Co-localization of nestin and insulin and expression of islet cell markers in long-term human pancreatic nestin-positive cell cultures

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Abstract

Strategies to differentiate progenitor cells into β cells in vitro have been considered as an alternative to increase β cell availability prior to transplantation. It has recently been suggested that nestin-positive cells could be multipotent stem cells capable of expressing endocrine markers upon specific stimulation; however, this issue still remains controversial. Here, we characterized short- and long-term islet cell cultures derived from three different human islet preparations, with respect to expression of nestin and islet cell markers, using confocal microscopy and semi-quantitative RT-PCR. The number of nestin-positive cells was found to be strikingly high in long-term cultures. In addition, a large proportion (49.7%) of these nestin-positive cells, present in long-term culture, are shown to be proliferative, as judged by BrdU incorporation. The proportion of insulin-positive cells was found to be high in short-term (up to 28 days) cultures and declined thereafter, when cells were maintained in the presence of 10% serum, concomitantly with the decrease in insulin and PDX-1 expression. Interestingly, insulin and nestin co-expression was observed as a rare event in a small proportion of cells present in freshly isolated human islets as well as in purified islet cells cultured in vitro for long periods of time. In addition, upon long-term subculturing of nestin-positive cells in 10% serum, we observed reappearance of insulin expression at the mRNA level; when these cultures were shifted to 1% serum for a month, expression of insulin, glucagon and somatostatin was also detected, indicating that manipulating the culture conditions can be used to modulate the nestin-positive cell’s fate. Attempts to induce cell differentiation by plating nestin-positive cells onto Matrigel revealed that these cells tend to aggregate to form islet-like clusters, but this is not sufficient to increase insulin expression upon short-term culture. Our data corroborate previous findings indicating that, at least in vitro, nestin-positive cells may undergo the early stages of differentiation to an islet cell phenotype and that long-term cultures of nestin-positive human islet cells may be considered as a potential source of precursor cells to generate fully differentiated/functional β cells.


Introduction

Islet transplantation has the potential to become a widely applicable treatment for type 1 diabetes mellitus. Successful islet transplantation, accompanied by an alternative immunosuppressive scheme, has recently been reported (Shapiro et al. 2000). However, one of the most severe limitations of this approach is the scarcity of transplantation
material, namely, purified islets. Therefore, strategies to select for cells capable of differentiating in vitro into β cells have been viewed as attractive alternatives to increase β cell availability prior to transplantation.

Understanding the mechanisms of islet cell differentiation, together with a more detailed knowledge of the signals driving later steps in β cell differentiation, constitutes an important step towards in vitro growth and differentiation of precursor cells. However, the exact nature of the pancreatic stem cell is still not well defined (Berna et al. 2001). It has recently been suggested that nestin-positive cells present in rodent and human islets could constitute multipotential stem cells (Hunziker & Stein 2000, Zulewski et al. 2001, Abraham et al. 2002, Esni et al. 2004). However, this issue is still controversial, since several recent publications have correlated nestin expression with endothelial cells (Lardon et al. 2002, Humphrey et al. 2003, Klien et al. 2003, Treutelaar et al. 2003). Human and mouse pancreatic progenitor cells do not express nestin during development (Piper et al. 2002, Selander & Edlund 2002), whereas nestin was shown to be expressed in mesenchyma but not in epithelial cells of the developing pancreas (Selander & Edlund 2002, Humphrey et al. 2003). It has also been demonstrated that nestin is expressed in reactive stellate cells in both normal and regenerating rat pancreas (Lardon et al. 2002). Differentiation of nestin-positive cells is also controversial, with Zulewski et al. (2001), Abraham et al. (2002) and Huang & Tang (2003) showing expression of endocrine markers by these cells, whereas Humphrey et al. (2003) did not find them to be able to differentiate in vitro or in vivo.

