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Epithelial cell proliferation and islet neogenesis in IFN-g transgenic mice

Danling Gu and Nora Sarvetnick*

Department of Neuropharmacology CVN-10, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

*Author for correspondence

SUMMARY

We have identified a model system for the study of pancreatic islet development and regeneration in transgenic mice bearing the interferon-gamma (IFN-g) gene expressed in the pancreatic islets. Previous studies showed that the locally produced IFN-g causes lymphocyte infiltration and islet cell destruction. Here we demonstrate that new islet cells are formed continuously from duct cells as evidenced by (1) the dramatic proliferation of duct cells, (2) the appearance of primitive cells and (3) their subsequent differentiation to endocrine cells. The IFN-g induced islet neogenesis is similar to embryonic islet morphogenesis and offers a model system for studying factors modulating islet development. Additionally, the duct cells occasionally transdifferentiate to gastrointestinal-like cell types and hepatocytes. These results underscore the lymphokine's ability to initiate a complex 'transdifferentiation' pathway, providing a window for understanding lineage interrelationships within a terminally differentiated structure.

Key words: interferon-gamma, islet regeneration, transgenic mouse

INTRODUCTION

The endocrine cells of pancreatic islets of Langerhans develop from the epithelial cells of the ducts (Laguesse, 1894, 1896). The neogenesis of islets from duct epithelial cells occurs during normal embryonic development and in very early postnatal life. In the mouse, the pancreas takes on an 'adult' configuration with islets being well defined and distinct from duct structures by 2-3 weeks of age (when the animals are weaned). During the rest of adult life, no endocrine cells are observed in the ducts and very few mitotic figures are found. Additionally, the mitotic index of ducts and islet cells is very low (Githens, 1988). Therefore, the normal developmental process that generates the islets ceases following the early postnatal period. An analysis of the islet growth pattern reveals that the islet growth and the maintenance of islet mass are under strict regulatory controls (reviewed by Hellerstrom and Swenne, 1985; Hellerstrom et al., 1988).

During normal ontogeny, the first endocrine cell type observed is usually the glucagon-secreting cells (A cells) (Rall et al., 1973), although the earliest emergence of this endocrine cell type is quite controversial; in some studies, insulin- (B cells), somatostatin- (D cells), or pancreatic polypeptide-secreting cells (PP cells) are observed to appear prior to or coincidental with A cells (Grillo and Shima, 1966; Clark and Rutter, 1972; Assan and Boillot, 1973; Schaeffer et al., 1973; Dubois, 1975; Jarousse and Rosselin, 1975; Rall et al., 1979; Alpert et al., 1988; Herrera et al., 1991). Endocrine gene expression occurs prior to pancreas organogenesis, before the appearance of pancreatic diverticulum (Gittes and Rutter, 1992). The endocrine cells then appear in the duct wall (Dubois, 1975) and later form clusters. These clusters grow and become islets (Conklin, 1962; Liu and Potter, 1962; Bjorkman et al., 1966). The early embryonic endocrine cells in the duct wall transiently express tyrosine hydroxylase (TH), the first enzyme of the catecholamine biosynthetic pathway (Teitelman et al., 1981a,b, 1987; Teitelman and Lee, 1987).

Adult pancreatic beta cells are known to have a poor regenerative capacity (Lazarow, 1952). In pathogenic states where islet destruction occurs, such as insulin-dependent diabetes mellitus (IDDM), regeneration is not a noteworthy component. Regeneration has not been reported in the non-obese diabetic (NOD) mouse or the biological breeding (BB) rat, animal models of IDDM, where the islet cells are lost by presumed immunological mechanisms (Like, 1985). Furthermore, regeneration is not observed in diabetes induced by streptozotocin (Sz) injection of adult rats (Steiner et al., 1970; Logothetopoulos, 1972). In recent years, numerous transgenic mouse models for studying IDDM have appeared (Sarvetnick et al., 1988; Lund et al., 1990; Oldstone et al., 1991; Allison et al., 1992; Heath et al., 1992). These transgenic mice have achieved duplication of the various stages of human IDDM to different degrees and have provided a powerful tool in elucidating mechanisms that lead to IDDM. However, to our best knowledge, regeneration of islet cells has not been reported among these many transgenic mouse strains except in the very recently published report that a tumor necrosis factor

(TNF) transgene is linked to an insulin promoter (Higuchi et al., 1992). The transgene, TNF, causes insulitis and disorganization of islets and proliferation of pancreatic ductules, but little regeneration of endocrine cells.

Limited islet regeneration has been reported in several experimental conditions. One of the best documented is following partial pancreatectomy of young rats (Setalo et al., 1972; Bonner-Weir et al., 1981, 1983). Limited regeneration has also been reported following ligation of pancreatic arteries (Adams and Harrison, 1953), in steroid diabetes (Kern and Logothetopoulos, 1970), following injection of insulin antibody (Logothetopoulos and Bell, 1966), after alloxan administration (Johnson, 1950; Hughes, 1956; House, 1958; Bunnag et al., 1967; Boquist, 1968; Korcakova, 1971) and after cellophane wrapping (Rosenberg and Vinik, 1989). More impressive regeneration can be found in newborn and neonatal animals following islet destruction. When newborn rats are treated with Sz, the pancreas is able to repair itself (Portha et al., 1974, 1979; Cantenys et al., 1981; Dutrillaux, 1982). Two mechanisms account for the appearance of these new islet cells. In one system, the islets are regenerated from division of existing terminally differentiated islet cells (Jacob,1977). In another system, the new islet cells are thought to be generated by differentiation from duct epithelial cells (Cantenys et al.,1981). Since the islets originate from duct epithelial cells during development, the latter case is considered to be a recapitulation of the embryonic events. In systems showing regeneration from duct epithelial cells, the experimental animals are quite young, usually neonates and occasionally up to 4 weeks of age. It is possible that substantial regeneration only occurs in neonates since the precursor pool is still present. These islet progenitor cells do not remain in the adult animal and are lost in early life.

Occasionally, transdifferentiation to nonpancreatic cell types is found. In the db/db mouse proliferating duct cells were noted to give rise to ciliated cells, mucous or Paneth secretory cells (Like and Chick, 1970a,b). In rats, a copper-deficient diet leads to the development of pancreatic hepatocytes (Scarpelli and Rao, 1981; Rao et al., 1989). Thus cells within the pancreas may retain the potential to differentiate to nonpancreatic cell types under certain conditions (Rao et al., 1990; Reddy et al., 1991).

We report in this paper that a transgenic mouse strain with marked pancreatic inflammation exhibits extraordinary islet cell regeneration which originates from proliferating duct cells. This mouse strain carries the mouse IFN-g gene linked to the human insulin promoter and exhibits inflammatory-induced islet loss (Sarvetnick et al., 1988, 1990). The inflammatory lesions are replaced by numerous ducts of various sizes, which are not found in the islets of BALB/c or diabetic NOD mice. We provide evidence by immunolabeling and electron microscopic (EM) techniques that these duct cells proliferate and are progenitors of new islet cells. The ins-IFN-g transgenic mice, with their remarkable ability to regenerate new endocrine cells, provide a model system for understanding factors that regulate B cell growth and development in vivo.

MATERIALS AND METHODS

Histology and immunocytochemistry

Tissues were fixed in Zn-formalin (10%) or in Bouin's fixative and processed for paraffin embedding. Sections were stained with hematoxylin and eosin (H & E) for histological examination.

To identify proliferating cells, the mice were given a single dose of BrdU (Accurate Chemical & Scientific Corp), 100 μg/g body weight, dissolved in 0.007 N NaOH in normal saline and killed the next day. To make BrdU accessible to antibody, the sections were pretreated with 2.8 N HCl for 15 minutes and followed antibody labeling.

Immunolabelings were performed on 4% paraformaldehyde-fixed frozen or paraffin sections. The paraffin sections were deparaffinized in xylene, rehydrated in graded ethanol to distilled water. The entire labeling steps were carried out at room temperature. All washes and reagents were in PBS. Excessive aldehydes of the fixed sections were quenched in 0.2 M glycine for 30 minutes. The non-specific binding sites were blocked in 2% normal goat serum for 10 minutes. All antibody incubations were 30 minutes and washed two times, 3 minutes each, between two steps. The first antibodies were guinea pig anti-insulin (Dakopatts), rabbit anti-mouse glucagon (Linco Research), rabbit anti-human somatostatin (Dakopatts), rabbit anti-mouse albumin (Accurate Chemical & Scientific Corp), rabbit anti-mouse alpha- fetoprotein (ICN Immuno Biologicals) and rat anti-BrdU (Sera Lab). The second antibodies were biotinylated goat anti-guinea pig IgG for insulin detection, biotinylated rabbit anti-rat IgG for BrdU and biotinylated goat anti-rabbit IgG for the rest of antigens. The sections were then treated with 1% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase. Next, the sections were incubated in avidin-biotin peroxidase complex (ABC kit, Vector Labs) and carried out the reaction in 0.07% hydrogen peroxide using diaminobenzindine (DAB) as chromogen. Sections were countered stained in 1.5% methyl green or hemotoxylin and dehydrated in graded ethanol and mounted in Permount (Fisher Scientific) with a cover glass.

