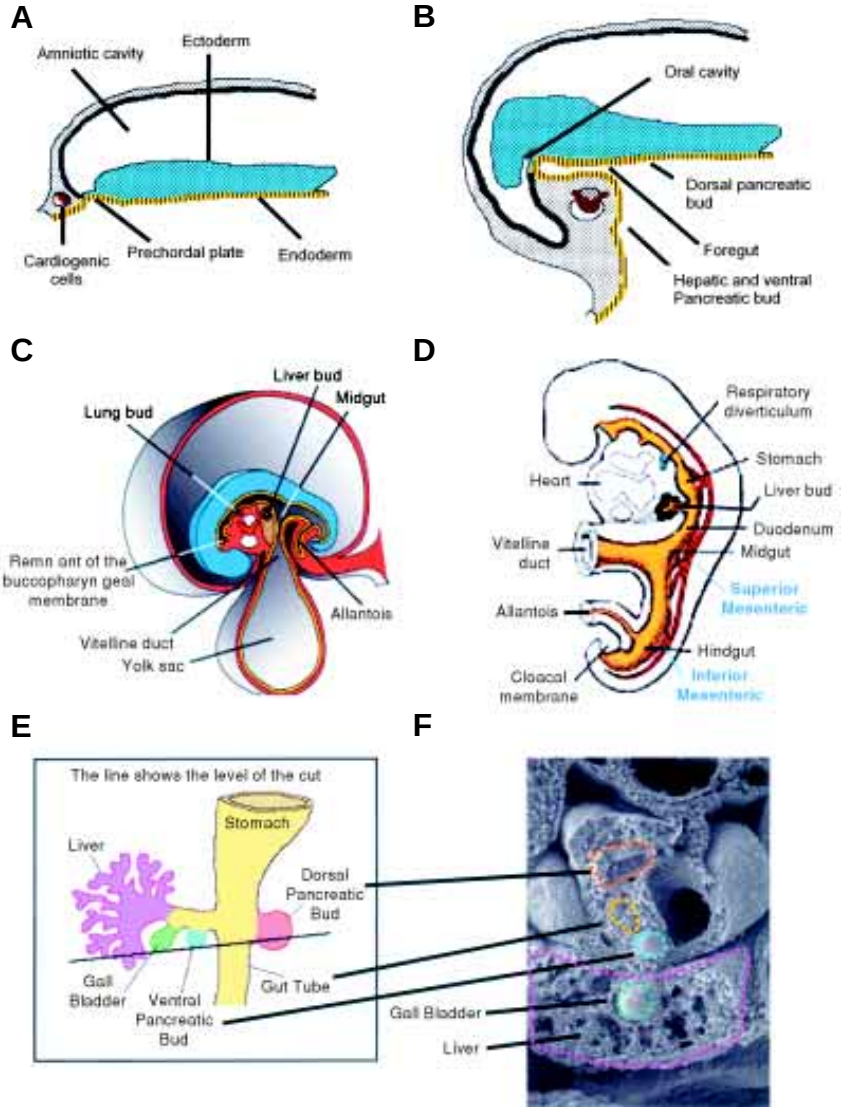

Fig. 1. The mammalian pancreas originates from the embryonic endoderm. *Different buds formed in separate areas of the endoderm* **(A,B)** *are brought together into the presumptive duodenal region as a consequence of the cephalic folding of the embryo* **(B-D)**. *The growing ventral pancreatic bud represents a third diverticulum in the liver primordium* **(E,F)**.