Here we analyzed the expression of β cell markers during long term culturing of nestin-positive cell populations expanded from primary cultures of purified human pancreatic islets by confocal microscopy and RT-PCR. We found co-localization of nestin and insulin as a rare event, both in freshly isolated islets and in long-term cultures enriched in nestin-positive cells. Low mRNA levels of insulin, glucagon and somatostatin were detected after prolonged subculturing in low serum medium without addition of differentiation-inducing factors. The attempt to induce differentiation of nestin-positive cells by subculturing onto Matrigel did not promote any increase in insulin expression, in spite of the formation of islet-like clusters. The expression of islet cell markers observed in long-term cultures corroborates previous findings that, at least in vitro, nestin-positive cells may undergo the early stages of differentiation to an islet phenotype, deserving further studies to address their potential role as an alternative β cell source.

Materials and Methods

Islet isolation and purification

In order to obtain highly purified human islet preparations, we followed the procedure based on that described by Shapiro et al. (2000), with minor modifications. Briefly, upon informed consent from the donors’ relatives, pancreata were removed from cadaveric donors in a multi-organ procurement program after in situ vascular perfusion with cold University of Wisconsin (UW) solution. Pancreata were immediately transported to the Human Pancreatic Islet Unit for processing. Pancreatic islets were isolated by perfusion and digestion with Liberaze-HI purified enzyme blend (Roche, Indianapolis, IN, USA) via the Wirsung duct. Digestion was monitored throughout the digestion procedure by staining samples of pancreatic tissue with dithizone (diphenylthiocarbazone, Sigma), which specifically stains Zn2+-containing insulin granules present in insulin producing β cells. Digestion was interrupted when the majority of the islets were free from the exocrine tissue and pancreatic islets were purified in continuous Ficoll gradients using an aphaeresis/cell separator system (Cobe 2991 cell processor, Gambro BCT, Inc, Lakewood, CO, USA). Islet-enriched fractions were pooled, stained with dithizone and quantified by counting under the microscope with the help of an optical net and then converted to the standard number of islet equivalents (IEQ), which represents the number of 150 μm diameter islets present in the sample. Islet cell viability, assessed by a fluorimetric cell viability assay (Live/Dead) according to Bank (1987), was usually greater than 80%. The purity of each preparation was assessed by comparing the amount of dithizone-stained endocrine tissue with the unstained exocrine (acinar) tissue.

The most purified islet fractions were pooled and cultured in plastic tissue culture flasks in the presence of CMRL 1066 (GIBCO) medium supplemented with 10% FCS (Cultilab, Campinas, São Paulo, Brazil). Adherent cells could be observed after 12 h in culture and, in a few days, cells formed a continuous monolayer, which was subcultured weekly at a 1:4 ratio.

To observe the effects of serum deprivation on the expression of islet cell markers, confluent cells grown on uncoated plastic maintained in CMRL 1066 medium supplemented with 10% FCS for 12 passages were washed with PBS and maintained for an additional 28 days in CMRL 1066 medium containing 1% FCS. After this period, insulin, somatostatin, glucagon, PDX-1 and nestin expression were evaluated by RT-PCR.

Immunocytochemistry

Cells were fixed and permeabilized with methanol for 10 min at 20 °C, followed by washing (three times) with cold PBS. After fixation, cells were incubated for 1 h at room temperature in blocking solution (2% BSA in PBS). After incubation with the primary antibodies, cultures were washed (four times) with PBS, incubated for 1 h at room temperature with the secondary antibody, and then washed (four times) with PBS. Coverslips were mounted using the Prolong Antifade Kit (Molecular Probes, Eugene, OR, USA).
Antibodies

The rabbit anti-human nestin polyclonal antiserum was a generous gift from Dr Conrad A Messam (National Institute of Neurological Disorders and Stroke, National Institute of Health, Bethesda, MD, USA) and used at a 1:200 dilution, as described in Messan et al. (2000). The mouse monoclonal antibody against human cytokeratin 19 (CK19) and the mouse monoclonal antibody against human insulin were obtained from ICN (Costa Mesa, CA, USA) and rabbit affinity purified polyclonal antibodies to insulin were obtained from Zymed (San Francisco, CA, USA) and diluted at 1:20 (ICN) and 1:50 (Zymed). Antibody against human vimentin (Amersham-Pharmacia) was diluted 1:50. Secondary antibodies were conjugated to either Texas Red or FITC (Molecular Probes) and used at 1:200 dilution.

