

Pancreatic duodenal homeobox-1 and islet neogenesis-associated protein: a possible combined marker of activateable pancreatic cell precursors

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Abstract

The aim of this work was to study the possible relationship between pancreatic duodenal homeobox-1 (Pdx-1) and islet neogenesis-associated protein (INGAP) during induced islet neogenesis. Pregnant hamsters were fed with (S) and without (C) sucrose, and glycemia, insulin secretion *in vitro*, and pancreas immunomorphometric parameters were measured in their 7-day-old offspring. S offspring had significantly lower glycemic levels than C animals. Insulin release in response to increasing glucose concentrations in the incubation medium (2–16 mM glucose) did not increase in pancreata from either C or S offspring. However, pancreata from S offspring released more insulin than those from C animals. In S offspring, β -cell mass, β -cell replication rate and islet neogenesis increased significantly, with a simultaneous decrease in

β -cell apoptotic rate. INGAP- and Pdx-1-positive cell mass also increased in the islets and among acinar and duct cells. We found two subpopulations of Pdx-1 cells: INGAP-positive and INGAP-negative. Pdx-1/INGAP-positive cells did not stain with insulin, glucagon, somatostatin, pancreatic polypeptide, or neurogenin 3 antibodies. The increment of Pdx-1/INGAP-positive cells represented the major contribution to the Pdx-1 cell mass increase. Such increments varied among pancreas sub-sectors: ductal>insular>extrainsular. Our results suggested that INGAP participates in the regulation of islet neogenesis, and Pdx-1/INGAP-positive cells represent a new stem cell subpopulation at an early stage of development, highly activateable in neogenesis.

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Introduction

The mass of pancreatic β -cells is controlled by two opposite processes: while β -cell mass expansion depends on β -cell replication and islet neogenesis (Bonner-Weir 2000), β -cell apoptosis (Shafir *et al.* 1999) leads to the opposite effect.

During embryonic gestation, differentiation into glucagon-, somatostatin-, pancreatic polypeptide (PP)-, and insulin-expressing cells is controlled by the expression of transcription factors such as pancreatic duodenal homeobox-1 (Pdx-1), neurogenin 3 (Ngn-3), NK homeobox 6.1 (Nkx 6.1), and others (Edlund 1998, Gradwohl *et al.* 2000, Jensen *et al.* 2000, McKinnon & Docherty 2001). At late fetal gestation and early neonatal life most new β -cells are formed by the process of neogenesis (Bouwens *et al.* 1994, Kaung 1994). In adult life, β -cells are highly differentiated, with a low proliferative capacity that decreases with age (Svenne 1983, Bouwens & Kloppel 1996, Montanya *et al.* 2000), and neogenesis is difficult to detect (Wang *et al.* 1995). However, neo-

genesis can be observed in animal models where pancreas regeneration has been induced by a variety of procedures such as alloxan and streptozotocin injection (Fernandes *et al.* 1997), partial pancreatectomy (Sharma *et al.* 1999), duct ligation (Wang *et al.* 1995), or cellophane wrapping of the pancreas head (Rosenberg *et al.* 1983). In most of these conditions there is an increased proliferation of isolated duct cells concomitant with the expression of Pdx-1, also known as IDX-1, IPF-1, STF-1 and GSF (Karlsson *et al.* 1987, German *et al.* 1995, McKinnon & Docherty 2001). However, little is known about the source of these putative ductal stem cells and their regulation.

We have previously shown that sucrose administration to normal young hamsters induced an increase in β -cell mass, ascribed to a simultaneous increase in β -cell replication rate and islet neogenesis (Del Zotto *et al.* 1999, 2000). These changes were more marked in the offspring of hamsters fed with sucrose during pregnancy (Gagliardino *et al.* 2000). In both circumstances, the increase in β -cell mass was accompanied by a significant increase in islet neogenesis-associated protein

(INGAP)-positive cell mass (Del Zotto *et al.* 2000). Thus, we have hypothesized that INGAP is involved in sucrose-induced islet cell growth and differentiation.

In an attempt to see whether Pdx-1 and INGAP could be related during the active process of islet neogenesis, we have studied the changes occurring in insulin secretion, β -cell-, Pdx-1-, and INGAP-positive cell mass, and in β -cell apoptotic rate, in offspring from normal hamsters receiving 10% sucrose in the drinking water during pregnancy.

Materials and Methods

Animals

Pregnant Syrian hamsters weighing around 60 g (8 weeks old) were housed in a temperature-controlled room (23 °C) with a fixed 12 h light:12 h darkness cycle (lights on 0600–1800 h). At day 1 of pregnancy (defined as the morning of the discovery of a vaginal plug), they were randomly divided into two groups: the control group (C) had free access to a standard commercial diet and tap water, whereas the treated group (S) received the same diet plus 10% sucrose in the drinking water. After delivery (lactating period), both groups of animals were maintained on their respective regimens, and their offspring were killed by decapitation 7 days after birth. Water intake was measured daily in both groups, while the body weight of each offspring was recorded at the time of death.

Blood glucose

Serum glucose levels were measured in every offspring at the time of death (around 0900 h) using the Medisense glucometer (Abbott Laboratories Company, Bedford, MA, USA).

Insulin secretion studies

Due to the small amount of tissue available in 7-day-old animals, we incubated minced pancreata rather than

isolated islets (Massa *et al.* 1997). Briefly, two small pieces of minced pancreas were dropped into a plastic tube (0.7 cm diameter) with a permeable bottom (Whatman filter-paper disk stuck to the walls of the tube); this tube was inserted into another (1.0 cm internal diameter) containing Krebs–Ringer bicarbonate buffer, 400 U/ml aprotinin, and different concentrations of glucose (2, 8, and 16 mM). The pieces of pancreas were then incubated for 60 min at 37 °C; at the end of this period, the inner tube containing the pieces of pancreas was removed, allowed to settle overnight in an oven at 37 °C, and then weighed. The incubation medium was collected for insulin determination by radioimmunoassay (Herbert *et al.* 1965). For this procedure, we used an antibody against rat insulin and rat insulin standard (Linco Research Inc., St Charles, MI, USA), and highly purified porcine insulin labeled with ¹²⁵I (Linde *et al.* 1980). Parallel tracer displacement curves were obtained using either the rat insulin standard, or partially purified insulin extracted from hamster pancreata.