To colocalize each endocrine hormone and BrdU in the same cells, deparaffinized sections were first incubated with antibody to the hormone and visualized with alkaline phosphatase using alkaline phosphatase substrate kit III as chromogen (Vector Labs). Sections were then digested in 1.4 N HCl for 2 hours and labeled with a mixture of mouse monoclonal anti-BrdU antibodies (seralab and Chemicon) for 2 hours at room temperature and futher incubated overnight at 4°C. The label were detected by alkline phosphatase using Fast-Red TR salt (Sigma) as chromogen.

Electron microscopy

For EM observations, tissues were fixed in modified Karnovsky's fixative (2% paraformaldehyde, 1.5% glutaraldhyde in 0.1 M cacodylate buffer, pH 7.4) for 1-4 hours, post fixed in 1% OsO_4 for 1 hour, dehydrated in graded ethanol and embedded in Epon. Thick sections were cut 0.5-1.0 μm and stained in toluine blue. Thin sections were stained in uranyl acetate and lead citrate and viewed with a Hitachi HU 12 A electron microscope. For EM immunolocalization of BrdU and insulin, tissues were initially fixed in 4% paraformaldehyde for 1-4 hours, at 4°C, then transferred to 0.2% glutaraldehyde and 4% paraformaldehyde for 0.5 hours at 4°C. The tissues were postfixed in OsO_4, dehydrated and embedded as described above. Nickel grids with thin sections were pretreated with Targe (Electron Microscopy Sciences) and 1% H_2O_2 for 5 minutes each to etch epon. To localize BrdU sections are digested with 250 μg/ml trypsin for 2 minutes at 37°C. The immunolabeling followed the same procedure outlined for light microscopy except BrdU was marked by 10 nm colloidal gold particles complexed with goat anti-rabbit IgG.

RESULTS

Ductal hyperplasia in the pancreas of ins-IFN-g transgenic mice

The pancreas in young adults of 6-8 weeks old was characterized by the presence of peri-islet and interstitial lymphocytes, although the majority of islets appeared normal. A proportion of the islets, however, was surrounded by the massive accumulation of inflammatory cells. In exocrine tissue, some acini in the adjoining regions were infiltrated by lymphocytic cells. Ductules next to the inflamed islets appear more prominent than those not immediately adjacent to the inflamed islets (Fig. 1B). In 9- to 12-week-old mice, many more of the above described inflamed islets and associated ducts were found. In addition, severely damaged islets and large ducts with obvious local wall thickening appeared in some of the advanced lesions (Fig. 1C). Exocrine components near the inflamed lesions, again, were filled with small ductules. Adipocytes and fibroblasts also became noticeable. When the mice reach 14-16 weeks, the inflamed lesions spread to encompass large portions of the pancreas. Few, if any, intact islets remained. The ducts became much larger and fused to form an extensive array of ductal system. Small ductules continued to form in the acinar tissue next to the inflamed lesions. The interstitial space was filled with inflammatory cells, fibroblasts and adipocytes (Fig. 1D). In the old mice of 10 months, the inflamed regions consisting of ductal system continued to expand. Acinar tissue was also markedly decreased in the surrounding areas and replaced by the inflammatory cells and the fibrous tissue. Adipose tissue occupied the outer margin of the inflamed lesions (Fig. 1E).

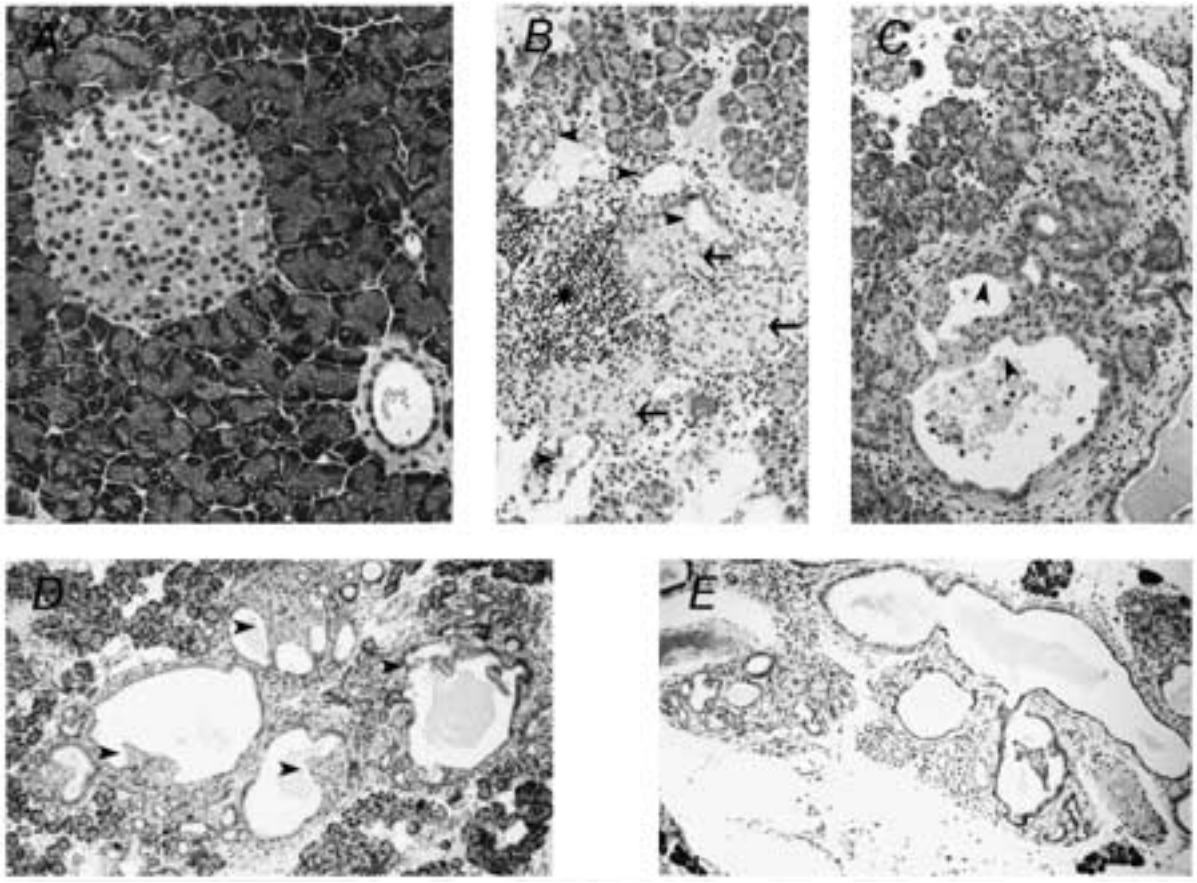

Fig. 1. Histological features of the pancreas from a BALB/c mouse (A) and from ins-IFN-g transgenic mice (B-E). (A) Normal morphology of an islet. Note the typical spherical shape of the islet and the intercalated duct surrounded by the exocrine acinar units. Mouse age, 4 months. ×200. (B) The early stage of an inflamed islet lesion. Note the massive inflammatory cells accumulate on one side of the islet tissue (star). The islet tissue in this micrograph originally may have consisted of three islets (arrows). Their integrity is lost due to the damage by inflammation. Note also the presence of ducts adjacent to the inflamed islets and ductules in the acinar tissue (arrowheads). Mouse age, 7 weeks. ×120. (C) The intermediate stage of an inflamed islet lesion. Note the absence of the previously existing islet tissue (arrow) and the increase in the number and the size of the ducts. The wall of the large ducts has buds (arrowheads) resembling rudimentary islets. Note also the fibrous tissue in the interstitial space. Mouse age, 9 weeks. ×120. See also Fig. 11E for details in a higher magnification micrograph. (D) The advanced stage of an inflamed islet lesion. The ducts have become more numerous and larger and have fused to appear as a branching ductal system. Several buds and islet-like structures (arrowheads) are present. Many ductules appear in the acinar tissue. The interstitial space is filled with adipocytes and fibroblasts in addition to the monocytic lymphocytes. Mouse age, 4 months. ×60. (E) The very advanced stage of an inflamed islet lesion. The ducts have become an elaborate system. The acinar tissue in the adjoining areas have all but disappeared and is replaced by adipose and fibrous tissue. Mouse age, 10 month. ×60.

Endocrine cells are observed budding from ducts

The duct walls, which consist of single-layered cuboidal epithelial cells, are observed to lobulate and branch as the ducts rapidly increase their length and diameter (Fig. 1). The lobulated parts of the duct contain more than single-layered epithelial cells, or 'buds', as referred to in earlier work (Cantenys et al., 1981; Dutrillaux et al., 1982). In the mature mice of 3-4 months old, the lobulation and branching of the ducts were so extensive that it was difficult to identify the individual buds.