Several different strains of genetically modified mice, i.e. lacking or on the contrary overexpressing different transcription factors, have added insights into the ontogeny of the pancreas (Table 1). The notochord induces dorsal pancreas development through the inhibition of Shh (sonic hedgehog) in pre-pancreatic endoderm (Wells and Melton, 1999). This repression is sufficient for expression of pancreatic genes such as the homeodomain gene *Pdx1 (Pancreatic and duodenal homeobox gene-1)* (Hebrok *et al.,* 1998). PDX1 is the earliest marker, found in the duodenal wall, of pancreatic bud cells (Edlund, 1998; Larsson, 1998; Sander and German, 1997; St-Onge *et al.,* 1999; Yamaoka and Itakura, 1999). *Pdx1* is necessary for the development of the pancreas, as *Pdx1*-deficient mice lack pancreas at birth. However, insulin and glucagon cells are present in early embryonic buds of *Pdx1*-/- mice, and their pancreatic mesenchyme develops normally (Ahlgren *et al.,* 1996). *Isl1* (LIM homeodomain protein islet-1)-null mice are not viable and lack the dorsal pancreatic mesenchyme; pancreatic buds from -/- embryos cultured for 7 days showed that ductal *Isl1* expression is required for the differentiation of islet cells (Ahlgren *et al.,* 1997). *Pax-4* (paired-box domain 4) -/- mice have no insulin nor somatostatin cells, whereas *Pax-6*-deficient mice do not have glucagon-producing cells (Sander and German, 1997; Sosa-Pineda *et al.,* 1997; St-Onge *et al.,* 1997); together, the two genes regulate the differentiation of all proximal gastrointestinal endocrine cells and reflect common pathways for pancreatic and gastrointestinal endocrine cell differentiation (Larsson *et al.,* 1998). Homeodomain transcription factor *Nkx2.2*-deficient mice have incompletely differentiated β-cells and become diabetic (Sussel *et al.,* 1998). Inactivation of the winged helix factor *Hnf3-α* (hepatocyte nuclear factor 3-α) in mice greatly impairs islet glucagon gene expression (Kaestner *et al.,* 1999). Mice bearing the homeobox gene *Hlxb9* null mutation fail to develop the dorsal pancreas, and in the ventral part have reduced numbers of insulin cells (Harrison *et al.,* 1999; Li *et al.,* 1999). Cell adhesion molecules are also important regulators of pancreas organogenesis: *N-cadherin*-deficient mice have selective agenesis of the dorsal mesenchyme and pancreas, like *Isl1*-KO embryos (Esni *et al.,* 2001).

Two basic helix-loop-helix (bHLH) transcription factors seem to be implicated in determining whether an early pancreas cell is to become exocrine or endocrine: *PTF1-p48* and *neurogenin3 (ngn3)*. Mice in which *PTF1-p48* is mutated have no exocrine pancreas (Krapp *et al.,* 1998), whereas inactivation of *ngn3* results in agenesis of endocrine pancreas (Gradwohl *et al.,* 2000). *ngn3* is a pro-neural gene, and appears to be required for the specification of the four pancreatic endocrine cell types. Furthermore, overexpression of *ngn3* under control of the *Pdx1* promoter in transgenic mice is sufficient to drive early and ectopic differentiation of islet cells, mostly α-cells (Apelqvist *et al.,* 1999; Schwitzgebel *et al.,* 2000). In the early pancreatic bud, *ngn3* is detected in proliferating cells expressing *Pdx1* but not in hormone-containing cells (Schwitzgebel *et al.,* 2000). This strongly suggests that *ngn3*+ cells could represent an islet cell precursor population, although it remains to be proven by a strict cell lineage analysis (Herrera, 2000).

The study of cell lineages is hampered by the fact that a precursor cell may express a gene at one developmental stage and turn it off later; in addition, as cells can move, their location at the end of morphogenetic movements is rarely the position of origin. Therefore, despite the important findings obtained using a gene KO approach, without the ability of tracking the fate of a cell over time, the problem of islet cell lineages would likely remain largely unresolved; in particular, *in vivo* cell labeling techniques (in transgenic mice) are indispensable to discriminate between paracrine/endocrine and lineage relationships.

In this review we summarize our own studies on the development of the mouse endocrine pancreas, with special emphasis on the cell lineage relationships between the four islet cell types.