Culture of nestin-positive cells on Matrigel substrate

To test the differentiation-inducing ability of Matrigel, nestin-positive cells originating from different islet preparations obtained from different pancreas donors were maintained in culture dishes coated with Matrigel (50 µl solution/cm²) for 3 to 120 h. Matrigel is a preparation of murine sarcoma basement membrane (Collaborative Biomedical Products, Bedford, MA, USA). For RT-PCR analysis, cells cultured for 44 and 95 days were subcultured in Matrigel for 48 h before RNA purification. Control cultures were kept in uncoated plastic plates.

Confocal analysis

Coverslips were observed under a BioRad 1024-UV confocal system attached to a Zeiss Axiovert 100 microscope, using a ×40 NA 1·2 plan apochromatic water immersion objective. All confocal immunofluorescence microscopy images presented here correspond to single optical sections.

DNA synthesis assay

To assess proliferation of nestin-positive cells, BrdU incorporation was measured. Cells (5·0 × 10⁴) were subcultured in the presence of 10% FCS onto coverslips in 60 mm plates. After incubation for 12 h with 10 µM BrdU, cells were fixed in cold methanol for 10 min and washed twice in cold PBS. BrdU incorporation was revealed with an anti-BrdU antibody, using the Amersham-Pharmacia Kit. Triple staining was achieved by staining with the anti-BrdU and anti-human nestin antibody plus 0·5 µg/ml DAPI (4,6-diamidino-2-phenylindole; Sigma); an anti-mouse antibody conjugated to Texas Red for the anti-BrdU and an anti-rabbit antibody conjugated to FITC for the anti-nestin were used. The percentage of BrdU-positive nuclei and BrdU/nestin-positive cells was determined by counting at least six different microscope fields.

Basal insulin secretion

Cells were plated into 24-well plastic dishes or in dishes coated with CMRL 1066 medium:Matrigel solution/cm² (50:1) at a density of 10⁴ cells/well and cultured for 48 h in CMRL 1066 (Mediatech Cellgro, Miami, FL, USA) supplemented with 100 U/ml penicillin and 5% FCS. On the following day, the cells were incubated with CMRL supplemented with 0·5% FCS for 4 days. Supernatants were collected and stored at −20 °C until assayed for insulin. Secreted insulin was quantified in conditioned media from each well by electrochemiluminescence assay ELECSYS (Roche). The reagent is highly specific for the detection of human insulin and cross reaction to proinsulin or C-peptide is <0·01%. Nondetectable insulin levels were verified in the control samples. Results were normalized to cell number. Data are presented as the mean ± s.e.m. Each experiment was repeated with triplicate values within each group. The statistical differences between group means were tested by an unpaired two tailed t-test. A P value <0·05 was considered statistically significant. The calculations were performed using the Prism software version 3·03 (Graph Pad Software Inc., San Diego, CA, USA).