The larger buds took the shape of islet-like structures and seemed to detach from the duct, a process described as 'budding'. The budding in embryonic islet morphogenesis and other reported islet neogenesis systems normally result in the islet-like structures in the interstitial space. We have observed, in addition to the normal budding (Fig. 2B), an unusual phenomenon exhibited by these transgenic mice in which the buds very often protruded into the duct lumen (Fig. 2A,C,D). We describe this process as 'reversed budding' as opposed to the normal budding. The islet-like structures frequently seen in the duct lumen most likely resulted from the reversed budding. In some instances, the bud actually formed a bridge across the lumen between the duct walls.

Ultrastructural analysis of islets and ducts in the ins-IFN-g pancreas

The EM observations of small pancreatic ducts demonstrated that the wall consisted of cuboidal epithelial cells, which bordered the lumen and were encased by a basal lamina on the abluminal side. These epithelial cells were characterized by the presence of numerous microvilli on the plasma membrane facing the lumen and the cells formed junctional complexes with the neighboring cells at the luminal face. Single endocrine cells, especially of B type, were readily recognized within the duct wall based on the morphology of hormone-containing granules (Fig. 3A,B).

Demonstration of pancreatic cell proliferation

The appearance of numerous pancreatic ducts of various sizes in the inflamed pancreatic lesions suggested that the duct cells might be dividing. To ascertain which cell types contributed to the increase in the number and the size of the ducts, the replicating cells were identified by the incorporation of thymidine analog 5′-bromodeoxyuridine (BrdU)

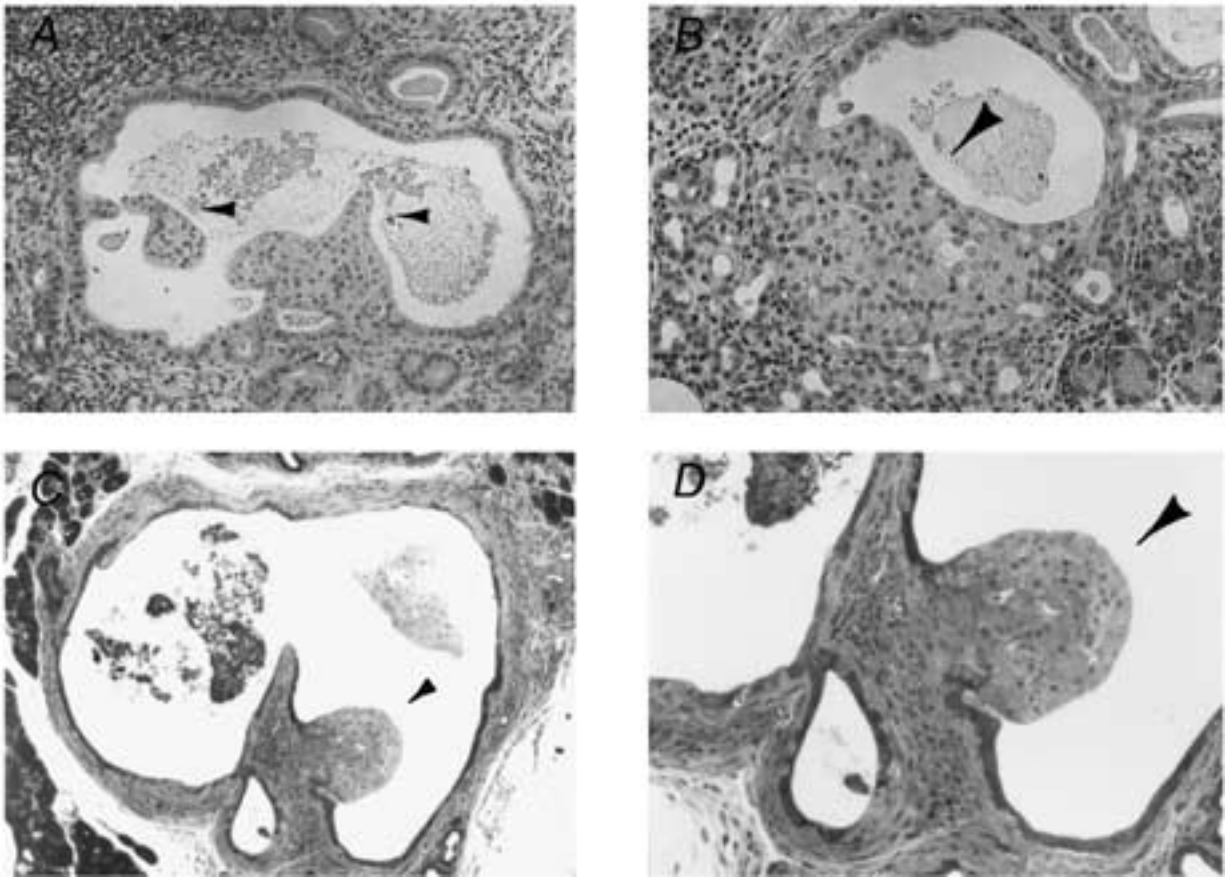

Fig. 2. (A) A large duct with two lumen-bound buds (arrowheads). Note the large size of the duct, which is normally not found in the BALB/c pancreas and the islet tissue within the lumen. Such buds usually contain immunoreactive insulin (see Fig. 6D). Mouse age, 10 weeks. ×60. (B) A large bud (arrowhead) in the midst of surrounding lymphocytes. This bud grows toward the interstitial tissue as normally observed in the islet morphogenesis in embryos. Note the extensive array of ducts in the field. Mouse age, 10 weeks. ×200. (C) A large duct showing a bud in reversed-budding (arrowhead). This well-formed bud points to the lumen. Mouse age, 7 weeks. ×70. (D) A high-powered micrograph of C showing the normal appearance of the endocrine cells in this reversed bud (arrowhead). ×130.

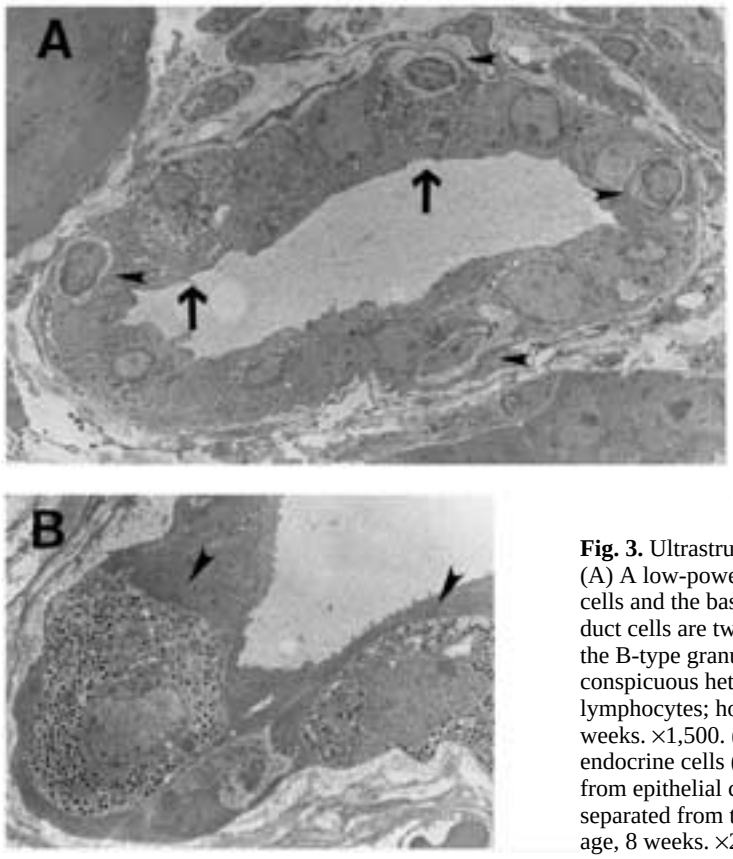

Fig. 3. Ultrastructure of pancreatic ducts from ins-IFN-g transgenic mice. (A) A low-powered EM of a small duct. Note the cuboidal duct epithelial cells and the basal membrane surrounding the duct. Prominent among the duct cells are two endocrine cells (arrows). These two cells contain mainly the B-type granules. Also present are four cells with scanty cytoplasm and conspicuous heterochromatin in the nucleus (arrowheads). They resemble lymphocytes; however, their exact identity is not certain. Mouse age, 6 weeks. ×1,500. (B) A medium-powered EM of part of a duct containing two endocrine cells (arrowheads). Note that microvilli project into the lumen from epithelial cells. The two endocrine cells contain B granules and are separated from the lumen by a thin layer of epithelial cell cytoplasm. Mouse age, 8 weeks. ×2,160.

in the nucleus. The mice received a single i.p. injection of BrdU, 100 μg/g body weight, the night before they were killed. The incorporated BrdU was then visualized by immunoperoxidase technique for LM studies or immunogold for EM localization.