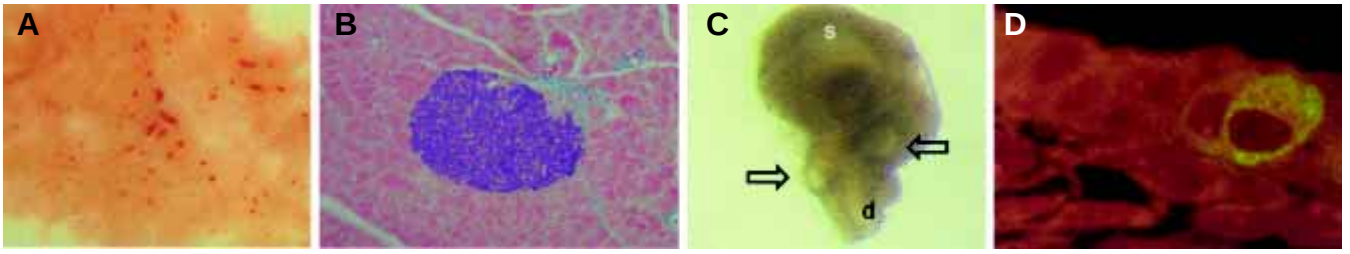

Fig. 2. The pancreas is both an exocrine and an endocrine gland. (A) *Whole mount adult mouse pancreas perfused with dithizone, which specifically stains insulin cells. Islets appear as red dots scattered in the exocrine tissue.* **(B)** *Mouse pancreas paraffin section stained so that insulin-containing cells are purple (aldehyde-fuchsin method). In islets, large proportions of centrally located endocrine cells do produce insulin. Exocrine acini and ducts constitute the pink tissue surrounding the islet.* **(C)** *The two pancreatic buds (dorsal and ventral) embedded in mesenchyme can be easily distinguished in 11.5-day old mouse fetuses (arrows); s, primordium of the stomach; d, duodenum.* **(D)** *Epon semi-thin section of a 17.5-day old mouse fetal pancreas. An insulin-containing epithelial cell can be seen using a fluorescein-conjugated antibody.*

Timing of the Appearance of Differentiated Endocrine Phenotypes

By immunofluorescence on cytospin preparations of trypsinized total mouse pancreatic buds and on semi-thin sections of epon-embedded mouse pancreatic buds, we have found glucagon and pancreatic polypeptide (PP) family-containing cells at embryonic day 10.5 (E10.5) in dorsal buds and at E11.5 in ventral buds (Herrera *et al.,* 1991). Insulin-containing cells appear in dorsal buds at E11.5, and one to two days later in ventral buds. Somatostatin-containing cells are detectable from E13.5 in both dorsal and ventral buds. A quantitative analysis showed that up to E15.5, PP family (mostly PYY and PP)-containing cells are relatively abundant in both buds. By PCR amplification of cDNAs prepared from total pancreatic RNA, we also detected PP mRNA from E10.5 onwards, thus confirming the early expression of the PP gene in the developing mouse pancreas.

Analysis of endocrine cells *in situ* suggested three major patterns of cell distribution in embryonic pancreas. First, individual hormone-containing cells are located within the epithelium of pancreatic ducts. In both dorsal and ventral buds, the majority of these endocrine cells contain PP family peptides, but many also contain glucagon, insulin or somatostatin. Secondly, clusters of endocrine cells are found in the pancreatic interstitium. Many of these cells contain simultaneously peptides of the PP family and glucagon, which, by immunogold labeling of consecutive thin sections, can be shown to coexist within individual secretory granules. Finally, starting on E18.5, typical islets are formed with centrally located β-cells and with the adult "one cell-one hormone" phenotype. These results suggested that PP family-containing cells might occupy an unexpected place in the lineage of endocrine islet cells.