Immunohistochemical studies

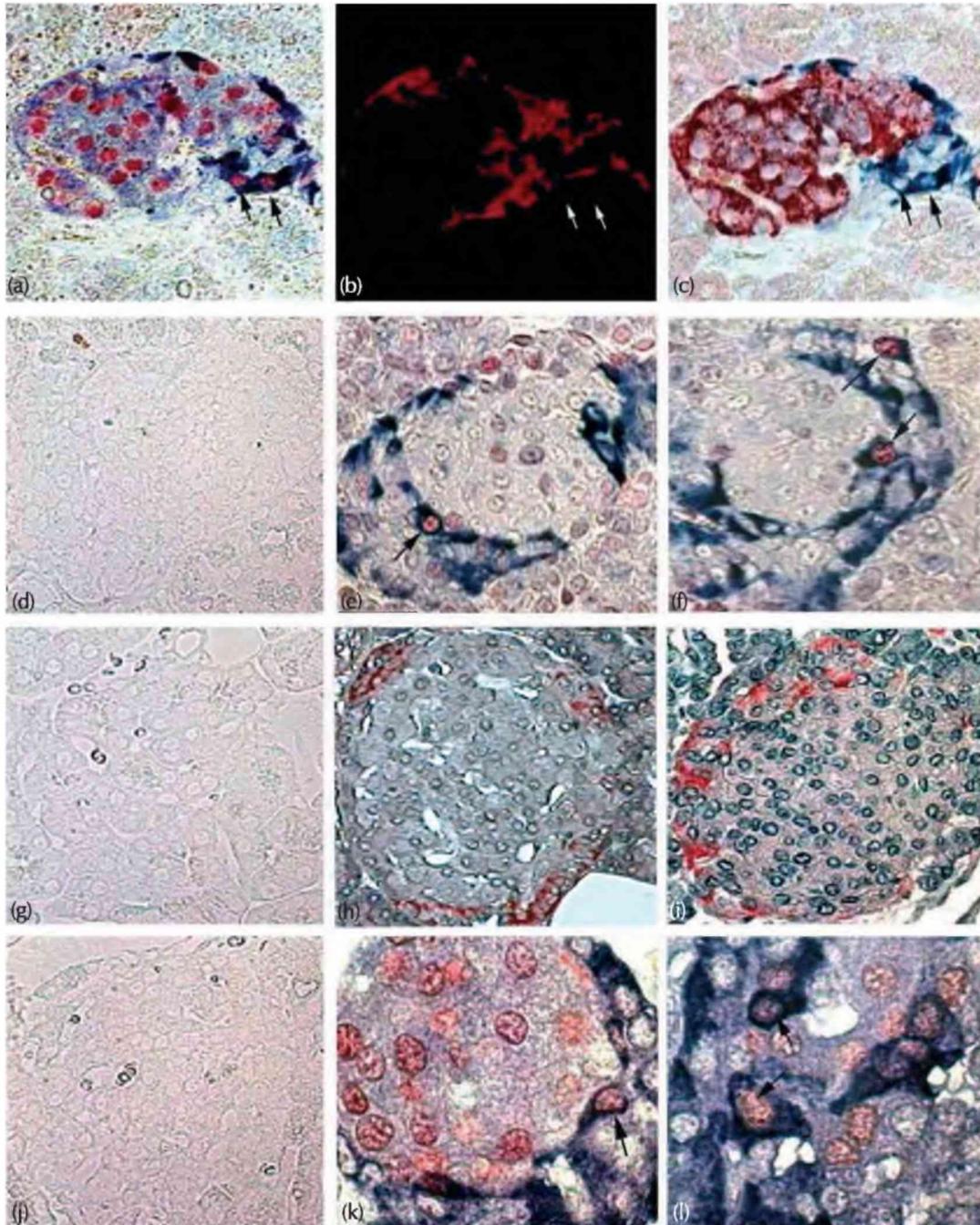
The whole pancreas was removed and its wet weight was recorded. The pancreas was then fixed in Bouin's fluid and embedded in paraffin. Serial sections of fixed pancreas (5 μ m) were obtained from three different levels of the blocks with a rotatory microtome, and mounted on silanized slides (3-amino-propyltriethoxy-silane; Sigma Co., St Louis, MO, USA) (Hsu *et al.* 1981). Sections were deparaffinized, incubated for 30 min in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity, and rehydrated in a descending ethanol series, followed by an incubation in 2.5% porcine serum to reduce non-specific binding. The slides were then incubated for 24 h at 4 °C in a humidified chamber with different appropriately diluted primary antibodies. For the final staining of all these cell types, we used the streptavidin–biotin complex (1:40 and 1:20 respectively; Sigma), or alkaline phosphatase (1:40; Sigma). The incubation period for this step–procedure was 30 min with the secondary biotinylated antibodies or alkaline phosphatase.