The ductal cells, from the pancreas of our transgenic mice ranging from 1.5 to 13 months old, all exhibited very strong mitotic activity as illustrated in Fig. 4A-C. We assume that the BrdU labelling corresponds to DNA synthesis preceding mitotic activity since doublets of cells are frequently seen and mitotic figures are found in the duct cells of ins-IFN-g transgenic mice in the sections designated for other experiments (see Fig. 2A,B). In particular, in the small ducts ranging approximately from 30 to 50 μm in diameter, a characteristic of interlobular ducts, as high as 73% of the duct cells were observed to incorporate BrdU. A comparable quantification of BrdU-stained cells among the duct cells and in islets was made between ins-IFN-g transgenic, BALB/c, non-obese diabetic (NOD) mice and 6-month-old Obese (ob/ob) mice. The BrdU-positive duct cells from the transgenic mice were 42% based on 20 ducts with a total of 1,765 cells counted. Although neither BALB/c, NOD, or ob/ob mice contained an equivalent number of duct cells as the transgenic mice, in more than 240 sections of pancreas surveyed, there were no positively stained nuclei found in the pancreatic duct cells of BALB/c, NOD or Obese mice. The BrdU-incorporating cells in islets were also examined. The IFN-g transgenic mice had a mitotic index of 1.5% from a total of 5,368 cells in islets. The islets in this study probably included mainly newly formed islet-like structures because the previously existing islets in all likelihood no longer existed. No BrdU-incorporating cells were found in the islets of BALB/c, NOD or obese mice. The results of the BrdU quantitative study are summarized in Table 1. Nearly all the labeled cells, excluding the lymphocytes in the extensive inflammatory lesions, were located in the ductal regions, including the duct cells and islet-like structures. In addition, a few scattered cells in the interstitial space, possibly fibroblasts and lymphocytes, were stained.

Electron microscopy of immunolabeled proliferating cells

The results of EM immunogold experiments were in good

Table 1. Mitotic index of duct cells and islet cells in ins-IFN-g transgenic, BALB/c and NOD mice

		Mitotic index* (BrdU-positive cells/total cells)	
Animals	(*n*)	Duct cells	Islet cells
ins-IFN-g	(5)	42% (742/1,765)	1.5% (81/5,368)
BALB/c	(5)	0 (0/375)	0 (0/2,123)
NOD	(3)	0 (0/143)	0 (0/1,080)

*Mitotic index was expressed as a percentage of BrdU-positive nuclei divided by total nuclei scored. The mice were injected with BrdU 100 μg/g body weight, i.p. and killed the following morning. The incorporated BrdU was visualized by the immunoperoxidase method. The data were collected from a minimum of 360 pancreas sections for each mouse strain. The duct cells included in this study were from the ducts in the interstitial tissue space, excluding the exocrine parenchyma.

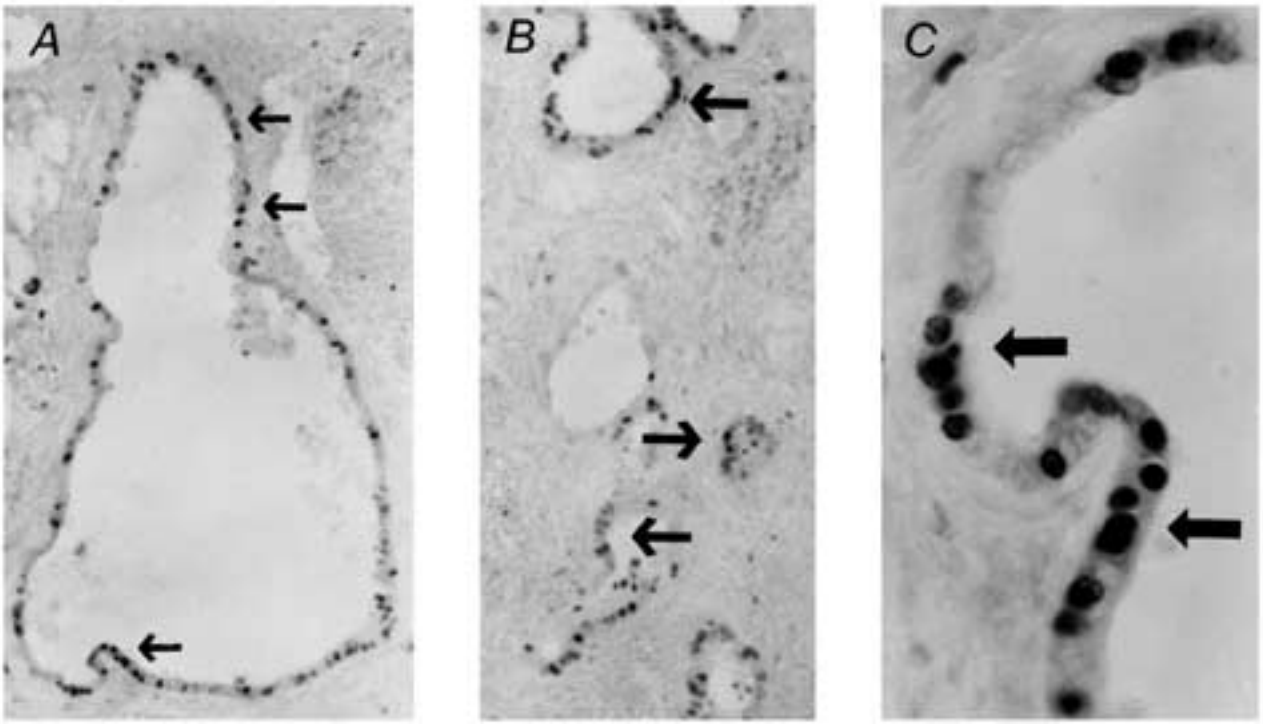

Fig. 4. Demonstration of duct cell proliferation in the ins-IFN-g transgenic mice. The mouse has received BrdU 100 μg/g body weight, i.p. overnight. The incorporated BrdU is visualized by anti-BrdU immunoperoxidase technique. (A,B) Low-powered micrographs. Note the numerous duct cells stained positively for BrdU in the large duct (arrows; A) and in the numerous various sizes of ducts (arrows; B). The results demonstrate a strikingly high mitotic activity exhibited by the duct cells. Mouse age, 8 months. ×150 (A, B). (C) A high-powered micrograph of A. Note that only the nucleus of labeled cells, not the cytoplasm, is stained (arrows). ×630.

agreement with the data of LM immunoperoxidase observations in that there were numerous proliferating duct cells in the ins-IFN-g transgenic mice. Some of these labeled duct cells also contained B insulin granules suggesting that the B-cell is capable of cell division (Fig. 5). It is noteworthy that, at higher resolution, the EM studies revealed that many of the labeled cells that would have been considered bordering the lumen under LM were, in fact, separated by a thin layer of epithelial cell cytoplasm. The presence of the proliferating cells not lining the lumen implied that the proliferating epithelial cells contributed not only to an increase in the diameter of the duct lumen but also to the thickness of the duct wall, which results in the bud formation.

Identification of endocrine cell subtypes

We wished to determine whether the duct wall, especially the buds, and the islet-like cell mass contained endocrine cells. All four major endocrine cell types of the islets, A, B, D and PP cells, were identified by the respective immunoreactive hormone present in each cell type. The A cells, as revealed by the specific antibody against glucagon, were localized as isolated single cells and small clusters consisting of three to six cells within the duct wall or in the outer margin of the islet-like structures (Fig. 6A). The insulin-containing B cells were most numerous. They were also found in the duct wall, as single cells or more often in clusters (Fig. 6C). They occupied the major portion of the islet-like structures (Fig. 6D). The D cells were identified by specific antibody to somatostatin. They were most likely seen as isolated single cells within the duct wall and on the periphery of the islet-like structures. They appeared with long cellular processes and were larger than either A or B cells (Fig. 6B). The PP cells were scattered in the duct wall and as single cells in the islets (data not shown). The distribution pattern of the endocrine cell types in the islet-like structures resembled that of fetal islets, rather than adult islet morphology. The mantle-like arrangement of islet organization found in the adult pancreas, where B cells are encircled by A and D cells, was not present.

Demonstration of proliferating endocrine cells

Of great significance is whether newly formed endocrine cells in the buds and the islet-like structures are capable of cell division. All three of the major four endocrine cell types (A, B and D cells) examined incorporated BrdU as demonstrated by double-labeling with antibodies to each respective hormone in conjunction with BrdU (Fig. 7A-C). The incorporation of BrdU by all three cell types indicates that the division of new endocrine cells is a part of islet regeneration process in the ins-IFN-g transgenic mice. Interestingly, somatostatin cells incorporating BrdU far outnumbered both insulin and glucagon BrdU-labelled cells. These data are consistent with recent studies demonstrating the early expression of this islet hormone in the developing foregut (Gittes and Rutter, 1992). The ductal/periductal location of the BrdU-positive cells indicates that the majority of cell division occurs within the