TABLE 1

SOME FACTORS INVOLVED IN PANCREAS DEVELOPMENT AS DETERMINED USING KO MICE

Targeted gene	Dorsal pancreas	Ventral pancreas	Reference
Pdx1	Aplasia	Aplasia	(Ahlgren *et al.,* 1996; Jonsson *et al.,* 1994; Offield *et al.,* 1996)
Ngn3	No islets	No islets	(Gradwohl *et al.,* 2000)
PTF1-p48	No exocrine cells	No exocrine cells	(Krapp *et al.,* 1998)
Pax4	No β- nor δ-cells	No β- nor δ-cells	(Sosa-Pineda *et al.,* 1997)
Pax6	No α-cells	No α-cells	(St-Onge *et al.,* 1997)
Isl1	Agenesis; no mesenchyme	No endocrine cells	(Ahlgren *et al.,* 1997)
Hlxb9	Agenesis	Fewer β-cells	(Harrison *et al.,* 1999; Li *et al.,* 1999)
Hes1	Hypoplasia	Hypoplasia	(Jensen *et al.,* 2000)
N-cadherin	Agenesis; no mesenchyme	Normal	(Esni *et al.,* 2001)
Beta2/NeuroD	Fewer β-cells; distorted islets	Fewer β-cells; distorted islets	(Naya *et al.,* 1997)
Nkx2.2	Immature β-cells	Immature β-cells	(Sussel *et al.,* 1998)

Role of Diffusible Factors in Pancreatic Morphogenesis

We cultured pancreatic rudiments from E12.5 mouse embryos in three-dimensional gels of extra cellular matrix proteins and examined their development and differentiation for up to 12 days (Sanvito *et al.,* 1994). Addition of cytokines to cultures of pancreatic buds in collagen gels modified the relative proportions of the epithelial components of the gland. In the presence of EGF the proportion of the tissue occupied by ducts overrided that of acinar structures, whereas the endocrine portion of the tissue was not significantly modified. TGF-β1 inhibited the development of acinar tissue without decreasing the amount of ducts, mesenchyme, and endocrine cells. These results showed that cytokines can modulate the development of the pancreas and suggested a role for TGF-β1 in regulating the balance between the acinar and endocrine portions of the gland *in vivo*. This hypothesis was further explored by generating transgenic mice over expressing TGF-β1 in islet β-cells (Sanvito *et al.,* 1995), which resulted in massive fibrosis of both islets and exocrine pancreas. The number of β-cells was not decreased and the mice were normoglycemic. We concluded that the lesions developed in the TGF-β1 transgenic mice are reminiscent, in many aspects, of those found in chronic pancreatitis, suggesting that the cytokine may be involved in this disease. These animals thus represent a good experimental model to study the fibrotic lesions characteristic of this disorder.

Ablation of Islet Endocrine Cells by Targeted Expression of Hormone-Promoter-Driven Toxigenes

We performed a study (Herrera *et al.,* 1994) of the ontogenetic relationships between the different types of endocrine cells in the islets of Langerhans by generating transgenic mouse embryos in which cells transcribing the glucagon, insulin, or PP genes were destroyed through the promoter-targeted expression of the diphthe-

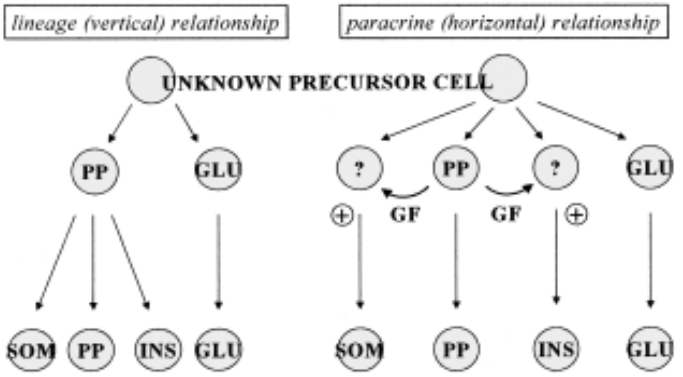

Fig. 3. Toxigene-mediated ablation of cells in which a 548-bp sequence of the 5′ region of the pancreatic polypeptide gene is active, prevents the differentiation of insulin- and somatostatin-producing cells *(from Herrera* et al., *1994; see text). This could be due to an either lineage or paracrine relationship between these cells. PP, pancreatic polypeptide; GLU, glucagon; SOM, somatostatin; INS, insulin; GF, putative growth or differentiation factor.*