Figure 1 (opposite) (a) Paraffin section of a C pancreas treated with antibodies against INGAP and Pdx-1. Pdx-1-positive cells can be seen in islet β -cells. Arrows show INGAP (blue cytoplasm)/Pdx-1-positive (red nucleus) cells in islet non- β -cells (\times 200). (b) The same islet section, stained with glucagon antibody and Texas red. INGAP/Pdx-1-positive cells (arrows) did not react with the glucagon antibody (\times 200). (c) The same islet section as in (a) and (b), stained with insulin antibody and peroxidase–carbazole. In this case, the Pdx-1 antibody was not used to avoid stain overlapping. INGAP/Pdx-1-positive cells (arrows) did not react with the insulin antibody (\times 200). (d) Negative control of combined immunostaining by omission of the first antibody (PCNA and INGAP). (e) C offspring pancreas section treated with INGAP (blue) and PCNA (red) antibodies. The arrow shows a cell stained positively with both antibodies at the islet periphery (\times 200). (f) S offspring pancreas section treated with INGAP (blue) and PCNA (red) antibodies. The arrows show two cells stained positively with both antibodies at the islet periphery (\times 200). (g) Negative control of immunostaining by omission of the first antibody (CK). (h) C offspring pancreas section treated with the CK 19 (clone 4-62; Sigma) antibody (peroxidase–carbazole) counterstained with hematoxylin. Scanty CK-positive cells are seen at the islet periphery (\times 100). (i) S offspring pancreas section treated with the CK 19 (clone 4-62; Sigma) antibody (peroxidase–carbazole) counterstained with hematoxylin. Numerous CK-positive cells are seen at the islet periphery (\times 100). (j) Negative control of combined immunostaining by omission of the first antibody (INGAP and Pdx-1). (k) C offspring pancreas section treated with antibodies against INGAP and Pdx-1. The arrow shows INGAP (blue cytoplasm)/Pdx-1-positive (red nucleus) cells in the periphery of the islet (\times 400). (l) S offspring pancreas section treated with antibodies against INGAP and Pdx-1. The arrows show INGAP (blue cytoplasm)/Pdx-1-positive (red nucleus) cells in the islet (\times 400).

Cell replication rates

We performed sequential double staining for proliferating cell nuclear antigen (PCNA; 1:4000; Sigma) and β -cells (1:20 000; our own guinea pig-insulin antibody), PCNA and non- β -cells with a mixture of three different rabbit antibodies against somatostatin (1:6000; DAKO, Glostrup, Denmark), glucagon (1:400; Peninsula Laboratories,

Belmont, CA, USA), and PP (1:10 000; Novo Nordisk A/S, Bagsvaerd, Denmark), and PCNA and INGAP cells with rabbit INGAP antibody (IgG1246; 1:600; Eli Lilly Co., Indianapolis, IN, USA). First, we performed PCNA staining as described above using carbazole as chromogen; the same section was then immunostained for β -, non- β - and INGAP cell identification as already described, except



that alkaline phosphatase and fast blue (Sigma) were used as chromogens. Alkaline phosphatase conjugate was applied to each section for 30 min at room temperature; the sections were then washed, and alkaline phosphatase substrate was applied for another 30 min. Sections were further washed and mounted in aqueous medium (DAKO). Within a given cell type, the replication rate was quantified and expressed as the percentage of PCNA-labeled cells among the total β -, non- β -, and INGAP cells counted (no less than 3000 each). On the other hand, at ductal level immunostained cells (PCNA and INGAP) were expressed as the number of positive cells per area (mm^2). Immunocytochemical stainings have been validated in previous studies (Del Zotto *et al.* 1999, 2000). Further, for every immunostaining, simple controls were done by omitting the primary antiserum.

Apoptotic rate

To identify apoptotic bodies, the propidium iodide technique was used (Scaglia *et al.* 1997). For this purpose, deparaffinized and rehydrated sections were washed in phosphate-buffered saline before incubation for 30 min in a dark humidified chamber with a solution of propidium iodide (4 $\mu\text{g}/\text{ml}$; Sigma) and ribonuclease A (100 $\mu\text{g}/\text{ml}$; Sigma). A Zeiss AxioLab epifluorescence microscope equipped with an HBO50 mercury lamp, together with two different filters, was used for visualization of auto-fluorescent labeling (propidium iodide) to detect apoptotic bodies. Glucagon immunofluorescence staining (fluorescein isothiocyanate; FITC) was used to reveal peripheral islet cells.

For quantitative evaluation, positively labeled apoptotic endocrine cells were counted under a $\times 40$ objective lens in sections obtained from different levels of the blocks. The number of apoptotic cells was expressed as the percentage of the total number of β - and non- β -cells counted.

Cytokeratin (CK) immunostaining

For antigen retrieval, deparaffinized sections were pretreated with 250 ml antigen-retrieval solution (Vector Laboratories, Burlingame, CA, USA) for 10 min in a 500 W microwave oven (Madsen *et al.* 1997). To reveal the presence of CK-positive cells, we used a panspecific cocktail of antibodies against human CK clone AE1-AE3 (DAKO) and CK 19 (Sigma clone 4:62).

Detection of Pdx-1- and INGAP-positive cells

Sequential double staining for Pdx-1 and INGAP detection in pancreatic cells was as follows. We first stained Pdx-1 cells with the Pdx-1 antibody (1:1200; kindly provided by Dr C Wright, Department of Cell Biology,

Vanderbilt University, Nashville, TN, USA), and revealed them as described above using carbazole as chromogen; the same section was then immunostained with the INGAP antibody (1:250), except that alkaline phosphatase and fast blue (Sigma) were used as chromogens. Later, the percentage of cells expressing separately or co-expressing these two factors was quantified within each subsector of the pancreas, i.e. islet, extrainsular, and duct cells (no less than 1000 each). In addition, glucagon (fluorescein) and somatostatin (Texas red) were used to reveal co-expression of these hormones with Pdx-1 and INGAP.