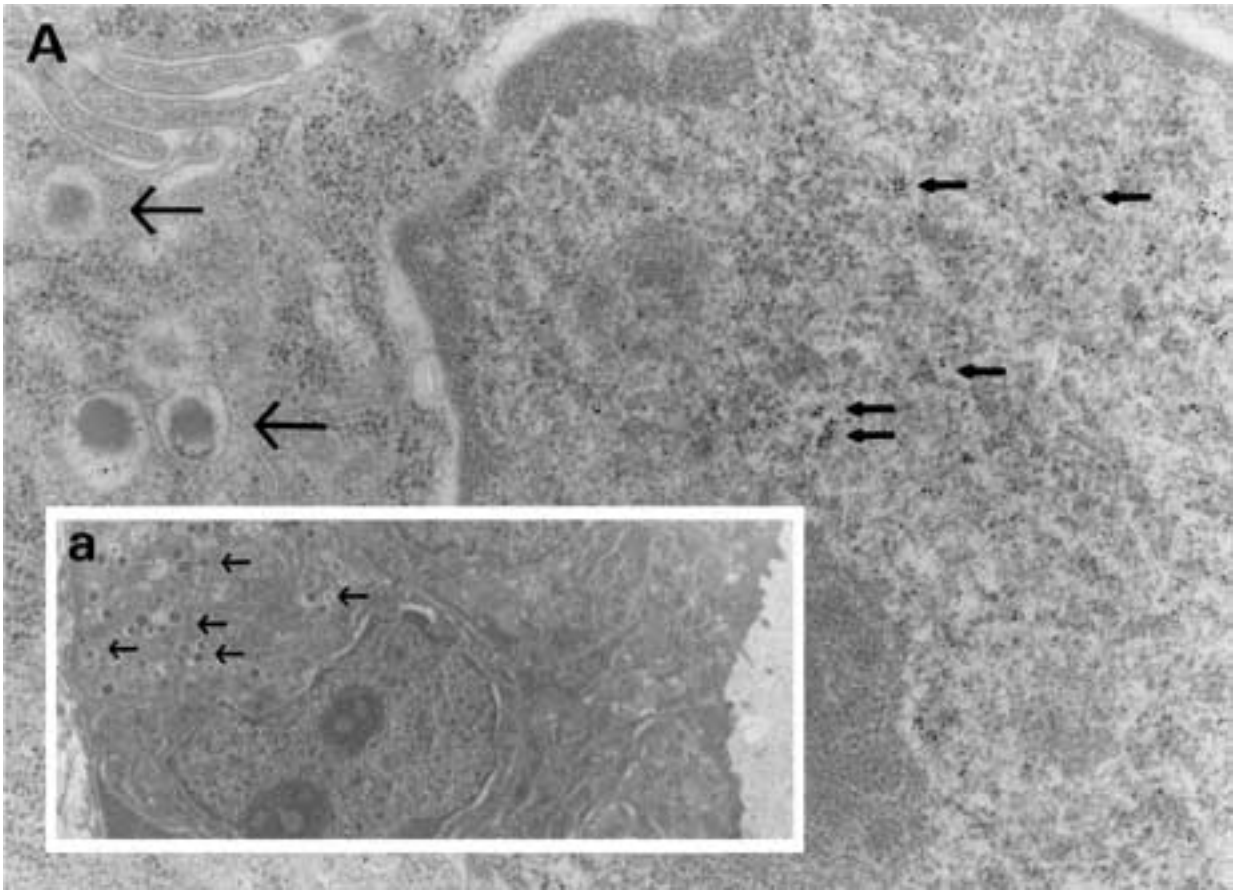

Fig. 5. Identification of duct cells proliferation in the ins-IFN-g transgenic mice by anti-BrdU immunogold method. The incorporation of BrdU is performed as in Fig. 4. (a) A low-power EM showing the duct cell containing endocrine granules (arrows) in the cytoplasm in the duct wall. Note that this endocrine cell is not bordering the lumen. Mouse age, 7 weeks. ×6,300. (A) A high-power EM of the same cell shown in (a). Note the nucleus is decorated with gold particles (small arrows) indicating the incorporation of BrdU. The cytoplasm contains endocrine granules (large arrows) with central dense core and peripheral lucent halo, thereby indicating an insulin-producing B endocrine cell. ×55,200.

duct structure, the new endocrine cells then continue to differentiate as they migrate away from the duct wall. With the observed mitotic index we would expect very large numbers of endocrine cells to be found in the transgenic mice; however, these new endocrine cells are destroyed by the inflammatory cells (Sarvetnick et al., 1990). Mature islets were only observed in the reversed budding location suggesting that these structures escaped the very aggressive immune surveillance, perhaps because of their unique location.

Identification of unusual non-pancreatic cell types

Others have demonstrated that pancreatic duct cells can 'transdifferentiate' into hepatocytes (Scarpelli and Rao, 1981; Rao et al., 1986, 1989). We undertook immunoperoxidase studies using antibodies against albumin and alpha-fetoprotein to identify hepatocyte-like cells. Single cells located along the ductal regions were found to react with both the alpha-fetoprotein and albumin antibodies (Fig. 8A,B). Other albumin- and alpha-fetoprotein-containing cells were also detected in the interstitial tissue in the affected lesions. These hepatocyte-like cells were observed singly or in group of two to three cells. These hepatocytes were among the largest cells observed as compared with endocrine cells and epithelial cells. Additionally, we observed secretory epithelial cells resembling Paneth cells of gastrointestinal tract within the ducts (data not shown) (Like and Chick, 1970a,b; Satoh et al., 1990).

Identification of cells containing tyrosine hydroxylase

During ontogeny, the epithelial cells that differentiate into endocrine cells synthesize tyrosine hydroxylase (TH) (Teitelman and Lee, 1987). The immunolabeling of TH revealed that many positively stained cells were closely apposed to the ductal lumen in the transgenic mice. This enzyme was restricted to the apical cytoplasm facing the lumen (data not shown).

We also studied the regenerating pancreas for the expression of glutamic acid decarboxylase (GAD), which is found in pancreatic endocrine cells. The GAD-positive cells were found in the duct walls, the buds and the islet-like structures of the transgenic mice. The islets from BALB/c pancreas also contained GAD-positive cells. These

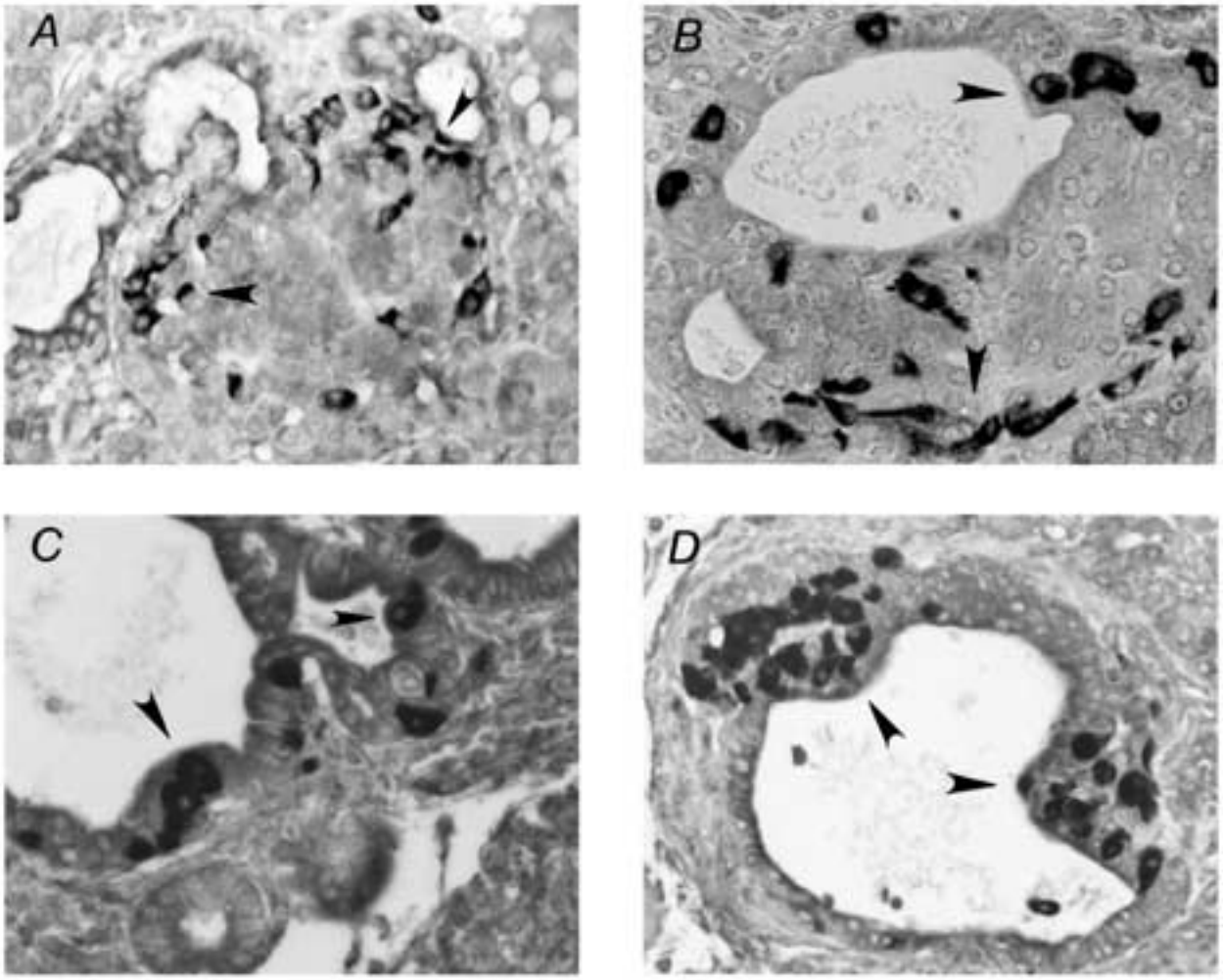

Fig. 6. Demonstration of A, B and D islet cells in the ins-IFN-g transgenic mice by the immunoperoxidase method. (A) Glucagon-containing cells are distributed in the ducts and in the peripheral region of the islet-like structure. Numerous A cells are seen in the duct wall of the small ducts and also in the fringe area of this well-formed islet-like structure (arrowheads). Mouse age, 4 months. ×200. (B) Somatostatin-containing cells are observed in the ducts and in the islet-like structures. Note the peripheral locations of D cells in the islet-like structure and their characteristic long cellular processes (arrowheads). Mouse age, 3 months. ×380. (C) Single insulin-containing cells are localized in the ducts (arrowheads). Mouse age, 3 months. ×250. (D) Another field of an insulin-labeled duct at a later stage of islet differentiation than shown in C. Note two buds formed by this duct contain many insulin-positive cells (arrowheads). Note also that these two buds are budding into the lumen. ×200.