RT-PCR

Total RNA was prepared from cell cultures maintained until the 12th passage in CMRL 1066 medium supplemented with 10% FCS and then shifted to low (1%) FCS concentration for an additional 28 days, and also from freshly isolated islets, by guanidine thiocyanate/mercaptoethanol lysis followed by ultra centrifugation on CsCl cushions (Chirgwin et al. 1979). Reverse transcription was carried out using SuperScript II RT and oligodT
priming and 5 µg of total RNA, according to manufacturer’s instructions (Invitrogen). First strand cDNA products were treated with RNase H and diluted 1:20 in Tris-EDTA buffer. PCR amplification for each gene studied was performed under the following cycling conditions: initial denaturation at 95 °C for 1 min, denaturation at 95 °C for 30 s, annealing at 57–66·5 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. Reactions were carried out in 20 µl final volume, containing 1U of Taq polymerase (Biolase, Bioline Inc., Reno, NV, USA), 1 × PCR reaction buffer, 1·5 mM MgCl2, 0·2 mM dNTPs and 0·4 µM primers. Betaine was added to PCRs for nestin and PDX-1 cDNAs to 0·5 and 0·75 M final concentrations respectively, in order to improve amplification of these GC-rich templates. The number of cycles for the exponential phase of PCR amplification were determined to be 34 for PDX-1, 30 for nestin and 20 for GAPDH (Fig 8), and both 18 and 32 cycles were used in order to analyze insulin mRNA expression in samples with very high or very low levels of this transcript. Annealing temperatures were 66·5, 57, 65 and 60 °C respectively. GAPDH was used as an internal control to normalize for the amount of template used in the amplification reaction. The oligos used for nestin were based on Zulewski et al. (2001) (HNF: 5′-AGAGGGGAATTCCTGGAG-3′ and HNR: 5′-CTGAGGACCAGGACTCTCTA-3′). For GAPDH the oligos were those from the PCR-Select kit from Clontech. Primers INSF: 5′-CATCACTGTCTTCTGCCAT-3′ and INSR: 5′-TCCACCACCC TGTTGCTGTA-3′ were used for insulin, PDX1F: 5′-CATGAACGGCGAGGAGCAGTA-3′ and PDX1R: 5′-GTT GAAGCCCTCAGGCCAGG-3′ for PDX-1, SMTF: 5′-ACTCTCCAGCTCGGCTTTC-3′ and

Figure 2 Confocal microscopy of triple-labeled immunofluorescence of freshly isolated human pancreatic islet cells cultured for 24 h, depicted for (A) insulin (red), (B) cytokeratin 19 (green) and (C) DAPI staining for nuclei (blue). The superposition of A, B and C is shown in D. Note the strong staining for insulin in the center of the islet (A) and the presence of ductal cells restricted to the islet periphery (B). Scale bar in µm.
SMTR: 5’-TCAGAGGTCTGATATGGACAATAC-3’ for somatostatin and GLUCF: 5’-GCACACTACCA GAAGACA GCA-3’ and GLUCR: 5’-AAGCAATGT GGCCTCAGAAT-3’ for glucagon. Densitometry of at least three independent experiments was made by ImageQuant software (Amersham), normalized and plotted into XY graphs as mean ± S.E. To analyze gene expression in confluent cells maintained in CMRL 1066 medium supplemented with 1% FCS for 28 days, the primers for PDX-1 were changed as follows: PDX1F: 5’-GCCTTT CCCATGGATGAAGTCT-3’ and PDX1R: 5’-AAGT TCAACATGACGCCAGCT-3’. In these samples, the number of cycles were 20 for GAPDH, 35 for glucagon, somatostatin, PDX-1 and nestin and 39 for insulin.

To analyze gene expression in Matrigel compared with control (uncoated surfaces), the PCR cycling was at 95 °C for 2 min followed by 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min (aliquots were removed every three cycles between cycles 23 and 29 for nestin, 30 and 39 for insulin and between 22 and 28 for GAPDH), with a final extension step of 72 °C for 5 min. The amount of first strand template for PCR used for all reactions was normalized relative to GAPDH.

