Cell and Tissue Research

DOI 10.1007/s00441-002-0614-z

Regular Article

Identification and characterization of small cells in the adult pancreas: potential progenitor cells?

Maria Petropavlovskaia(✉) · Lawrence Rosenberg

M. Petropavlovskaia · L. Rosenberg
Department of Surgery, McGill University, and Research Institute of the McGill University Health Center, 1650 Cedar Ave., Montreal, Quebec, Canada, H3G1A4

✉ E-mail: maria.petropavlovskaia@mail.mcgill.ca
Fax: +1-514-9348289

Received: 12 December 2001 / **Accepted:** 10 July 2002 / **Published online:**

This study was supported by The Canadian Institutes for Health Research. L.R. is a Chercheur national of the Fonds du Recherche Scientifique du Quebec

Abstract. In this report we describe the identification of a novel cell type in human and canine pancreas using tissue culture techniques. These cells, representing less than 1% of total islet cells, are of a small size (7-10 μm) and highly quiescent. They display a fairly immature morphology, which is characterized by a weakly developed protein synthesis machinery, a few mitochondria and a small number of neuroendocrine granules. These cells, which we have termed "small cells," are usually organized into small clusters, which can be identified within the islets of predominantly small size. They can also be collected as separate structures from preparations of freshly isolated islets. Immunohistochemically, small cells are positive for PDX-1, synaptophysin, insulin, glucagon, somatostatin, pancreatic polypeptide, α -fetaprotein and Bcl-2 and negative for cytokeratin 19 and nestin. Insulin secretion studies demonstrated that these cells secrete insulin in a glucose-responsive fashion, although do not respond to secretagogues such as IBMX and arginine as do mature beta cells. Although this study does not provide evidence of the proliferative and differentiation potential of small cells, their immature morphology, along with a small size and quiescence, let us hypothesize that these cells may serve as progenitors contributing to the islet growth.

Keywords. Pancreatic islet - Culture - Human - Canine - Progenitor cell

Introduction

There has been increasing interest in recent years in the identification of stem or progenitor cells in the pancreas. The availability of such a cell for expansion in vitro and for bioengineering would have a significant therapeutic potential for diabetes mellitus (Soria et al. *2001*). Stem and progenitor cells in adults have been isolated primarily from bone marrow and the nervous system and are being extensively studied (Weissman *2000*; Fuchs and Segre *2000*). Although it is generally believed that similar cells exist in other tissues and organs as well, actual proof for this is very limited.

Evidence for the existence of a progenitor cell in the pancreas rests primarily on the phenomenon of islet neogenesis, which can be experimentally induced by cellophane wrapping of the pancreas (Rosenberg et al. *1983*), partial pancreatectomy (Bonner-Weir et al. *1993*), and streptozotocin-induced diabetes (Fernandes et al. *1997*) and which has also been observed during pregnancy (reviewed in Bonner-Weir *2000*). Although a protein that induces islet neogenesis (islet neogenesis associated protein, INGAP) has been purified and characterized and its cDNA cloned (Rosenberg *1998*; Rafaeloff et al. *1997*), a potential target-progenitor cell remains unknown.

At present, most of the studies favor the pancreatic duct as a potential source of progenitor cells in adult pancreas (Rosenberg *1998*; Bonner-Weir *2000*). This is based on the established information that development of islets during embryogenesis is closely associated with ductal epithelium (Madsen et al. *1996*). Secondly, scattered endocrine cells have been found within the adult ductal system (Gu and Sarvetnick *1993*) and the budding of new islets from the small ducts has been described (Bouwens and Pipeleers *1998*). The ability of ductal cells to expand in vitro and to form insulin-producing islet-like structures has also recently been demonstrated (Bonner-Weir et al. *2000*; Ramiya et al. *2000*).

A few recent publications indicate that another potential source of progenitor cells is the islet itself. For example, analysis of islet regeneration in the mouse pancreas after administration of streptozotocin suggests the presence of beta progenitor cells in the islets that differentiate into insulin-producing cells following injury (Guz et al. *2001*). This study proposes the existence of two presumptive progenitor cells, one of which expresses Glut-2 and another coexpressing insulin and somatostatin. Another report demonstrates that rat and human islets contain a distinct population of nestin-positive and hormone-negative immature cells, which proliferate extensively in vitro and appear to be multipotential (Zulewski et al. *2001*). Whether these cells participate in islet regeneration and neogenesis in vivo has yet to be determined.

It should be noted that the number of progenitor cell types in the pancreas may not be limited to the cells already described. It is possible that the pancreas harbors an as yet unidentified cell that remains dormant and undifferentiated most of the time, similar to a hematopoietic stem cell or a hepatic oval cell, and that resumes proliferation when a need for islet neogenesis arises.

In this report we describe a novel cell population isolated from adult canine and human islet preparations. These cells are of neuroendocrine lineage and are characterized by their very small size (7-10 μm in diameter), immature morphology and quiescence. Their presence in small, growing islets suggests that they may have the potential of progenitor cells.

Materials and methods

Islet isolation

Pancreata from mongrel dogs (2-4 years old with body weight 20-25 kg) were removed with the animals under general anesthesia in accordance with Canadian Council for Animal Care (CCAC) guidelines. Human pancreata were obtained from heart-beating cadaveric donors following in situ flush with UW solution at the time of multiorgan harvest for transplantation. Prior consent for organ donation was obtained by the local procurement organization Quebec-Transplant. Cold ischemia time varied between 4 and 8 h.

Islet isolation from both canine and human pancreas was carried out using enzymatic digestion with Liberase CI and Liberase HI (Roche Diagnostics, Laval, Que., Canada) for canine and human pancreas respectively, mixed with 0.1 mg/ml Dnase I (Roche Diagnostics, Laval, Que., Canada). Digestion was followed by semiautomated dissociation and EuroFicoll purification as previously described (Paraskevas et al. *1999*; Wang and Rosenberg *1999*). The final islet preparations were 70-90% dithizone positive. Islets were resuspended in the regular culture medium CMRL-1066 (Gibco Brl, Burlington, Ont., Canada) supplemented with 10% fetal bovine serum (FBS) (Montreal Biotech, Montreal, Que., Canada), penicillin, streptomycin and fungizone (Gibco Brl, Burlington, Ont., Canada) and counted under an inverted microscope.