Detection of Ngn-3-positive cells

Deparaffinized sections pretreated for antigen retrieval (see above) were incubated for 24 h at 4 °C in a humidified chamber with anti-rabbit Ngn-3 antibody (1:2000, provided by Dr M German, University of California, San Francisco, CA, USA). The final staining was accomplished with the streptavidin-biotin complex (1:40 and 1:20 respectively; Sigma). The incubation period for this step-procedure was 30 min with the secondary biotinylated antibodies, and they were revealed using carbazole as chromogen. Sections of normal mouse (gestational age, 15.5 days; E15.5) were used as positive controls.

Morphometrical analysis

The morphometrical analysis was performed by videomicroscopy using a Jenamed 2 Carl Zeiss light microscope and an RGB CCD Sony camera in combination with OPTIMAS software (Bioscan Incorporated, Edmons, WA, USA). We measured the following parameters: total pancreas area excluding connective tissue; endocrine pancreas area; exocrine pancreas area; β - and non- β -cell area; number of β - and non- β -cells; the areas and number of cells with and without Pdx-1/INGAP co-expression; the number of islets per unit area; and islet volume. We also estimated the volume density of β - and non- β -cells, Pdx-1/INGAP and Pdx-1 cells, as well as the size of β - and non- β -cells. To estimate islet β -cell, non- β -cell, INGAP/Pdx-1, extrainsular, ductal, and CK mass, we multiplied the respective volume densities by the total weight of the pancreas (Bonner-Weir & Smith 1994). We also estimated the relationship between the islets and duct cells, expressed as a percentage of the total number of islets in contact with ducts (Bertelli *et al.* 2001).

Statistical analysis

Quantitative data are expressed as means \pm S.E.M. The statistical significance was determined by Student's *t*-test. A *P* value of <0.05 was considered statistically significant.

Results

Body weight, daily food ingestion and water intake of pregnant hamsters

Comparable body weights were recorded in C and S pregnant hamsters at the end of the experiments (78.3 ± 7.2 vs 76.1 ± 8.6 g). S animals drank a significantly larger volume of water than C animals (16.0 ± 0.7 vs 10 ± 0.1 ml/day; $P < 0.005$). Equivalent amounts of food (and caloric intake) were consumed daily by C and S hamsters (17 ± 2 vs 14 ± 1 g, and 79.0 ± 0.9 vs 79.9 ± 1 Cal respectively). Due to the presence of sucrose in the drinking water, the S group received a qualitatively different daily intake of nutrients as compared with C animals (carbohydrates:proteins:lipids; 66:26:8 vs 45:43:12 respectively).

Body weight and pancreas weight of the offspring

Significant differences were recorded in the body weight of C vs S offspring (7.3 ± 0.3 vs 8.4 ± 0.2 g ($n = 25$); $P < 0.005$). In contrast, no differences in pancreas weight were recorded between the two groups (19 ± 0.9 vs 20.8 ± 0.5 mg ($n = 25$)).

Serum glucose levels in the offspring

Similar fasting serum glucose levels were found in C and S normal adult hamsters (128 ± 7 mg/dl). While values from C offspring were in the same range as those measured in adult hamsters, they were significantly lower in S offspring (128 ± 3.4 vs 88.5 ± 3.8 mg/dl ($n = 16$) respectively; $P < 0.001$).

Insulin released in vitro by pancreas pieces from the offspring

The release of insulin in pancreata from either C or S offspring did not increase in response to increasing glucose concentration in the incubation medium (2, 8, and 16 mM glucose). However, the pieces of pancreas from S offspring released more insulin than did those from C ones (C vs S, $n = 18$) at any concentration tested: 2 mM glucose: 0.8 ± 0.2 vs 1.7 ± 0.4 , $P < 0.025$; 8 mM glucose: 0.5 ± 0.2 vs 1.8 ± 0.4 , $P < 0.02$; 16 mM glucose: 0.7 ± 0.1 vs 1.7 ± 0.4 ng/mg wet weight ($P < 0.025$).

Morphological studies

In both groups, pancreatic islets of variable shape and size showed β -cells occupying the central zone, and non- β - and INGAP-positive cells located at the islet periphery. The exocrine pancreas was composed of characteristic serous acini surrounded by loose connective tissue. The ducts were lined by cubic or cylindrical cells, embedded in dense connective tissue.

A small number of ductal cells reacted positively with the insulin-specific antibody and with the pooled mixture

of antisera against non- β -cell hormones. In the islets, while Pdx-1-positive cells were located in the central zone, INGAP-positive cells were only seen at the islet periphery (Fig. 1a and c).

There were two subpopulations of Pdx-1-positive cells, only one of which co-stained with INGAP. The Pdx-1/INGAP-positive cells did not exhibit positive staining with anti-insulin, glucagon (Fig. 1b and c), somatostatin, or PP antibodies (Fig. 2). Conversely, cells only positive for Pdx-1 stained positively with insulin, while 40% of INGAP-positive cells co-stained with glucagon antibodies (Del Zotto *et al.* 2000). Neither of these two subpopulations of Pdx-1-positive cells reacted with the Ngn-3 antibody in either group of animals (Fig. 3). Pancreata from C and S offspring showed Pdx-1/INGAP-positive cells in the islets (Fig. 1k and l), as well as at extrainsular (Fig. 4b and c) and ductal (Fig. 4e, f, and g) levels.

PCNA antibody labeled nuclei from exocrine-, ductal-, β -, and INGAP-positive cells in C and S offspring (Fig. 1e and f).

CK-positive cells were depicted at the islet periphery (Fig. 1h and i), being more abundant in S islets (Fig. 1i: 320 ± 20 vs 190 ± 10 μ g; $P < 0.04$), and in close proximity to duct cells (Fig. 4i).

Morphometrical studies

Pancreatic β -cell mass increase in S offspring (Table 1) was 114% ($P < 0.005$), with a concomitant 59% increase in the PCNA index of these cells ($P < 0.02$), and a 350% decrease in β -cell apoptotic rate ($P < 0.01$). Apoptotic β -cells stained with propidium iodide were not accompanied by any detectable inflammatory reaction. Both exocrine and duct cells with fragmented or condensed nuclei were also observed, but they were not quantified.