results indicate that both the primitive endocrine cells and the fully differentiated endocrine cells expressed GAD throughout their life cycle.

Hyperglycemia is not required for regeneration

To determine whether the observed cell proliferation correlates with hyperglycemia, we studied the clinical status of the transgenic mice. Duct cell proliferation and islet budding was observed in transgenic mice that had blood glucose levels in the normal range (80-120 mg/dl) as well as in diabetic mice. We find that continued backcrossing onto the BALB/c strain has caused delay in the clinical diabetes in these transgenic mice, whereas outcrossing causes early severe diabetes in a high proportion of animals. Our studies of the ins-IFN-g/SCID transgenic mice (see below) have revealed that the cell proliferation in these mice is also not correlated with hyperglycemia.

Infiltrating lymphocytes are not required for duct cell proliferation

Severe combined immunodeficient (SCID) mice have a deficiency in immune functions mediated by T and B lymphocytes (Bosma et al., 1983). Ins-IFN-g/SCID mice do not have lymphocyte infiltration in the pancreas and do not suffer from islet loss as a result (Sarvetnick et al., 1990). The majority of the pancreas in the young adult ins-IFN-g/SCID mice, age 6-8 weeks old, showed no signs of abnormality. Pancreatic ducts within the islets (intra-islet ducts) and in the surrounding acinar tissue were observed in a few islets (Fig. 9A). Aggregation of inflammatory cells, in contrast to the islets in young adults of ins-IFN-g mice, had not occurred. As the mice grew to 9-12 weeks old, more islets were seen to have intra-islet ducts and an increase in the number and the diameter of the ducts in the acinar tissue. Larger and more numerous intra-islet ducts were

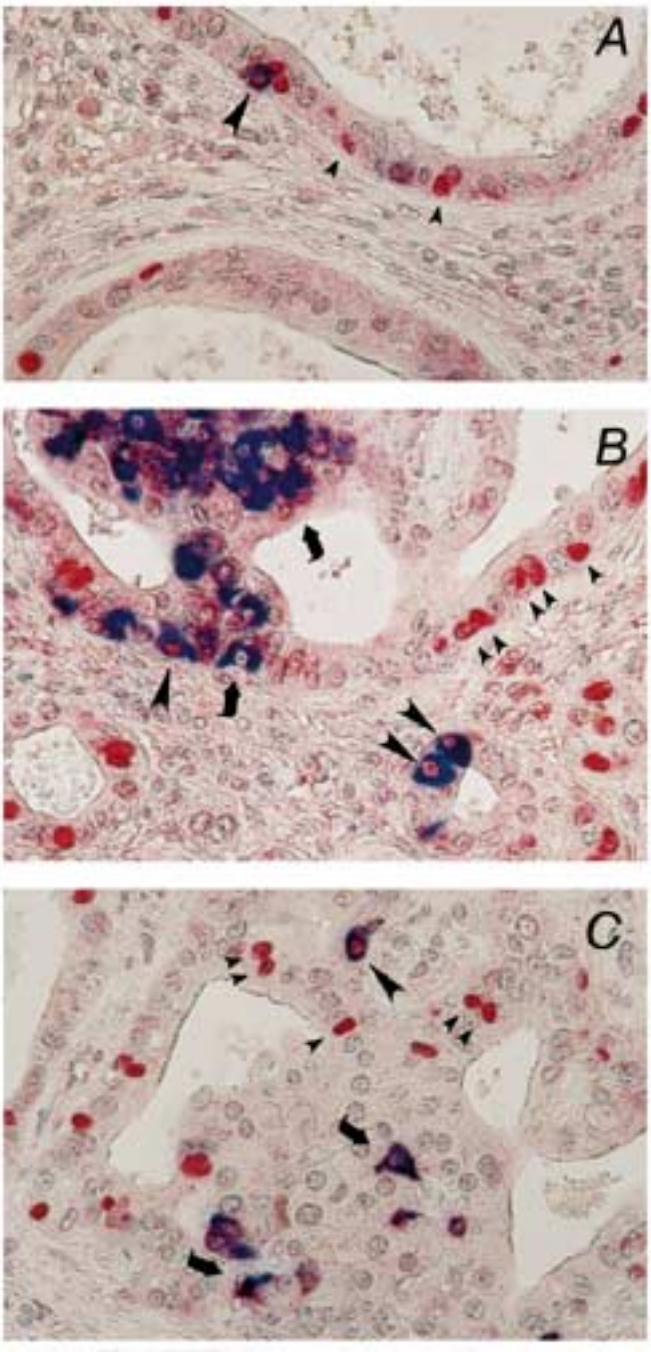

Fig. 7. Identification of proliferation of the endocrine cells in the regenerating islets of the ins-IFN-g transgenic mice. Cells producing each of the three endocrine hormones examined showed incorporation of BrdU. Double immunolabelings are perfomed on paraffin-embedded sections to reveal each hormone in the cytoplasm and BrdU in the nucleus. (A) Proliferation of glucagon-containing A cells. Note several duct cells stained positively for BrdU in the nucleus (small arrowheads). One of the cells also labeled with glucagon antibody in the cytoplasm (large arrowhead). Mouse age, 3 months. ×380. (B) Proliferation of insulin-containing B cells. Numerous cells contain insulin as revealed by the staining in the cytoplasm in part of a reversed bud shown and in the duct wall (arrows). Three of the insulin-stained cells in the ducts also incorporate BrdU in the nucleus (large arrowheads). Additionally, many cells, mainly in the duct, stain only with BrdU antibody (small arrowheads). Mouse age, 3 months. ×380. (C) Proliferation of somatostatin-containing D cells. Note numerous duct cells incorporate BrdU in the nucleus (small arrowheads). A few cells in the islet-like structure stained for somatostatin in the cytoplasm (arrows). A single cell adjacent to the duct wall contains somatostatin and also incorporates BrdU (large arrow). Mouse age, 3 months. ×380.

found in some islets which lost the integrity of the original islet morphology (Fig. 9B,F). Fibroblasts, adipocytes and a few monocytic lymphocytes occupied the interstitial space. In 16-week-old ins-IFN-g/SCID mice, the ducts continued to grow in size and joined to form an elaborate ductal system similar to the one described in the pancreas of ins-IFN-g mice. (Fig. 9C. See Fig. 1D for comparison). Small ducts continued to appear in the acinar tissue adjacent to the ductal system. In the very old ins-IFN-g/SCID mice at age of 10 months, the ducts continued to proliferate and enlarge. Very few islets not yet populated by the intra-islet ducts could be found. Fibrosis was a constant feature around the large ducts. Adipose tissue filled a large area of the interstitial space previously occupied by the acinar tissue (Fig. 9D. See Fig. 1E for comparison). However, the pancreatic ducts of ins-IFN-g/SCID exhibited a very strong mitotic activity as shown by the presence of numerous mitotic figures (Fig. 9G) and the incorporation of BrdU (Fig. 9H). The pancreas was also populated by the numerous ducts of various sizes as described for the pancreas of non-immune deficient transgenic mice. The proliferating intra-islet ducts in addition to the proliferating acinar ducts are a distinction between the ins-IFN-g/SCID and the immunocompetent transgenic mice. Intra-islet ducts are not a noteworthy feature of the lesions in the ins-IFN-g transgenic mice whereas they are quite common in the ins-IFN-g/SCID mice.