Culture of human and canine islets

Aliquots of islets (1,000 islets/10 ml) were transferred into 10-cm tissue culture plates and allowed to adhere to plastic for 48-72 h. The cultures were maintained for different time intervals (up to 4 months) with a media change every 3 days. In a number of experiments freshly isolated islets were sieved through stainless steel meshes (Bellco Biotechnology, Vineland, NJ) with pore size 25, 94 and 140 µm to fractionate islets and cell clusters by size.

Sieved islets were cultured on either plastic or extracellular matrices such as collagen type I (rat tail, prepared according to Richards et al. *1983*) and bovine, a gift from Dr. S. Sullivan (Organogenesis, Canton, MA), pig gelatin 0.1% (Sigma-Aldrich, Oakville, Ont., Canada), human fibronectin 5 µg/cm^2, mouse laminin 5 µg/cm^2 and Matrigel, thin gel method (all from Becton-Dickinson, Mississauga, Ont., Canada), prepared according to the manufacturer's instructions. In a number of experiments islets were embedded into collagen, as described (Yuan et al *1996*) or Matrigel (thick gel method).

Immunocytochemistry

Immunostaining was performed for insulin, glucagon, somatostatin (BD PharMingen, Mississauga, Ont., Canada) (dilution 1:100), CK-19 (1:50), α -fetaprotein (1:100), c-kit (1:100), CD34 (1:50), Bcl-2 (1:80), pancreatic polypeptide (1:500), synaptophysin (1:100) (Dako Diagnostics, Mississauga, Ont., Canada), nestin (1:200) (Chemicon International, Temecula, CA), LIF receptor, and transforming growth factor (TGF) β receptor types I and II (Santa-Cruz Biotechnology, Santa Cruz, CA, 1:200).

Antibody for PDX-1 was a kind gift from Dr. C. Wright, Vanderbilt University, Nashville, TN. The insulin, c-kit, CD34 and Bcl-2 antibodies were monoclonal mouse anti-human IgG1 type, while the rest of the antibodies were rabbit anti-human. Monolayers were fixed with either 4% paraformaldehyde at 4°C, 10% formalin at room temperature or methanol at -20°C, 10 min, depending on the suggestions of the antibody manufacturers. Plates were stained with multiple primary antibodies using greased 8-mm cloning cylinders (Bellco Biotechnology, Vineland, NJ), which were attached to the areas of interest after plates were washed with phosphate-buffered saline (PBS) and blocked with 5% lamb serum (Gibco Brl, Burlington, Ont., Canada). Primary antibodies (50-100 µl/cylinder) in

appropriate dilutions were added for overnight incubation at 4°C. Normal rabbit serum and IgG1 isotype antibodies (same dilution) were used as negative controls to exclude non-specific staining. After washing several times with PBS, antigens were detected using a biotin-HRP-based Histostain-plus bulk kit and an AEC (red) chromogen/substrate system (Zymed Laboratories, San Francisco, CA). Alternatively, an ABC detection kit (Vector Laboratories, Burlington, Ont., Canada) was used, followed by diaminobenzidine (DAB) staining.

BrdU immunostaining

To assess DNA replication in the small cells 0.1 mM bromodeoxyuridine (BrdU) (Sigma-Aldrich, Oakville, Ont., Canada) was added to the culture medium for 24, 72 h, 7, 14 and 28 days. In the case of a long-term labeling, medium with freshly added BrdU was changed every 2 days. Incorporated BrdU was detected with monoclonal anti-BrdU antibody, purchased from Sigma-Aldrich or Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada), followed by Histostain-plus/AEC system (Zymed Laboratories, San Francisco, CA) as described above.

Electron microscopy

Processing of samples for electron microscopy was performed in the Department of Pathology, Montreal General Hospital. Cell clusters were fixed in 1.0% glutaraldehyde/4% formaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C, for 30 min or longer, and postfixed in 1.0% osmium tetroxide in 0.1 M phosphate buffer for 30 min at 4°C. Following dehydration through a graded series of acetones, samples were embedded into Epon 812 and polymerized at 60°C overnight. Ultrathin 60-nm sections were cut on an Ultracut-5 ultramicrotome, stained with uranyl acetate and lead citrate and examined on a Philips CM10 electron microscope.

Insulin secretion

To measure the basal secretion of insulin, samples of culture media were harvested 24 h after media change and collected at -20°C until further testing. Quantitative determination of insulin was performed using a 1-2-3 porcine insulin ELISA kit and a 1-2-3 human insulin ELISA kit (Alpco Diagnostics, Windham, NH) for canine and human samples respectively.

Glucose responsiveness of cultured cells was studied in the course of glucose challenge experiments, carried out in RPMI supplemented with 0.5% bovine serum albumin (BSA) and containing glucose in either low (2.2 mM) or high (22 mM) concentration as previously described (Wang and Rosenberg *1999*). The course of glucose stimulation was as follows: low glucose 60 min - high glucose 30 min - high glucose + 50 µM 3-isobutyl-1-methylxanthine (IBMX)/or 20 mM arginine (Sigma) for 30 min - low glucose 60 min.

Results

Cell isolation and appearance of primary cultures

Freshly isolated islets, plated into tissue culture plates, immediately attach to the plastic. Within a couple of days cells start migrating from the islets, forming rounded areas of polygonal and elongated cells. The dynamics of cell attachment and spreading are basically the same for canine and human islets. In both species this cell population appears to be quite heterogeneous at first but becomes more homogeneous as cells form a monolayer after approximately a week in culture. As cells spread out of the islet core, they usually increase in size and lose their endocrine phenotype.

Among the variety of cell types forming a monolayer, we found scattered groups of small cells, which are either flattened or clustered into small rounded clusters and are very distinct from the other cells (Fig. 1). Single cells of this type can also be seen. These cells are scarce and represent less than 1% of the total islet cell population. Distinctive features of these cells become more obvious with prolonged periods in culture - unlike most cells, they remain small, rounded or hexagonal in shape and usually maintain close cell contacts with each other (Fig. 1). Because of their small size, about 7-10 µm, we termed these cells "small cells." To characterize these cells we used a number of tissue culture techniques, along with immunocytochemistry and electron microscopy.