There were no marked differences between C and S offspring either in non- β -cell mass (260 ± 10 vs 250 ± 10 μ g) or in the percentage of PCNA-labeled non- β -cell nuclei (1.33 ± 0.03 vs $1.3 \pm 0.2\%$).

CK-positive cell mass increased significantly in pancreata from S offspring (320 ± 20 vs 190 ± 10 μ g; $P < 0.04$). These animals also showed a significant increase in the number of PCNA-reacting ductal cells (S vs C: 11.9 ± 0.8 vs 5.8 ± 1.6 cells/mm²; $P < 0.002$). Taking advantage of the fact that ductal cells stained with the CK antibody, we counted the percentage of islets in close contact with these cells, and showed that sucrose treatment increased the percentage of such cells significantly (88.6 ± 5.0 vs 64.0 ± 7.2 ; $P < 0.02$) (Fig. 4h and i).

The mass of INGAP-positive cells increased significantly in the islet, the extrainsular, and the ductal pancreas compartments in S offspring (Table 2). The magnitude of such increases was different in each level: ducts, 350%; islet, 300%; extrainsular, 86%. The PCNA index of overall INGAP-positive cells also increased by 168% ($P < 0.002$).

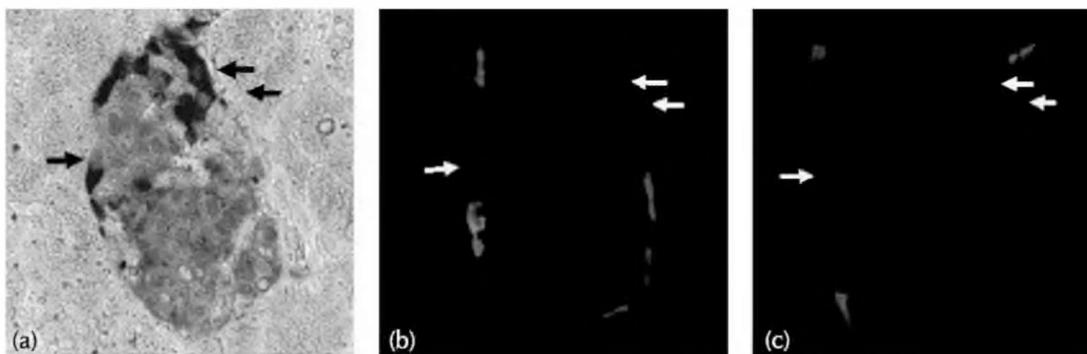


Figure 2 (a) Paraffin section of a C pancreas treated with antibodies against Pdx-1 and INGAP. The islet shows Pdx-1 localization in β -cells (center), while the INGAP (black cytoplasm)/Pdx-1- (gray nucleus) positivity (arrows) is only seen in cells located in the periphery of the islet ($\times 200$). (b) The same section as in (a) treated with an antibody against somatostatin, detected by FITC (arrows show no INGAP/Pdx-1 colocalization) ($\times 200$). (c) The same section as in (a) treated with an antibody against PP, detected by Texas red (arrows show no INGAP/Pdx-1 colocalization) ($\times 200$).

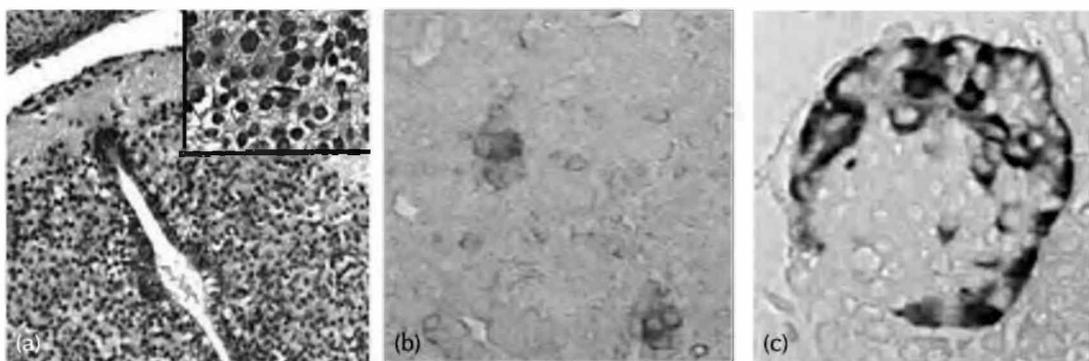


Figure 3 (a) Paraffin section of medulla spinalis from an E15.5 mouse (control staining) treated with Ngn-3 antibody and revealed by carbazole. The black nucleus represents Ngn-3-positive cells ($\times 100$). Inset: section of the slide at a higher magnification ($\times 400$). (b) Paraffin section of the pancreas from an E15.5 mouse sequentially treated with Ngn-3 and INGAP antibodies, stained with phosphatase-fast blue (Ngn-3) and peroxidase-carbazole (INGAP). While no Ngn-3-positive nuclei are depicted in the pancreas, some INGAP-positive cells can be seen within small islets ($\times 100$). (c) Paraffin section of a hamster offspring treated as in (b). No Ngn-3-positive nuclei are seen in the pancreas, while a ring of INGAP-positive cells is seen in the islet ($\times 200$).

Most of the Pdx-1-positive cells were located at islet level (98%), with a smaller proportion at extrainsular (1.8%) and ductal (0.2%) level. Sucrose administration induced an overall 111% increase in the mass of Pdx-1-positive cells, from 886 ± 103.2 to $1872 \pm 205 \mu\text{g}$ ($P < 0.005$; Table 3), with an uneven increment in each subsector: ductal, 350% ($P < 0.001$); islet, 112% ($P < 0.005$); extrainsular, 69% (not significant).