ria toxin A chain (DT)-coding region. These results unambiguously demonstrated that neither glucagon nor insulin gene-expressing cells are essential for the differentiation of the other islet endocrine-cell types; they are thus incompatible with a model of islet development according to which cells co expressing glucagon and insulin would be precursors to all islet endocrine cells. They also suggested that PP gene-expressing cells are indispensable for the differentiation of islet β and δ cells, either because they produce a necessary paracrine or endocrine factor and/or because of a cell-lineage relationship (Fig. 3).

Islet Cell Lineage Analyses

In vivo cell ablation is one approach to depict ontogenic relationships, but it does not allow to discriminate between lineage and paracrine/endocrine relationships. Similarly, a gene KO approach is not suitable for demonstrating a cell lineage connection. Only the ability of irreversibly labeling a cell permits to follow its fate over time. Developments in gene recombination technology allowed only recently setting up an approach to "mark" irreversibly cells at one developmental stage using a given promoter, and therefore to identify all their descendants. In the context of our work, this involved the genetic labeling of cells that transcribe the insulin, glucagon, PP or PDX1 genes early during development, and the analysis of islet α- and β-cells later in development or in the adult, to determine whether they are derived from these early insulin, glucagon, PP or PDX1 gene-expressing cells.

To study the cell lineages of the endocrine pancreas we have used a version of the Cre/loxP system (Barinaga, 1994; Ramirez-Solis and Bradley, 1994; Sauer, 1993; Sauer, 1994; Zou *et al.,* 1994), whereby a reporter transgene is "activated" in a tissue-specific manner (Jacob and Baltimore, 1999). The experimental design consists in generating mice bearing two transgenes (summarized in Fig. 4). One transgene, "reporter", placed under the control of either the insulin (β-specific) or the glucagon (α-specific) gene promoter, contains a hGH coding region placed downstream of a "floxed" (i.e., loxP-flanked) transcription termination site (STOP sequence) (Lakso *et al.,* 1992; Sauer, 1993; Tsien *et al.,* 1996). The native reporter transgene cannot be expressed in β- or α-cells because of the STOP sequence; its expression requires the deletion of this sequence, which can be obtained in the presence of Cre recombinase. hGH is a good marker for immunohistochemical detection of gene expression, and has been used by others in a number of transgenic studies (Artavanis-Tsakonas *et al.,* 1995; Lardelli *et al.,* 1995; Tsien *et al.,* 1996; Weinmaster, 1997).

A second, or "tagger" transgene, consists of the Cre recombinase gene placed under the control of a promoter active in putative progenitor cells; its expression results in the deletion of the STOP site of the reporter transgenes, thus allowing hGH synthesis. This approach has been used by others to activate the transcription of either the SV40 T antigen, the LacZ, or the placental alkaline phosphatase genes in transgenic mice (Jacob and Baltimore, 1999; Lakso *et al.,* 1992; Tsien *et al.,* 1996), but not to analyze cell lineages.

Using this genetic approach allowing to follow cell lineages through the expression of Cre recombinase, we showed that: 1) adult α- and β-cells derive from cells that have never transcribed insulin or glucagon, respectively, 2) β-cell progenitors but not α-cell progenitors transcribe the PP gene, and 3) the PDX1 gene, whose expression characterizes the earliest pancreatic stem cells, and which is expressed by adult β-cells, is also expressed by α-cell progenitors (Herrera, 2000). Thus the islet α- and β-cell lineages appear to arise independently during ontogeny, probably from a common precursor (Fig. 5).

The power and elegance of this innovative approach also resides in the versatility of the transgenic mice encoding Cre recombinase in different subsets of pancreatic cell types. Thus, after these mice were employed to determine cell lineage relationships, they are now unique tools to inactivate specific genes in these same cell lineages ("conditional" KO mice).