Results

Insulin and nestin expression in freshly isolated human pancreatic islets

The human islet preparations, obtained from cadaveric donors as previously described (Shapiro et al. 2000), were well preserved, as judged by their high viability (greater

Figure 3 Confocal microscopy of triple-labeled immunofluorescence of freshly isolated islets cultured for 96 h depicted for (A) insulin (green), (B) nestin (red) and (C) DAPI labeling for nuclei (blue). The superposition of A, B and C is shown in D. Note the strong staining for insulin in the center of the islet (A), the presence of nestin-positive cells among the insulin-positive cells and of regions of co-localization of both insulin and nestin (in yellow). Scale bar in µm.
than 85%) via assay by the Live/Dead method (data not shown), and the high degree of enrichment for dithizone-positive islet cells in culture, as shown in Fig. 1A and B. When examined by confocal microscopy, freshly isolated islets cultured for 24 or 96 h (Figs 2 and 3 respectively) display a reasonably high insulin staining in the core (Figs 2A, D and 3A) with CK19-positive cells on the periphery (Fig. 2B and D). A large proportion of cells were nestin-positive (Fig. 3B) and regions of co-localization of both insulin and nestin can be seen as yellow spots (Fig. 3D).

Characterization of long-term cultures of human pancreatic islets enriched for nestin-positive cells

Due to the observation of co-expression of the precursor cell marker nestin and the β cell marker insulin in a small population of cells from freshly purified islet preparations, we investigated whether co-expression of these markers would persist upon prolonged sub-culturing.

Three highly purified human islet preparations were cultured in tissue culture flasks immediately after isolation. The majority of the islets adhere to the surface in 24 h and, after 48 h, a cell monolayer radially growing from the islets and enriched for β cells is observed. β cells stained by dithizone were present for up to 14 days in culture (Fig. 4). When islet cell monolayer cultures were maintained in 10% FCS the insulin marker decreased with time, whereas after 60 days in culture nestin-positive cells constituted the great majority (Fig. 5A and B). The data presented in Table 1 shows that 30 day cultures replated onto uncoated plastic and maintained for another 3 days proliferate at high rates, as judged by the high proportion of BrdU-positive cells, and almost all of these proliferating cells are nestin-positive. Upon reaching confluence, at 5 days culturing,
the overall proliferative index is lower, but nestin-positive cells are still proliferating.

In long-term cultures, where nestin-positive cells predominate, some rare cells exhibited co-localization of insulin and nestin (Fig. 5B). In spite of the high proportion of ductal cells found around fresh islets (Fig. 2), very few CK19 positive cells were observed even in short-term (Fig. 6) or in long-term cultures. Vimentin immunostaining, which labels mainly cells of mesenchymal origin such as fibroblasts and endothelial cells, was rarely observed during the entire observation period (data not shown).

After 12 to 14 passages (depending on the islet preparation), confluent cells grown on uncoated plastic and maintained in 10% FCS, formed islet-like structures (Fig. 7A and B). The islet cell cultures established from all three human islet preparations behaved similarly in terms of adherence to plastic substrate, optical morphology and growth in culture.

### Table 1

Percentage of BrdU labeling and BrdU/nestin-positive cells in monolayer cultures obtained from adult islet cells, maintained for 30 days and subcultured for 3 and 5 days. Data represent the average ± S.D. of six different microscopic fields.

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>BrdU/nestin-positive cells (%)</th>
<th>BrdU labeling index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>49.7 ± 0.4</td>
<td>57.1 ± 1.4</td>
</tr>
<tr>
<td>5</td>
<td>31.6 ± 0.8</td>
<td>42.9 ± 0.6</td>
</tr>
</tbody>
</table>

**Islet cell marker expression in long-term cultured human islets enriched for nestin-positive cells**

We set up to systematically analyze how subculturing would affect the expression of insulin and nestin markers in these primary cultures of human pancreatic islets enriched for nestin-positive cells.

![Figure 6](image1.png)

*Figure 6* Confocal microscopy of triple-labeled immunofluorescence of human pancreatic islet cell cultures maintained for 14 days on uncoated plastic, stained for nestin (green), CK19 (red), and DAPI for nuclei (blue). Scale bar in μm.