The two subpopulations of Pdx-1-positive cells (Pdx-1/INGAP-positive and Pdx-1/INGAP-negative) participated in a significantly different manner in the sucrose-induced increase of Pdx-1-positive cell mass, particularly when considering their distribution at the three pancreas compartments. Comparing Pdx-1/INGAP-positive vs Pdx-1/INGAP-negative cells, their mass increased 500 vs 200% at ductal level, 114 vs 114% in

the islets, and 100 vs 17% at extrainsular level (Fig. 5). Similar changes were observed when the number of Pdx-1 cells instead of their mass was considered (Table 4). These results suggested that, except for the islets, the sucrose-induced increase of the Pdx-1 cell mass mainly affected the Pdx-1/INGAP-positive cell subpopulation.

Discussion

Sucrose administration to young normal hamsters for up to 24 weeks did not significantly affect either body weight or glycemic levels (Del Zotto *et al.* 1999, 2000). Similarly, no significant differences in these two parameters were recorded in the mothers of C and S offspring at the time of delivery. Conversely, 7-day-old S offspring were

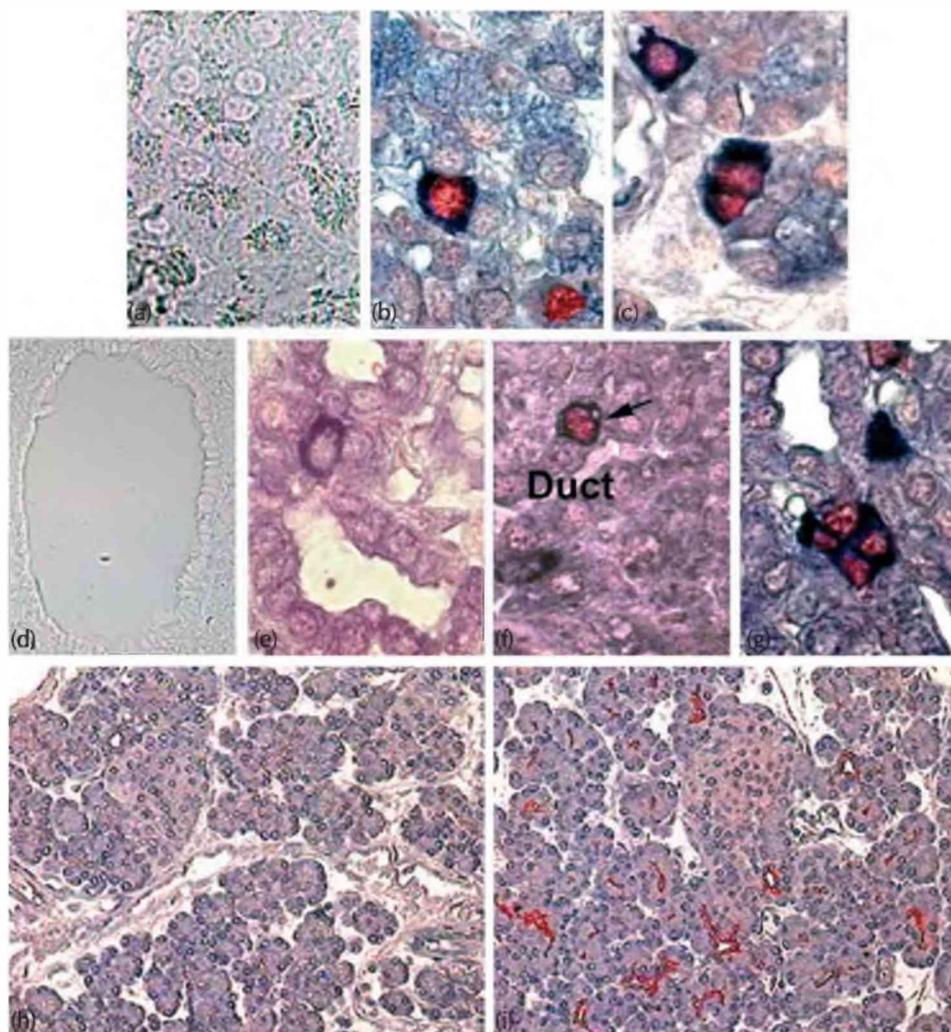


Figure 4 (a) Negative control of combined immunostaining of extrinsular cells by omission of the first antibody (INGAP and Pdx-1). (b) C offspring pancreas section double treated with INGAP (blue) and Pdx-1 (red) antibodies. Note an extrinsular cell reacting positively with INGAP (blue cytoplasm) and Pdx-1 (red nucleus) ($\times 400$). (c) S offspring pancreas section double treated with INGAP (blue) and Pdx-1 (red) antibodies. Three extrinsular cells reacting positively with INGAP (blue cytoplasm) and Pdx-1 (red nucleus) can be seen ($\times 400$). (d) Negative control of ductal cells obtained by omission of the first antibody (Pdx-1 and INGAP). (e) C offspring pancreas section double treated with INGAP and Pdx-1 antibodies. A single INGAP-positive ductal cell (blue cytoplasm) can be seen ($\times 400$). (f) C offspring pancreas section double treated with INGAP (blue) and Pdx-1 (red) antibodies. Note one INGAP-positive (blue cytoplasm) and Pdx-1-positive (red nucleus) ductal cell ($\times 400$). (g) S offspring pancreas section double treated with INGAP (blue) and Pdx-1 (red) antibodies. Four INGAP-positive (blue cytoplasm) and Pdx-1-positive (red nucleus) ductal cells can be seen ($\times 400$). (h) C offspring pancreas section stained with the CK antibody (pool against CKs AE₁–AE₃). No positive CK reaction can be detected either at ductal or islet level ($\times 100$). (i) S offspring pancreas section stained with the CK antibody (pool against CKs A-1/A-3). Note CK-positive duct cells in close contact with the islet ($\times 100$).

significantly heavier and had significantly lower serum glucose levels than C offspring. The greater release of insulin displayed *in vitro* by pancreata from S offspring, together with their inability to differentiate increasing glucose concentrations in the incubation media, could account for the above-mentioned changes. Such inability

of C and S offspring to recognize different glucose concentrations in the incubation media was not totally unexpected, since in normal hamsters a typical glucose: insulin secretion curve is obtained at 8 weeks, whereas maximal insulin release is only attained at 24 weeks of age (Massa *et al.* 1997).