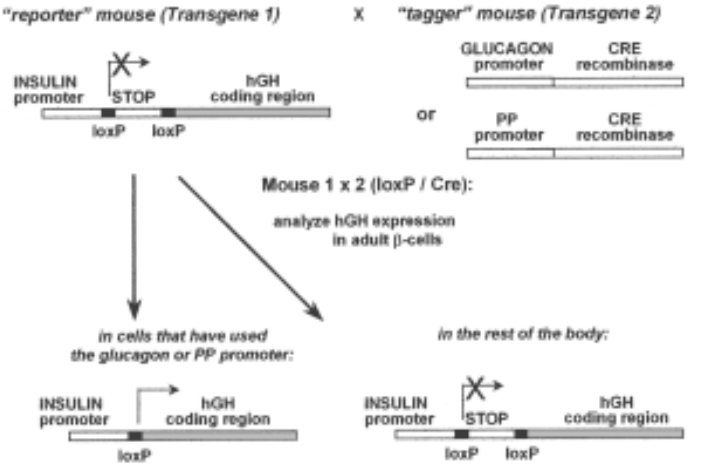

Fig. 4. General Cre/loxP-based strategy to genetically label islet endocrine cell precursors *(from Herrera, 2000; see text). For simplicity, only one example is illustrated: the experiment designed to assess whether adult insulin-producing β-cells descend from glucagon- and/or PP-producing progenitors. If Cre is made in the precursor cell, then mature insulin cells will produce hGH (Schwitzgebel* et al., *2000).*

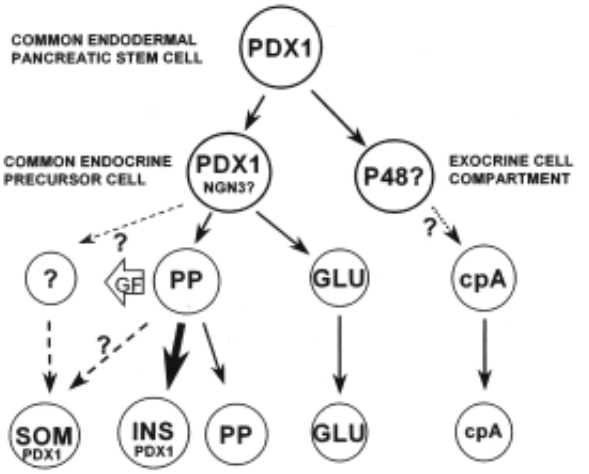

Fig. 5. Proposed pancreatic cell lineages, *as deduced from the cell ablation and labeling experiments in transgenic mice (Herrera* et al., *1994), data from the literature (Krapp* et al., *1998) and the cell lineage analyses (Herrera, 2000) (see text). p48, one of the two subunits of the exocrine pancreas-specific transcription factor PTF1, is the earliest exocrine marker known to date. That somatostatin-producing cells could descend from PP-expressing precursors is deduced from the cell ablation experiments in transgenic mice (Herrera* et al., *1994). cpA, carboxypeptidase A; GLU, glucagon; SOM, somatostatin; INS, insulin; GF, undefined growth factor.*

Role of Notch1 in Pancreas Differentiation: Generation and Study of Pancreas-Specific Notch1 Knock-Out Mice