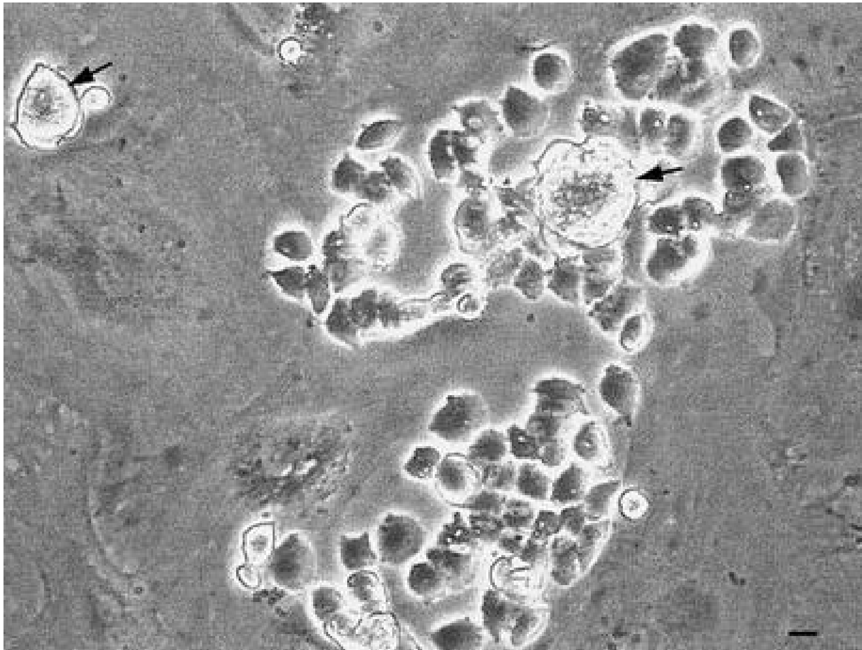


Fig. 1. Phase-contrast micrograph of a 2-month-old primary culture of canine islets. Two groups of small cells are clearly visible among large cells. The three-dimensional clusters of small cells (*arrows*) are typical of these cultures. *Scale bar* 10 μm

Morphology of the small cells

Ultrastructurally, the appearance of small cells is consistent with a fairly undifferentiated phenotype - the cytoplasm contains poorly developed endoplasmic reticulum and Golgi complex, several small mitochondria and a small number of neuroendocrine granules (Fig. 2A). The neuroendocrine granules are of different types, including typical insulin, glucagon and somatostatin granules as well as an undefined type of granules. Typically, one cell contains predominantly one type of granule but sometimes granules of different type can be observed in the same cell. For example, the cell in the center of Fig. 2A contains only typical insulin granules characterized by a dense, often crystal-like core (black arrows). Two adjacent cells on the right contain predominantly glucagon-like dense granules without a core (white arrows) but a few insulin granules as well. Coexistence of somatostatin and insulin granules in the same cell has also been observed (Fig. 2B). Some cells also contain a few mucin droplets, but exhibit no other ductal markers. A distinctive feature of small cells grouped in clusters is that they are tightly packed together with finger-like invaginations between cell membranes (Fig. 2A, arrowheads) and desmosomes. This fact is consistent with the observation that cells in the small clusters are very difficult, sometimes impossible, to dissociate by enzymatic treatment and/or ethylenediaminetetraacetic acid (EDTA).

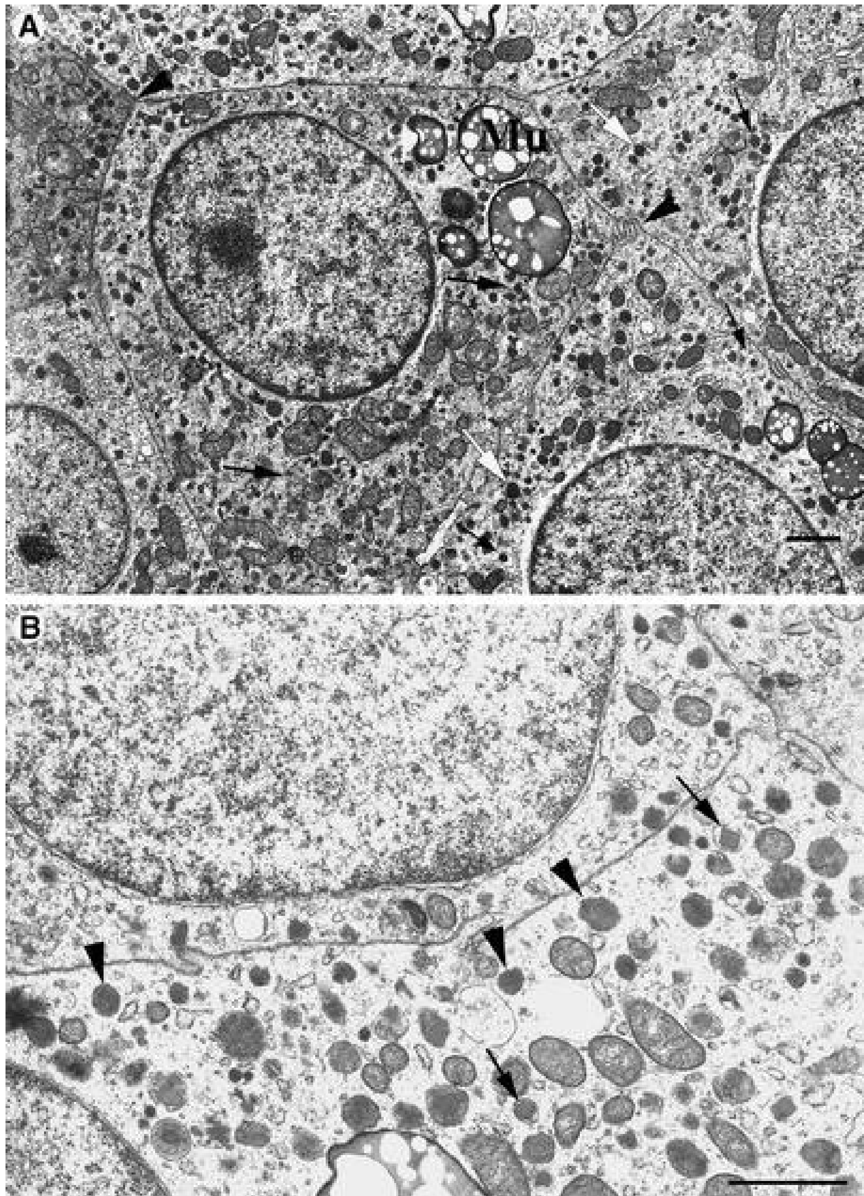


Fig. 2A, B. Electron micrographs of a small cluster (human) obtained by sieving the islets through a 25-µm pore-size mesh as described in "Materials and methods." **A** Small cells are characterized by cytoplasm of low density containing a small number of organelles, secretory granules (*arrows* indicate insulin granules containing a crystal core) and mucin droplets (*Mu*). *White arrows* indicate glucagon granules. Finger-like membrane invaginations (*arrowheads*) can be seen. **B** A fragment of a small cell coexpressing insulin (*arrows*) and somatostatin (*arrowheads*). *Scale bars* 1 µm