Table 1 Morphometrical changes induced in the β -cell area of offspring from sucrose-fed hamsters during pregnancy. Each value represents the mean of three animals and three section levels in each group \pm S.E.M.

Group	β -cell mass (μ g)	PCNA β -cells (%)	Apoptotic index (%)
Control	700 \pm 100	2.9 \pm 0.3	0.9 \pm 0.01
Sucrose	1500 \pm 200 ^a	4.6 \pm 0.1 ^b	0.2 \pm 0.01 ^c

^a $P < 0.005$; ^b $P < 0.001$; ^c $P < 0.01$.**Table 2** Morphometrical changes induced in INGAP-cell mass in the three pancreatic subsectors. Values are the means of three animals and three section levels in each group \pm S.E.M.

Group	INGAP-cell mass (μ g)			PCNA of INGAP+ cells (%)
	Insular	Extrainsular	Ductal	
Control	100 \pm 2	14 \pm 4	2 \pm 0.2	2.5 \pm 0.2
Sucrose	400 \pm 2 ^a	26 \pm 2	9 \pm 0.1 ^a	6.7 \pm 0.9

^a $P < 0.000001$.**Table 3** Compartmentalization of Pdx-1-positive cell mass. Each value represents the mean of three animals and three section levels in each group \pm S.E.M.

Subsector	Control	Sucrose	Increase (%)
	Insular (μ g)	868 \pm 100 (98%)	
Extrainsular (μ g)	16 \pm 3 (1.8%)	27 \pm 4 (1.4%)	69
Ductal (μ g)	2 \pm 0 (0.2%)	9 \pm 1 (0.6%) ^b	350

^a $P < 0.005$; ^b $P < 0.001$.

Offspring from S hamsters showed a significant 114% increase in the mass of β -cells, conditioned by a simultaneous increase in the replication rate of β -cells (59%), a decrease in β -cell apoptotic rate (350%), and increased islet neogenesis. Objective evidence supports the occurrence of the latter process, namely, increased CK-positive cell mass (Bouwens *et al.* 1994), islet percentage in close contact with the ducts (Bertelli *et al.* 2001), ductal cell replication rate (Sharma *et al.* 1999), and Pdx-1-positive cell mass (McKinnon & Docherty 2001).

The strong concomitant changes observed in INGAP cell mass and replication rate would also indicate that INGAP could be effectively involved in the regulation of such neogenetic processes rather than being simply casually related. The fact that sucrose administration only induced an increase in INGAP cell mass when islet neogenesis occurred (Del Zotto *et al.* 2000), together with our recent report of its transcription at islet level (Flores *et al.* 2003) would reinforce this assumption.

Sucrose administration to 8-week-old hamsters induced a significant increase in glucose-induced insulin secretion, β -cell mass (Del Zotto *et al.* 1999), and INGAP-positive cell mass (Del Zotto *et al.* 2000), but not in β -cell apoptotic rate or in the replication rate of ductal or islet INGAP-positive cells, as it currently occurred in S offspring. These facts might indicate that sucrose administration at this early stage of pancreatic development affects all the processes involved in pancreas remodeling and insulin secretion.

Although Pdx-1 plays an important role in the development of the pancreas and is expressed in pancreatic duct, endocrine, and acinar cells, its distribution becomes more restricted in the mature pancreas (Stoffers *et al.* 2000, McKinnon & Docherty 2001). Using different experimental approaches and detection methodologies, however, other authors have also found a concomitant increase of islet neogenesis and Pdx-1 in adult life: Sharma *et al.* (1999) measured a fivefold increase of Pdx-1 protein levels (Western blot analysis) in rat islets 3 days after partial pancreatectomy. At that period, most epithelium cells of the common pancreatic duct stained positively for Pdx-1, this effect being preceded by an increase in the replication rate of duct cells. An increase in islet size together with a three- to fourfold increase in the expression of Pdx-1 of total pancreatic protein (Western blot analysis) was also reported during a 2-week glucagon-like peptide-1 administration to control non-diabetic and db/db mice (Stoffers *et al.* 2000). Using semiquantitative fluorescence immunocytochemical analysis, the authors also showed an approximately sevenfold increase of Pdx-1 expression in exocrine tissue, but not within the islet in control non-diabetic mice treated with exendin-4 for 2 weeks. None of these authors reported the presence of transcription factors other than Pdx-1. Despite these reports on the association between Pdx-1 and islet neogenesis in postnatal life, our results represent the first quantitative determination of mass increase of these cells, and of their compartmentalization in the pancreas, concomitant with a process of postnatal islet neogenesis induced by dietary manipulation during fetal development.

Our report provides additional novel data regarding Pdx-1-positive cells and islet neogenesis: (1) a different increment of these cells at the three pancreas compartments (with the main increase occurring in ductal cells), and (2) the existence of two cell subpopulations, Pdx-1/INGAP-positive and Pdx-1/INGAP-negative, the former being those that underwent the highest increase (cell mass and number) in S animals, indicating their greater capacity to react in response to an increased insulin demand.