The generation of a multicellular organism, or of a single organ, results from a cascade of coordinate developmental decisions taken by cells as a consequence of the interactions between them and their neighbors. Many regulative events of this nature are mediated by Notch, a family of transmembrane proteins (Artavanis-Tsakonas *et al.,* 1995; Lardelli *et al.,* 1995; Weinmaster, 1997); these receptors are components of a highly conserved signaling mechanism present in the whole Animal Kingdom. As a consequence of such signaling interactions, specific transcription factors are then induced to achieve the expression of cell-type specific products, such as the different islet hormones. Inactivation of Notch1 in mice by homologous recombination results in embryo death early after implantation (at about E9 - E9.5) (Swiatek *et al.,* 1994). Notch receptors are broadly expressed in embryos, and continue to be expressed in uncommitted and proliferative cells throughout life (Fehon *et al.,* 1991; Xu *et al.,* 1992). The Notch ligands belong to the Delta family of transmembrane proteins: activation of Notch receptors by Delta-like (Dlk) proteins either prevents or influences the differentiation program of a cell by modifying its ability to reply to other instructive signals (Weinmaster, 1997). When Notch is inactivated by Delta (expressed by differentiating neighboring cells), it triggers the expression of bHLH genes such as Hes-1, inhibitors of the expression of proneural genes. Notch1, 2 and 3 are expressed in developping pancreas, as well as the ligands Jag1, 2, Dll1, 3, and Hes-1 (Jensen *et al.,* 2000). It appears that the Notch signalling pathway is involved in pancreatic differentiation (Apelqvist *et al.,* 1999), so that mice lacking either Dll1 or RBP-jk (in the Notch cascade), which are not viable, have an accelerated differentiation of endocrine cells in pancreatic buds (Apelqvist *et al.,* 1999). Mice having a Hes-1 null mutation have a severe pancreatic hypoplasia due to a loss of epithelial precursor cells: in the absence of Hes-1 there is an accelerated differentiation of glucagon-expressing cells (Jensen *et al.,* 2000).

To precisely study the role of Notch1 in the ontogeny of the endocrine pancreas we have devised the pancreas-specific inactivation of Notch1, in doubly transgenic mice, using the Cre/LoxP system. This study is ongoing, in collaboration with M. Aguet and F. Radtke (Lausanne, Switzerland), which provided the Notch1 floxed (i.e. loxP-flanked) mice. We are using the Pdx1 promoter-Cre, Insulin promoter-Cre, and Glucagon promoter-Cre mice described earlier, as well as the Pax6 promoter-Cre animals, in which the recombinase is made in all islet cell types (Ashery-Padan *et al.,* 2000). The preliminary results suggest that the conditional inactivation of Notch1 specifically in the precursors of the endocrine pancreas results in a reduction of the proportion of endocrine tissue, and that the silencing of Notch1 in cells already transcribing the insulin or glucagon genes has no further consequences in their viability or function (manuscript in preparation).

Concluding Remarks

Besides the fact that embryonic stem (ES) cells might be in the future a source of differentiated cell types, one should not forget that the potential to form new such differentiated cells is not limited to ES cells. In particular, as an example, formation of pancreatic endocrine tissue is not restricted to the period of embryonic development. Interestingly, it is thought that islet neogenesis in adults is probably similar in its intrinsic mechanisms to that of the embryonic development. Future experiments should be directed at determining the extent of such similarities. In this context, there are efforts put on trying to obtain pancreatic pluripotent cell lines that should allow developing an *in vitro* model of β-cell development for transplantation in diabetes.

As a final consideration, we would like to suggest that the ontogenic relationship described here between different islet endocrine cells (Herrera *et al.,* 1994; Herrera, 2000) may be viewed in an evolutionary perspective. In particular, the relation between PP promoter activity and formation of new insulin-secreting cells. The endocrine pancreas has taken up, in the course of evolution, the task of participating in the control of nutrients' metabolism. In early Protostomians, this might have been achieved by subsets of nerve cells and mediated by the secretion of neuropeptides, for instance from the PP-fold family such as NPY (Falkmer *et al.,* 1985). In late Protostomians, like Insects, the four islet hormones are present in brain neurons (Falkmer *et al.,* 1985): these may be the evolutionary descendants of the putative earlier PP-family neuropeptide secreting cells. In this view, expression of the PP gene family would be a hallmark of the precursors to brain insulin cells during evolution, as it appears to be in the pancreas for islet β-cells during ontogeny (Herrera *et al.,* 1994; Herrera, 2000).

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