---

Results of immunostaining, summarized in Table 1 and Fig. 3, are overall consistent with the electron-microscopic observations, indicating that small cells belong to the neuroendocrine lineage. Thus, synaptophysin - a protein characteristic of neurosecretory granules, appears to be the most reliable marker for small cells (Fig. 3A). Small cells maintained in tissue culture plates are positive for insulin, glucagon somatostatin (Fig. 3B-D) and polypeptide P (Table 1). It should be noted that small cells maintain expression of synaptophysin and the islet hormones for a few weeks, whereas the rest of the cell monolayer, represented by dedifferentiated islet cells of a large size, lose it within a few days (Fig. 3A-D). Although some small cells contain more than one type of secretory granules as shown

electron microscopically, no double immunopositive cells were detected in our experiments (not shown). This fact suggests that despite an immature phenotype small cells are committed to different lineages. It cannot, however, be excluded that immunostaining is not sensitive enough to detect a small amount of a coexpressed marker. Importantly, most of the small cells express Pdx-1, a transcription factor required for insulin gene transcription and which is associated with pancreatic development and regeneration. The typical nuclear localization of PDX-1 is visible in small cells but not in the underlying cell monolayer (Fig. 3E). Although some small cells contain mucin droplets as shown electron microscopically, they do not react with antibodies to cytokeratin 19, a marker of ductal phenotype. Small cells did not immunoreact with antibodies for such stem cell markers as CD34, c-kit and nestin (Table 1, Fig. 3F), but stained positive for α -fetaprotein and Bcl-2 (Fig. 3G, H).

Table 1. Immunophenotyping of small cells

Marker	Staining
Insulin	+
Glucagon	+
Somatostatin	+
Polypeptide P	+
Synaptophysin	+
α -Fetaprotein	+
Bcl-2	+
Pdx-1	+
LIFR	+
TGF β 1 RI, RII	+
TGF β 1, 2, 3	-
Vimentin	-
Nestin	-
CK19	-
c-kit	-
CD34	-
Thy-1	-
Amylase	-

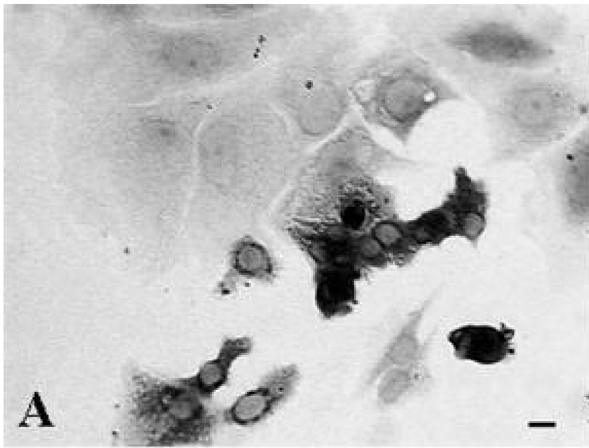
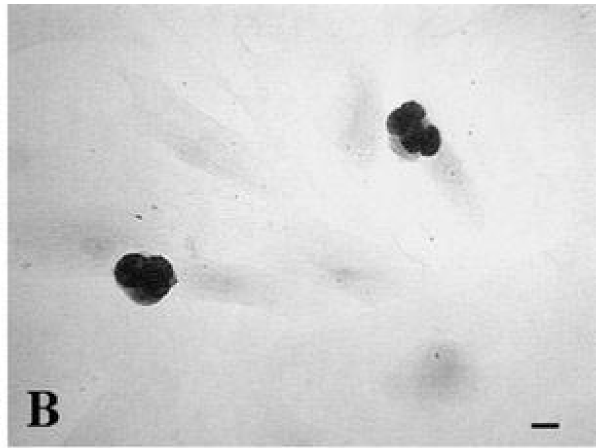
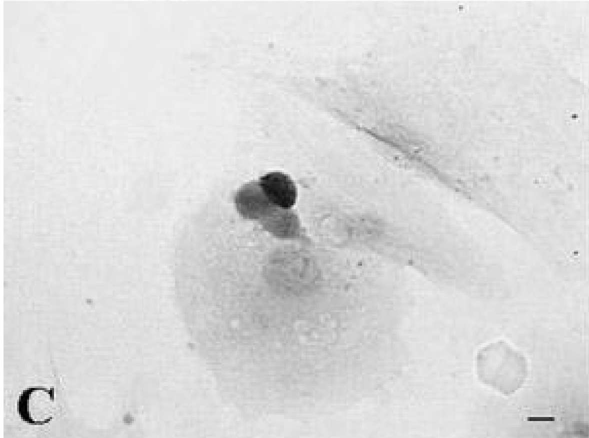
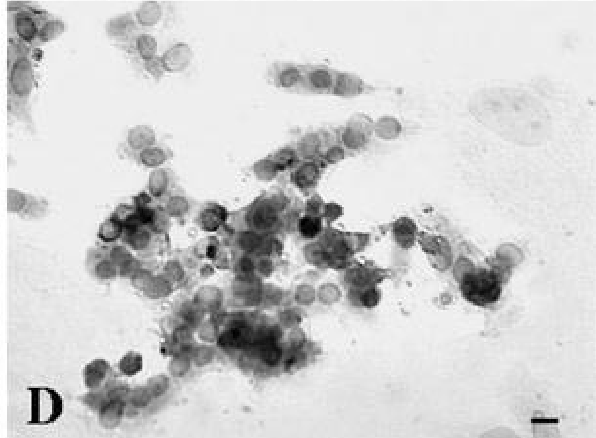
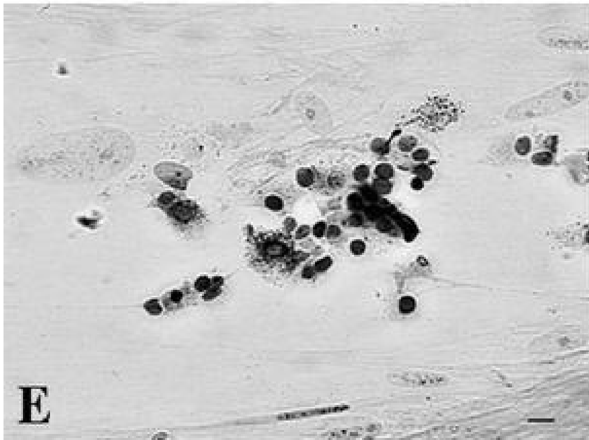
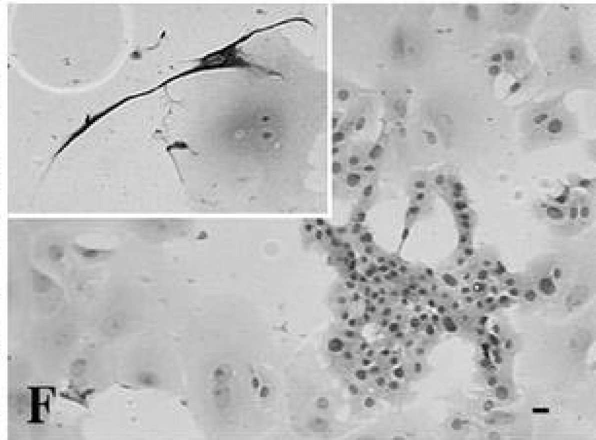
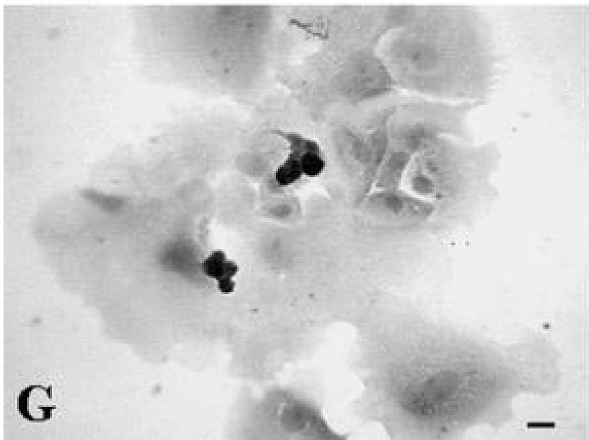
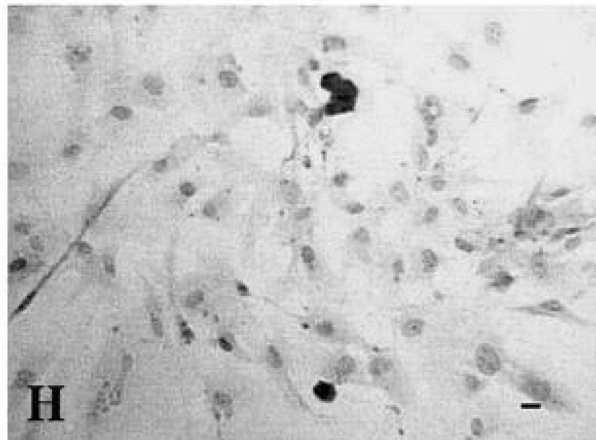