DISCUSSION

Several lines of evidence suggest that the duct cell proliferation and the duct-associated islet formation described in this ins-IFN-g transgenic mouse strain are recapitulating the islet ontogeny of embryos. The transient expression of TH exhibited by some duct cells in the transgenic mice is similar to that observed in the fetal gut during pancreatic development (Teitelman and Lee, 1987). Additionally, the frequent observation of a change in the axis of cell division of duct epithelial cells allowing one of the daughter cells to migrate away from the lumen and become a part of a bud is reminecent of the first step of islet formation (Fig. 2A,B) (Pictet and Rutter, 1972; Deltour et al., 1991). Thirdly, endocrine cells in the duct wall were demonstrated by both electron microscopic observations and specific endocrine antibody staining. The EM studies revealed that the endocrine cells existing either singly or in clusters are within the basal membrane surrounding the ducts. These EM observations do not support the notion that the ductal endocrine cells could be the remnants of the previously existing islet cells. Ductal endocrine cells also have been reported in new-born mice (Deltour et al., 1991) and in the cultured pancreas of new-born mice (Teitelman and Lee, 1987) and rats (Leduque et al., 1989) and considered by

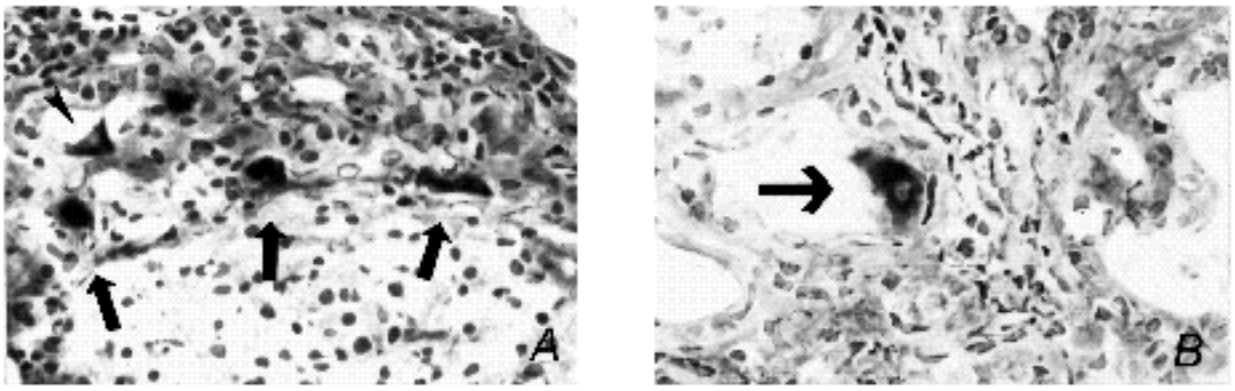

Fig. 8. Presence of hepatocyte-like cells in the regenerating islets of an ins-IFN-g transgenic mouse. Hepatocyte-like cells are identified by antibodies to alpha-fetoprotein (A) and to albumin (B). (A) Several alpha-fetoprotein-positive cells can be seen in this micrograph (arrows). One stained cell is located in the duct wall (arrowhead) and the rest are scattered in the interstitial tissue. (B) An albumin-containing cell is visualized to border the duct lumen (arrow). Note the extremely large size of the hepatocyte-like cells in both micrographs. Mouse age, 6 months. ×200 (A, B).

these authors as evidence suggestive of epithelial cell origin and as a part of the normal islet neogenesis. Taken together, these results provide evidence that there is an active endocrine regenerative process occurring in the pancreas of the IFN-g transgenic mice. The endocrine cells regenerate by neogenesis, recapitulating their ontogeny.

Similar experiments performed on other spontaneous diabetes models have provided both contrasts and similarities to our transgenic mice. BrdU-labeling experiments revealed no islet or duct cell proliferation in diabetic NOD mice. Our histological studies with older diabetic NOD mice revealed normal-sized duct structures and only islet remnants. This information is consistent with the cumulated data that, while hyperglycemia is preventable by therapy regimens, once diabetes occurs it is irreversible in this strain. Additionally, in the ob/ob mouse, we observed no duct cell proliferation in mice that exhibited mild hyperglycemia. Therefore, in this spontaneous model there is no ongoing cell proliferation analogous to what we have observed in the transgenics. However, in the late stage diabetic ob/ob mouse as well as another spontaneous diabetes mouse, the db/db strain, there have been reports of intra-islet ducts and duct cell metaplasia (Findlay et al., 1973; Like and Chick, 1970a,b) Indeed, these very interesting structures appear within islets in final stage diabetic animals and resemble the proliferating duct structures that we describe in this report. The main similarity is in the prominence of the duct structures in these models. However, islet regeneration by budding is not an obvious feature in either the ob/ob or the db/db models.

Comparison of our results with the spontaneous diabetes models leads us to several speculations. Disease states exhibiting common histopathological features could arise from quite distinct perturbations. Since the genetic lesion in the spontaneous mutations is undefined, it is not possible to determine the cause of the epithelial cell proliferation in these strains. However, if duct cell proliferation can occur in the absence of detectable islet cell regrowth then the proliferation and differentiation processes of the epithelial cells are separable. Since the regeneration that we are observing in our mice involves both cell proliferation and differentiation, this might arise from the collaboration of two or more separate processes. It is also possible that the proliferating duct cells in the db/db and ob/ob mice do not have the capacity to differentiate since they are not true ‘progenitor’ cells. Further characterization and comparison of these interesting spontaneous mutants with transgenic models is certainly warranted.

Fig. 9. Histological features of the pancreas from ins-IFN-g/SCID mice. (A) The pancreas of a young adult mouse showing two islets with the ducts within the islets and in the periphery (arrowheads). The appearance of ducts in association with islets alters the islet morphology resulting in the loss of its original spherical shape. The massive accumulation of inflammatory cells around the islets is notably absent. Mouse age, 6 weeks. ×120. (B) The pancreas of a matured adult mouse displaying hyperplasia of ducts in association with the islet (large arrowheads). The islet has lost its integrity as a result of the growth of the intra-islet ducts. The fibrous tissue surrounds the islet and its associated ducts. Adipocytes and monocytic lymphocytes appear in the interstitial space. Note that a cluster of ducts begin to form in the acinar tissue (small arrowheads). Mouse age, 10 weeks. ×120. See also Fig. 12F for details in a higher magnification micrograph. (C) The pancreas of a 4-month-old mouse showing the increase in the size and the number of ducts. Note that the islet-like structures bud into the lumen (arrows). ×60. (D) The pancreas of a 10-month-old mouse exhibiting enormous size of the ducts. The acinar tissue in the surrounding areas has been replaced by the fibrous tissue. The adipose tissue occupies the outer fringe areas. ×60. (E-H) A comparative pancreatic morphology of the ins-IFN-g transgenic mice (E) and the ins-IFN-g/SCID mice (F-G). (E) The pancreas of the ins-IFN-g transgenic mice. Note the morphological features are distinguished by the proliferation of ducts (arrows) and the massive invasion of the inflammatory cells (arrowheads). Mouse age, 12 weeks. ×60. See also Fig. 1B. (F) The pancreas of the ins-IFN-g/SCID mice. Note the proliferation of ducts (arrows), as in E, in the absence of inflammatory cells. (G) A high-powered micrograph of F showing several duct cells in mitosis (arrows). Mouse age, 9 weeks. ×80 (F) and ×500 (G). (H) Demonstration of duct cell proliferation in the ins-IFN-g/SCID mice by immunolabeling of incorporated BrdU. The mouse is treated identically as described in Fig. 4. Note several BrdU-incorporating cells in the three ducts shown (arrowheads). See Fig. 4 for comparison. Mouse age, 11 weeks. ×400.

Interestingly, a fundamental difference between our transgenic mice and the two spontaneous strains is that the observed proliferation is independent of the induced hyperglycemia. The ins-IFN-g transgenic mice exhibit both the destruction and the growth of islet cells, two opposing forces that apparently occur independently of each other. In some cases, a balance may be achieved allowing the maintenance of normoglycemia in animals where islets have succumbed to inflammatory cells. The fact that both the ob/ob and the db/db mice are diabetic prior to the observed duct cell metaplasia implies that the hyperglycemic change might be required for this process. It is known that hyperglycemia is not itself sufficient to stimulate duct cell proliferation; however, with other factors, it could contribute to cell proliferation indirectly. Another interesting distinction between our transgenic mice and the