While some cells showing positivity for either Pdx-1 or INGAP also stained with insulin and glucagon respectively (Del Zotto *et al.* 2000), Pdx-1/INGAP-positive cells were neither stained with insulin, glucagon, somatostatin, and PP antibodies, nor with the Ngn-3 antibody. Thus, based on these two conditions (absence of hormone

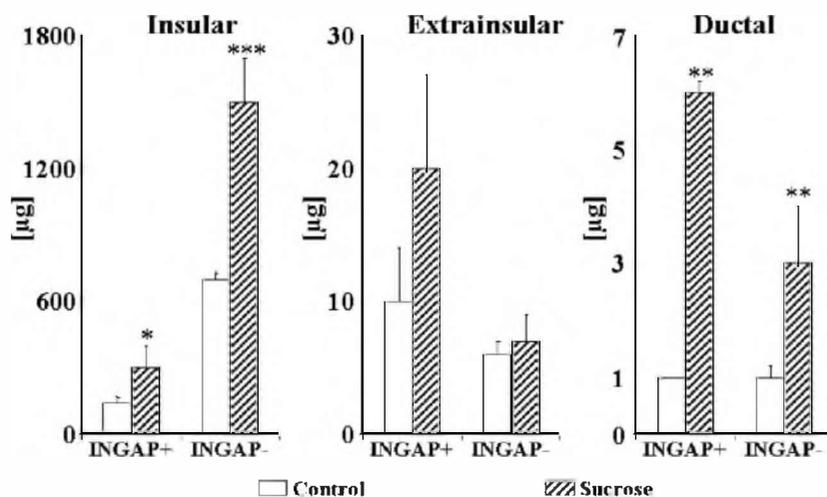


Figure 5 Changes induced by sucrose-feeding in Pdx-1-positive cells. Each value represents the mean of Pdx-1-cell mass \pm S.E.M. of three animals from each group and from two different levels of each paraffin block. *** $P < 0.005$; ** $P < 0.02$; * $P < 0.03$.

Table 4 Number of Pdx-1/INGAP-positive and Pdx-1/INGAP-negative cells. Each value represents the mean number of cells/area (10 mm²) from three animals and three different section levels \pm S.E.M.

Group	Insular			Extrainsular			Ductal		
	Pdx-1/ INGAP+	Pdx-1/ INGAP-	Total	Pdx-1/ INGAP+	Pdx-1/ INGAP-	Total	Pdx-1/ INGAP+	Pdx-1/ INGAP-	Total
Control	14 \pm 4.5	55 \pm 18.5	69 \pm 12	7.0 \pm 2.2	6.8 \pm 0.9	13.8 \pm 1.5	1.0 \pm 0.8	1.0 \pm 0.2	2.0 \pm 0.5
Sucrose	33 \pm 4.3 ^a	83 \pm 9.3	116 \pm 10	20 \pm 3.9 ^a	3.0 \pm 0.9	23 \pm 2.35	5.0 \pm 1.3 ^a	1.0 \pm 0.2	6.0 \pm 0.5

^a $P < 0.02$.

Total number of cells counted in each C and S pancreas subsector: insular, 2743 and 2995; ductal, 1003 and 1974; extrainsular, 486 and 505 respectively.

immunoreactivity and high growth reactivity), we have assumed that these cells represent a subpopulation of islet cell precursors at an early stage of development.

According to Grapin-Botton *et al.* (2001), the absence of Ngn-3 in these precursor cells could represent an advantage since it offers the chance to differentiate towards any of the islet cells according to demand. However, it is unlikely that Pdx-1 expression in all non-islet cells is sufficient *per se* to cause differentiation into endocrine cells (Habener & Stoffers 1998). The presence of other differentiation factors (INGAP?), and/or perhaps a specific topography within the pancreatic parenchyma (proximity with ductal structure and/or mature islets) may allow some Pdx-1-positive cells to progress towards a fully differentiated β -cell phenotype (Perfetti *et al.* 2000). In any case, our results suggest that INGAP would be a marker of a subpopulation of Pdx-1-positive cells that will undergo a more active differentiation in situations where neogenesis is stimulated.

We cannot explain at the moment the mechanism for any possible interaction between Pdx-1 and INGAP. Since complex phosphorylation and nuclear translocation

are steps involved in Pdx-1 activation (Stoffers *et al.* 2000), they could be alternative pathways where INGAP exerts its hypothetical effect.

The morphological and functional changes induced in the pancreas of normal adult rats and hamsters by sucrose ingestion has been ascribed to an insulin resistance state (Lombardo *et al.* 1996, Del Zotto *et al.* 1999). However, the main mechanism triggering the low tissue response to insulin in S hamsters is not completely clear. In the current experiment, despite the fact that the offspring were not fed directly with sucrose, they developed even more marked functional and morphological changes in their endocrine pancreas as compared with S adult animals. It could thus be assumed that some unidentified 'metabolic signal' (fructose?) could be present in both maternal circulation and breast milk, triggering the above-mentioned pancreatic changes.

In brief, our results have shown that the offspring from S hamsters present an apparent increased insulin secretion, and a simultaneous increase in β -cell mass. The latter was accompanied by an increase in the replication rate of β -cells as well as of their precursor duct cells, and, in islet

neogenesis, together with a decrease in β -cell apoptotic rate. These changes were associated with an increase in INGAP-positive cell mass in the islet and duct pancreas subsectors, and in the mass of Pdx-1-positive cells, particularly those which are INGAP-positive and located in the ducts. Altogether, these results reinforce the hypothesis that INGAP participates in the regulation of pancreas growth and secretory function, allowing the gland to cope with the peripheral insulin demand. Additionally, the appearance of INGAP immunoreactivity in Pdx-1-positive cells could become a useful marker for the identification of stem cells in the pancreas, which will follow a more active differentiation in the case of neogenesis.

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