A

B

C

D

E

F

G

H

Fig. 3A-H. Immunocytochemical staining of primary islet cultures grown in 10-cm tissue culture plates. Small cells are immunopositive for: **A** synaptophysin (shown are human cells, 1 week after plating); **B** insulin (canine, 1 week); **C** glucagon (canine, 1 week); **D** somatostatin (human, 2 weeks); **E** PDX-1 (human, 6 weeks); **F** nestin (human, 1 week-small cells are negative, *insert:* a nestin-positive cell from the same culture, same magnification); **G** α -fetaprotein (canine, 1 week); **H** Bcl-2 (human, 4 weeks). Small cells are surrounded by other islet cells, which stain negative for all these markers. *Scale bars* 10 µm

---

To assess the distribution of small cells among the islets, we fractionated freshly isolated islet preparations using a number of screens with different pore size. We found that small cells were present within most of the islets but their frequency appeared to correlate inversely with the islet size - they were predominantly found in small islets (diameter <150 µm), while big islets (>300 µm) contained very few if any such cells. Besides being found within islets, small cells can be collated from a fraction of small clusters that pass through a 25-µm pore size mesh. These clusters were present in all canine and human islet isolation preparations we examined, and appear to be independent structures rather than chipped islet fragments produced by overdigestion during islet isolation. The small cells in clusters have the same morphology as cells found in the islets.

Proliferative status of small cells in vitro

In tissue culture small cells appear to be extremely quiescent so that groups of these cells remain virtually unchanged for up to 4 months, when maintained in the same flask with regular media changes. Remarkably, when the cultures were exposed to continuous labeling with BrdU for up to 4 weeks, small cells failed to incorporate the label while the rest of the cells in the same culture plates (dedifferentiated islet cells) incorporated BrdU at a rate of almost 100%, as expected for such a prolonged labeling period (Fig. 4A). Quiescence of small cells presents a real challenge for their characterization in tissue culture. Because of their quiescence, low numbers and small size, they are easily lost in mixed cultures when monolayers are subcultured and other, rapidly proliferating cells take over. To isolate small cells from monolayers we used either cloning cylinders or picked them with a pipette tip and transferred them into a new plate with or without subsequent trypsinization and/or treatment with EDTA or dispase.