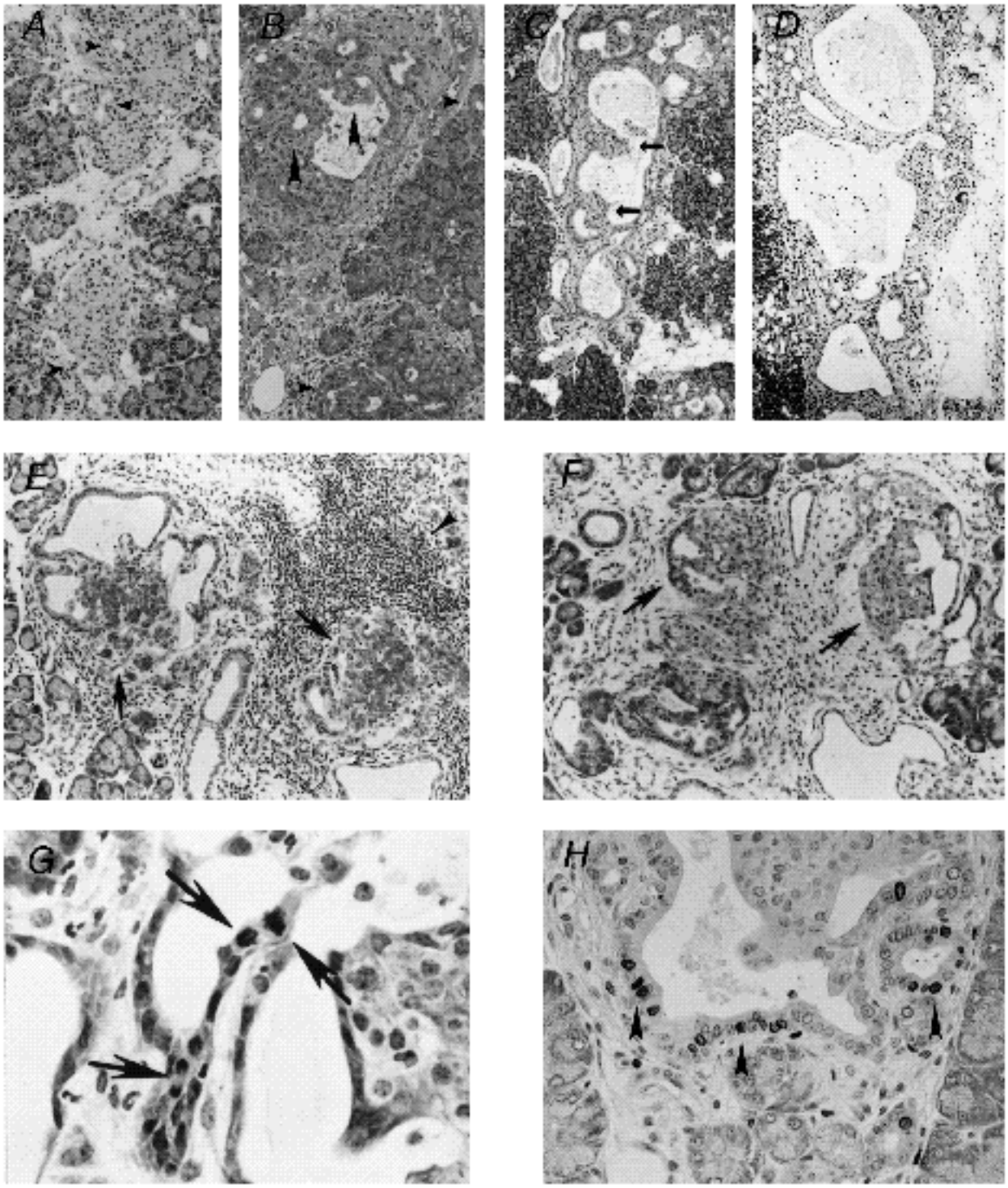

Fig. 9

spontaneous diabetes strains is the significant degree of exocrine tissue atrophy and fibrosis that we observe in the pancreas. Although the loss of endocrine tissue from the pancreas of adult mice does not induce regrowth, the exocrine tissue loss and fibrosis could potentially damage a subset of cells and stimulate a 'replenishment' response. Since we also observe significant fibrosis and exocrine tissue atrophy in the ins-IFN-g/SCID transgenic mice this becomes a common attribute between these and the immunocompetent transgenic mice since both exhibit the described proliferation. The presence of intra-islet ducts, however, in the ins-IFN-g/SCID transgenic mice is in contrast to the immunocompetent ins-IFN-g pancreas. This might reflect the continued presence of the islet structures in the absence of lymphocytes in the SCID strain, which allows for proliferation of the intra-islet epithelial cells.

Since the duct cell proliferation still occurs in the IFN-g/SCID transgenic mouse strain, we can conclude that (1) the proliferation occurs in the absence of mature B and T cells which constitute the greater part of the inflammation observed in immunocompetent animals and (2) the proliferation is not entirely dependent on the destruction of islet tissue. Our observations of tyrosine hydroxylase staining in regions of the duct wall of the adult transgenic pancreas allows us to speculate that a more primitive progenitor cell is present in these pancreata. This same progenitor cell, present transiently in neonatal mice, persists throughout adult life in the transgenic mice. When 'stimulated', this progenitor cell is capable of giving rise to mature insulin-producing cells.

We currently have two hypotheses as to how this progenitor cell perdures and is stimulated to divide. The first is that this primitive cell is immortalized by exposure to IFN-g. IFN-g certainly can stimulate the proliferation of cells and could have an effect on primitive cells as well. There are documented examples of nesidioblastosis, or uncontrolled differentiation of islet cells from duct cells and it is possible that this could be directly or indirectly induced by IFN-g or a factor with overlapping effects produced during an antiviral response. A second hypothesis is that a factor produced by infiltrating macrophages is inducing duct cell proliferation and islet regeneration. Since IFN-g is well known to activate macrophages, which make up a small but ubiquitous component of the inflammatory infiltrate, this notion seems particularly compelling. Additionally, macrophages are present in the ins-IFN-g/SCIDs where duct cell proliferation is observed. Furthermore, duct cell proliferation is not observed in transgenic pancreata expressing the cytokine IL-10 (unpublished observations) which exhibit intense inflammation but little islet destruction and no diabetes. Since IL-10 is reported to suppress macrophage function and antigen presentation (Malefyt et al., 1991), it is possible that while macrophages are present in the IL-10 infiltrates they are not stimulated to produce the proliferation-inducing factors. The exact role of macrophages in the islet regeneration in this IFN-g transgenic mouse strain remains to be defined further. With respect to other cytokine involvement in the replenishment of islet tissue, it is worthy to note that in TNF transgenic mice, ductal proliferation has not resulted in appreciable endocrine cell regeneration, even after the mice have been infused with IFN-g (Higuchi et al., 1992). It would be of great value to study double transgenic mice to clarify further the role of IFN-g and TNF in pancreatic morphology, especially with regard to duct proliferation and islet formation.

The reversed budding or the inward growth of islet-like structures accounts for up to two thirds of all the buds observed in the 3-4 months age group of ins-IFN-g transgenic mice. The inaccessibility of lymphocytes to the reversed buds could partly account for their abundance in this transgenic mice. The reversed budding is unique and has not been observed in other islet growth or development studies. In embryonic morphogenesis, the evagination of pancreatic primordium from the primitive gut requires the interaction of the gut epithelium and the adjacent mesoderm tissue (Spooner et al., 1977) and is also dependant on the presence of a collagen matrix (Montesano et al., 1983; Amory et al., 1988). Similar mechanisms may operate in guiding the regenerating islet budding. The change in polarity of budding may simply be due to the change in microenvironment of the islet primordium, which normally would have mesoderm-derived cells adjacent to the bud. The fact that islet tissue can differentiate in the absence of these mesoderm signals indicates that they are not necessary for islet development, only for the morphogenesis of the developing islet. It is possible that the interstitial fibroblasts or the activated macrophage in the pancreas of the transgenic mice might be producing a mesoderm-like signal to the differentiating duct cells. Alternatively, the reversed budding could result from the tropic actions of growth factor(s) present in the lumen. Similar reversed polarity of organization has also been observed in the retinal regeneration derived from the transdifferentiation of pigmented epithelium induced by basic fibroblast growth factor (bFGF) in chick embryos in vitro and in vivo (Park and Hollenberg, 1991; Pittack et al., 1991).

The newly formed ducts, especially the small ducts consisting of 5-10 cells, occasionally contain one or more acinar cells, raising the possibility that some duct cells may arise from transdifferentiated acinar cells. Indeed, the transformation of acinar cells to intercalated duct cells have been observed in human chronic pancreatitis (Bockman et al., 1982) and in pancreatic duct ligated rats (Walker, 1987). However, such transformations do not likely play a role in normal islet cell development (reviewed by Le Douarin, 1988; Dubois, 1989).

Although the origin of the duct progenitor cells are not known, the multipotential differentiation properties of duct cells have been clearly demonstrated. We have shown the presence of albumin- and alpha-fetoprotein-containing cells in the ducts, indicating that the duct cells transdifferentiate to hepatocyte-like cells. This is in agreement with the results of hepatocyte transdifferentiation in the rats after copper-deficient diet, although the transdifferentiation in the transgenic mice is much less extensive. We have also observed the appearance of Paneth-like cells in the ducts, another example of transdifferentiation of duct cells to phenotypes other than islet cells. In some human pancreas metaplasia, well-differentiated goblet cells (Walters, 1965) and other mucous cells (Oertel, 1989) have been reported to have a ductal origin. The question arises as to whether

these different cell types originate from a common progenitor as islet cells do or the ducts harbor a variety of predetermined cell populations. It would be of great interest to perform cell lineage analyses among these different cell types.

In conclusion, we have presented an islet regenerating system in ins-IFN-g transgenic mice which encompasses (1) the differentiation of islet cells from the proliferating progenitor cells in the duct, (2) the peculiar reversed budding of the islet-like structures and (3) occasional transdifferentiation to hepatocytes and Paneth-like cells. These results indicate the plasticity of the ductal differentiation pathways and offer a model system for studying growth factors modulating the islet cell differentiation.

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