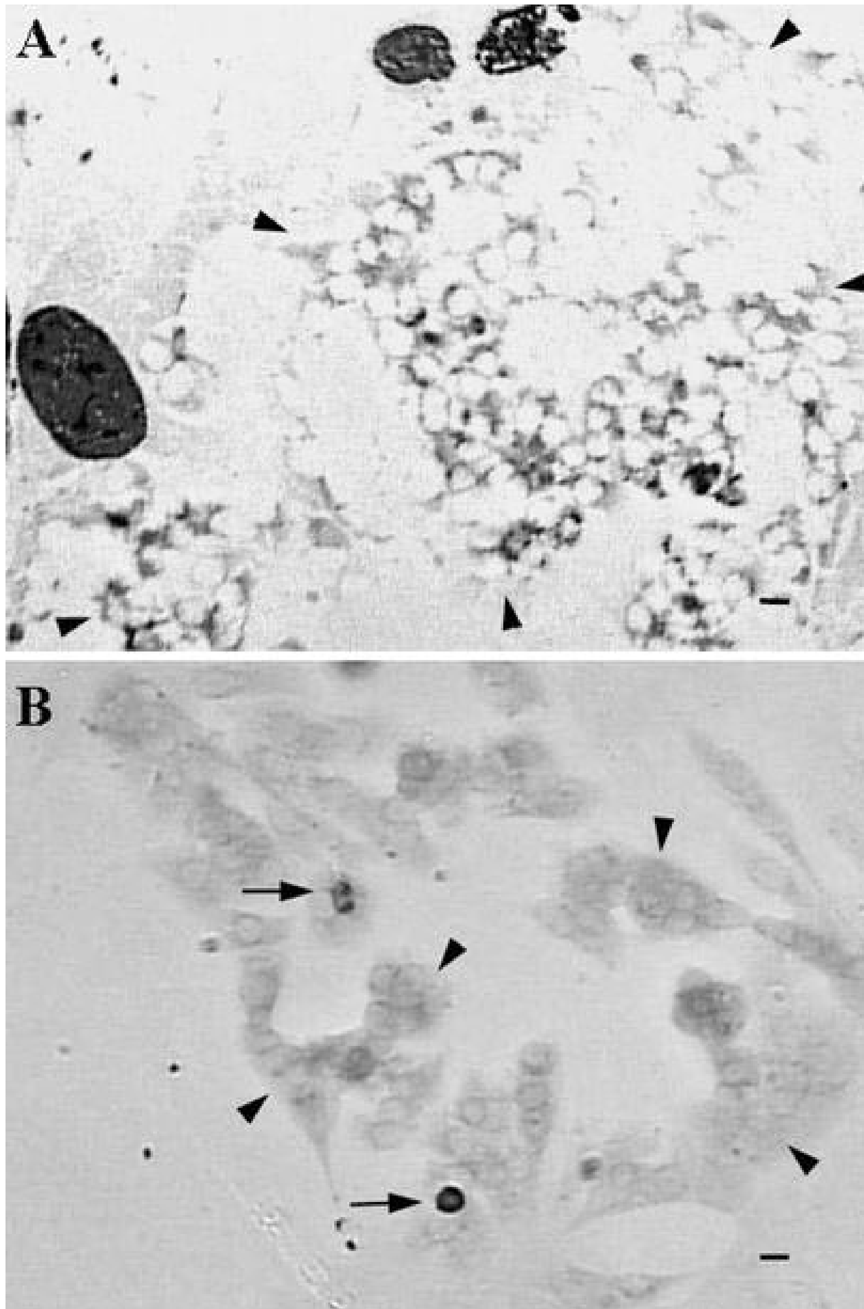


Fig. 4A, B. BrdU immunostaining. **A** Islet cell monolayers were continuously labeled with BrdU for 4 weeks. Incorporated BrdU was detected as described in "Materials and methods" without counterstaining. Shown are the areas of BrdU-negative small cells (*arrowheads*) and a few positive "big" cells; **B** islets were embedded into Matrigel and cultured with BrdU for 2 weeks, recovered with dispase and allowed to spread on plastic without BrdU. *Arrows* indicate BrdU-positive cells, *arrowheads* BrdU-negative cells. Cells were slightly counterstained with hematoxylin. *Scale bars* 10 μm

---

Our attempts to stimulate their proliferation by a number of factors, such as EGF, FGF-2, HGF, SCF, glucose (up to 22 mM), GLP-1, nicotinamide, LIF and INGAP, used alone or in combination and with and without serum have not been successful. Similarly, a number of extracellular matrices, including type 1 collagen, laminin, fibronectin and Matrigel, had no apparent effect on small cell quiescence. It

should be noted that the lack of proliferation does not seem to depend on the cell density. Thus, subculturing of small cells at different densities, both alone and in combination with other cells from monolayers, did not prove to be successful in stimulating proliferation.

Since small cells appear to prefer to stay clustered, it is possible that they depend on signaling mediated by direct cell-cell contacts and 3D islet structure, which is disrupted when the islets spread. To test whether small cells are able to proliferate in the intact islets, small islets (40-80 μm) were embedded into collagen (type I) or Matrigel to prevent spreading and to preserve the islet structure, and cultured for 2 weeks in the presence of BrdU. After 2 weeks, the cultures were treated with collagenase or dispase respectively, and collected islets were washed and allowed to spread on plastic for a week or two without BrdU in the medium, followed by fixation and immunostaining with a BrdU-specific antibody. Remarkably, as shown in Fig. 4B, we found single BrdU-positive small cells, usually one or two cells per cluster, although the frequency of the clusters containing positive cells was very low. This indicates that small cells do proliferate within the islets but they appear to be very slow cycling cells with a cell cycle of longer than 2 weeks. A more precise estimate has yet to be done. This also suggests that direct contacts with other islet cells are critical for small cell proliferation.

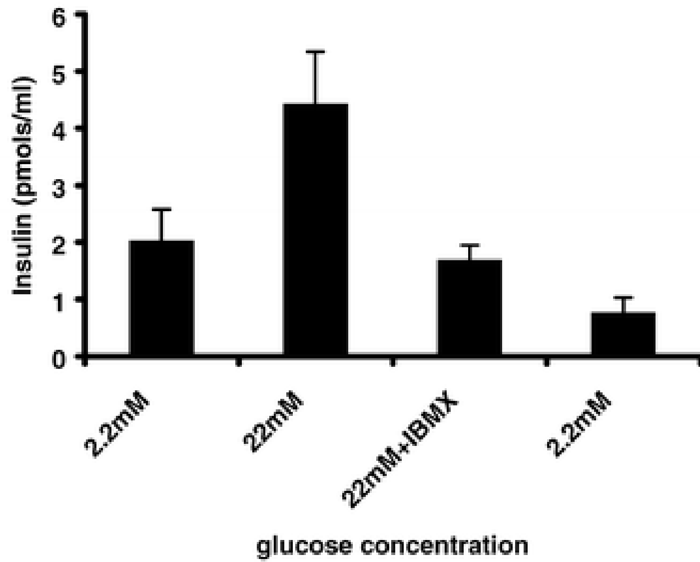
Insulin secretion

To determine if small cells secreted insulin, the culture medium was tested at different time intervals after islet isolation, using the insulin ELISA kit. For these experiments we used small human clusters, less than 25 μm in diameter, that were either sieved as described above, or handpicked and cultured in six-well plates in CMRL 1066, containing 10% FBS and 5.5 mM glucose. The number of dithizone-positive cells was counted after the samples of medium were taken. Roughly, small cells secrete 3 fmol/cell/24 h if tested within 72 h after islet isolation.

To determine whether small cells were glucose responsive, insulin secretion was studied in the course of glucose challenge experiments, carried out on small clusters 48-72 h after isolation in RPMI supplemented with 0.5% BSA and containing glucose in either low (2.2 mM) or high (22 mM) concentration.

The data of four experiments, summarized in Fig. 5A, show that insulin production by small clusters was glucose responsive - there was a 2.2-fold increase in insulin concentration after 30 min exposure to 22 mM glucose. Notably, this increase could not be further enhanced by either IBMX or another secretagogue, 20 mM arginine (not shown). In contrast, IBMX and arginine seem to inhibit the insulin secretion compared to high glucose alone. Human islets used as a control in this experiment responded to IBMX with higher or the same insulin release compared to high glucose alone (Fig. 5B). These data demonstrate the glucose responsiveness of small cells but suggest that mechanisms regulating the insulin release in small cells might be different from mature beta cells.

A



B

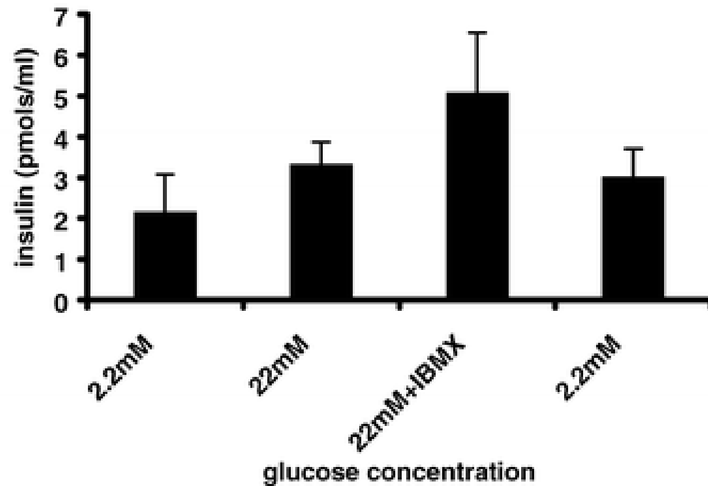


Fig. 5. Insulin release by human small cells **(A)** and islets **(B)** in response to glucose challenge. Approximately 5,000 small cells were plated into a six-well plate immediately after islet isolation, and 48 h later were subjected to a course of glucose stimulation: low glucose 60 min - high glucose 30 min - high glucose + 50 μM IBMX for 30 min - low glucose 60 min. Handpicked human islets (50) were maintained in non-tissue culture plates for 48 h after isolation and then underwent the same procedure as described above. Insulin content in the culture media is expressed as mean ± SEM

Unlike mature beta cells, which lose insulin expression immediately after spreading out of islets, the small cells continue secreting insulin for up to 8-10 weeks in culture, although in gradually decreasing amounts. This is another indication that the small cells represent a distinctive cell population within pancreatic islets.

Discussion

To the best of our knowledge, this is the first report describing a population of small, slow cycling or quiescent cells residing within normal canine and human pancreas. Our preliminary findings presented here show that these cells have a rather undifferentiated phenotype and belong to a neuroendocrine lineage as indicated by immunopositivity for synaptophysin and islet hormones.

These cells represent less than 1% of the total cultured islet cell population and can be found predominantly in primary cultures of spreading islets, although they become easily lost, or "diluted," by rapidly proliferating larger cells when cultures are split. This might explain why these cells have not been previously described when more traditional approaches to islet cell culture were used. Since these cells are quiescent, every consecutive passage makes detection of them in a culture plate less and less likely.

The origin and function of the small cells remain to be determined. Because small cells are immunopositive for α -fetaprotein, it is possible that they are the equivalent of the "oval cells" in the liver. Although they do not appear to express the markers found on the oval cells, such as CD34, c-kit, and Thy1, this possibility needs to be further explored.

We tried to identify small cells on histological sections of dog and human pancreas. One of the circumstances that make small cells more noticeable in tissue culture is that other cells increase in size right after spreading out from the islet, while small cells remain small. Hence, small cells may be less "visible" during histological examination of the pancreas where all cells maintain their original smaller size. Because markers specific for small cells have not been identified, they are not easily distinguishable from mature islet cells, when immunostained for islet hormones and synaptophysin. Identification of such markers, therefore, is important and could probably be done by genomic profiling of small cells. Interestingly, the pancreatic parenchyma contains scattered small areas of insulin-positive cells that do not seem to be connected to the ducts (our unpublished observation; Bouwens and Pipeleers *1998*). We think that they are likely to be the small clusters we isolated using 25-µm screens. Morphologically, small cells from these small clusters and those found in the islets look identical and stain for the same markers, but it is not clear whether they have the same differentiation potential.

It is not clear whether small cells in these clusters have a clonal origin. Our electron-microscopic observations indicate that although small cells have a similar phenotype, they are not identical and express different hormones. This may suggest that they belong to different lineages. However, the presence of small amounts of granules of a different type in the same cell may indicate a common origin of these cells.

It is important that the beta-lineage small cells secret insulin in a glucose-responsive fashion. However, the observed differences in their response to secretagogues such as IBMX and arginine, along with different behavior in tissue culture, suggest that these cells have different properties than mature beta cells. Another significant observation is that small cells retain the ability to secrete insulin for at least a few weeks in culture, while mature beta cells lose insulin expression within a few days. This pattern of insulin secretion correlates with expression of PDX-1, which transactivates insulin promoter and is required for insulin gene expression (Ohlsson et al. *1993*). If small cells could be

expanded in vitro without a loss in insulin secretion, it would have a significant therapeutic potential for the treatment of diabetes. Therefore, it is very important to try to induce proliferation of these cells, which, as this study shows, can be a difficult task. In our experiments small cells did not respond to the most common mitogens, used alone or in combinations. Obviously, this may simply indicate that the right growth factor(s) for these cells has not yet been found. Analysis of gene expression profiles may be a useful approach here to try to determine if small cells express receptors and/or signaling pathways that are absent in the other cells of the monolayer, and then to target those pathways specifically.

Another possibility is that small cells require the three-dimensional microenvironment of the islet, which includes direct contacts with other endocrine cells, blood vessels, nerves, fibroblasts and other islet components and which disappears when islets spread in a culture plate and endocrine cells dedifferentiate. Importantly, our results demonstrating some BrdU incorporation in small cells residing within the islets embedded in matrigel or collagen, support this possibility. Although embedding into collagen or Matrigel is a useful approach to preserve the islet structure, it should be noted that viability of the islets embedded in a matrix is limited to a few weeks. Therefore, to confirm these findings and to more precisely assess a cell cycle length of small cells, experiments on long-term BrdU labeling in vivo are required.

Success in stimulating proliferation of small cells would also be important with respect to determining their function in the islets. The observation that small cells possess a fairly immature morphology and are found predominantly in small, presumably growing islets, suggests that these cells may serve as progenitors. However, their apparent quiescence may contradict this idea. Although new experiments are needed to address this question, it should be noted that quiescence in vitro does not rule out the possibility that small cells are progenitors. First of all, quiescence in vitro does not necessarily reflect proliferative status of cells in vivo but may result from the absence of necessary growth factors in the culture medium. It may indicate that these cells require a particular governing signal or cascade of signals, which are present in vivo but not in vitro. Our data on BrdU incorporation demonstrate the importance of the intact islet structure for small cell proliferation. In this regard, transplantation experiments, as well as a long-term BrdU labeling, may provide an important insight into this issue.

It is interesting to note that quiescence is ascribed to many stem cells that may stay dormant for a long time and resume self-renewing cell divisions when the need arises (Fortunel et al. *1998*; Fuchs and Segre *2000*). Because a pancreatic stem cell has not been identified, it is tempting to hypothesize that the small cells described in this report are potential stem cells. Their small size, quiescence and immature morphology fit this hypothesis. Also, expression of PDX-1, which is associated with pancreatic development and regeneration of endocrine cells from pancreatic ducts in the adult pancreas (Kritzik et al. *1999*; Sharma et al. *1999*), supports the possibility that small cells are progenitors. Their ability to secret insulin in a glucose-dependent fashion does not contradict this notion either. Although a classic perception of stem cells is that they are undifferentiated cells, there is increasing evidence in recent years that stem cells possess certain morphological and biochemical features normally attributed to differentiated cell types (Anderson *2001*; Fuchs and Segre *2000*). Therefore, a potential pancreatic stem cell is likely to be a hormone-producing cell. Whether this is true is a question for future investigations.

Acknowledgements. The authors thank Maria Masluch of the Department of Pathology, Montreal General Hospital, for her assistance in the processing of cells for electron microscopy and Debby Agapitos for assistance with the study of insulin secretion.

References

Anderson DA (2001) Stem cells and pattern formation in the nervous system: the possible versus the actual. Neuron 30:19-35

Bonner-Weir S (2000) Perspective: postnatal pancreatic β cell growth. Endocrinology 141:1926-1929

Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE (1993) A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development. Diabetes 42:1715-1720

Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song K-H, Sharma A, O"Neil JJ (2000) In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci U S A 97:7999-8004

Bouwens L, Pipeleers DG (1998) Extra-insular beta cells associated with ductules are frequent in adult human pancreas. Diabetologia 41:629-633

Fernandes A, King LC, Guz Y, Stein R, Wright CVE, Teitelman G (1997) Differentiation of new insulin-producing cells is induced by injury in adult pancreatic islets. Endocrinology 138:1750-1762

Fortunel N, Batard P, Hatzfeld A, Monier M-N, Panterne B, Lebkowski J, Hatzfeld J (1998) High proliferative potential-quiescent cells: a working model to study primitive quiescent hematopoietic cells. J Cell Sci 111:1867-1875

Fuchs E, Segre JA (2000) Stem cells: a new lease on life. Cell 100:143-155

Gu D, Sarvetnick N (1993) Epithelial cell proliferation and islet neogenesis in IFN-γ transgenic mice.

Development 118:33-46

Guz Y, Nasir I, Teitelman G (2001) Regeneration of pancreatic β cells from intra-islet precursor cells

in an experimental model of diabetes. Endocrinology 142:4956-4968

Kritzik MR, Jones E, Chen Z, Krakowski M, Krahl T, Good A, Wright C, Fox H, Sarvetnick N (1999) PDX-1 and Msx-2 expression in the regenerating and developing pancreas. J Endocr 163:523-530

Madsen OD, Jensen J, Blume N, Petersen HV, Lund K, Karlsen C, Andersen FG, Jensen PB, Larsson L-I, Serup P (1996) Pancreatic development and maturation of the islet β cell studies of pluripotent

islet cultures. Eur J Biochem 242:435-445

Ohlsson H, Karlsson K, Edlund T (1993) IPF-1, a homeodomain-containing transactivator of the insulin gene. EMBO J 12:4251-4259

Paraskevas S, Aikin R, Maysinger D, Lakey JRT, Cavanagh TJ, Hering B, Wang R, Rosenberg L (1999) Activation and expression of ERK, JNK, and p38 MAP-kinases in isolated islets of Langerhans: implications for cultured islet survival. FEBS Lett 455:203-208

Rafaeloff R, Pittenger GL, Barlow SW, Qin XF, Yan B, Rosenberg L, Duguid WP (1997) Cloning and sequencing of the pancreatic islet neogenesis associated protein (INGAP) gene and its expression in islet neogenesis in hamsters. J Clin Invest 99:2100-2109

Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG (2000) Reversal of insulin-dependent diabetes using islets generated *in vitro* from pancreatic stem cells. Nature Med 6:278-282

Richards J, Larson L, Yang J (1983) Method for culturing mammary epithelial cells in rat tail matrix. J Tissue Cult Methods 8:31-36

Rosenberg L (1998) Induction of islet cell neogenesis in the adult pancreas: the partial duct obstruction model. Microsc Res Tech 43:337-346

Rosenberg L, Brown RA, Diguid WP (1983) A new model for the development of duct epithelial hyperplasia and the initiation of nesidioblastosis. J Surg Res 35:63-72

Sharma A, Zangen DH, Reitz P, Taneja M, Lissauer ME, Miller CP, Weir GC, Habener JF, Bonner-Weir S (1999) The homeodomain protein IDX-1 increases after an early burst of proliferation during pancreatic regeneration. Diabetes 48:507-513

Soria B, Skoudy A, Martin F (2001) From stem cells to beta cells: new strategies in cell therapy of diabetes mellitus. Diabetologia 44:407-415

Wang RN, Rosenberg L (1999) Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. J Endocrinol 163:181-190

Weissman IL (2000) Stem cells: units of development, units of regeneration, and units in evolution. Cell 100:157-168

Yuan S, Rosenberg L, Paraskevas S, Agapitos D, Duguid WP (1996) Transdifferentiation of human islets to pancreatic ductal cells in collagen matrix culture. Differentiation 61:67-75

Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Müller B, Vallejo M, Thomas MK, Habener JF (2001) Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 50:523-533