![Figure 7](image2.png)

*Figure 7* Phase contrast microscopy of nestin-positive cells maintained in culture in uncoated plastic for 12 to 14 passages shows spontaneously formed islet-like structures.
Insulin secretion, measured in culture supernatants, reproducibly decreased with time with similar kinetics in all islet preparations cultured in vitro, being detected for no longer than 28 days in culture (representative measures of one of these preparations are presented in Table 2).

Since insulin could no longer be detected in the culture medium after 4 weeks in culture, we measured insulin expression at the mRNA level. Relative levels of insulin cDNA were estimated by exponential amplification from reverse-transcribed total RNA collected at different passages of cultures maintained in CMRL 1066 medium supplemented with 10% FCS. Nestin and PDX-1 were also included in the RT-PCR analysis so as to allow determination of the relative expression levels of β cell markers and the nestin putative cell marker precursor during long-term in vitro culturing. The GAPDH housekeeping transcript was used as an internal control for normalization.

The results, shown in Fig. 8, indicate that nestin mRNA is detected in islet cultures from days 1–77 in culture. The insulin transcript was detected for up to approximately 21 to 42 days in culture, depending on the culture preparation, with extremely low levels of insulin transcripts being detected after this period. PDX-1 expression was detected only in the first 21 days in culture, falling below the threshold detection level thereafter. Interestingly, an increase in insulin expression was detected in the last observed time point (70 and 77 days of culture) in two out of the three islet preparations, but was not accompanied by PDX-1 re-expression after long-term culture.

In order to decrease the cell proliferation rate and to potentially favor differentiation, long-term (12 passages) confluent cultures were shifted from high (10%) to low (1%) serum concentration for another 28 days and the expression of insulin, somatostatin, glucagon, PDX-1 and nestin in these cultures was analyzed. No PDX-1 expression was found, while nestin, somatostatin and glucagon expression was found after 35 PCR cycles and insulin expression could be detected after 39 PCR cycles (Fig. 9).

Table 2: Ability of islet cell long-term cultures to produce insulin. Note the insulin production for up to 28 days in culture. Data represent the average ± S.E.M.

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>Insulin secretion (μU/cell)</th>
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<tbody>
<tr>
<td>7</td>
<td>9.5 × 10⁻³ ± 5.0 × 10⁻⁴</td>
</tr>
<tr>
<td>21</td>
<td>1.7 × 10⁻⁴ ± 3.0 × 10⁻⁵</td>
</tr>
<tr>
<td>28</td>
<td>8.0 × 10⁻⁶ ± 1.0 × 10⁻⁷</td>
</tr>
</tbody>
</table>

Matrigel does not induce differentiation of nestin-positive cells, but increases the basal insulin secretion of cultures enriched for β cells

Since co-localization of insulin and nestin was occasionally observed both in freshly isolated islets and in long-term cultures of islet-derived nestin-positive cells, we inquired whether Matrigel, a known differentiation inducer for many cell types, would be able to drive acquisition of a β cell-like phenotype by the nestin-positive cells. For this purpose, confluent long-term cultures of nestin-positive cells maintained in 10% FCS were subcultured onto uncoated plastic or Matrigel-coated plates.

Upon culturing, control cells grown on uncoated plastic display an elongated, fibroblastic morphology (Fig. 10A and C), whereas cells plated onto Matrigel actively migrate to form cell clusters (Fig. 10B). After 24 h it is already possible to observe the formation of these clusters, which were clearly formed by 120 h (Fig. 10D).

Evaluation of insulin and nestin expression in these two conditions showed that nestin mRNA can be detected in both monolayer and Matrigel-coated cultures. Very low levels of insulin transcripts, if any, are found under both conditions, and Matrigel does not seem to modulate its expression (Fig. 11).

To test the effect of Matrigel on islet cell monolayer cultures which still contained β cells (as previously shown by insulin secretion to the media and insulin expression shown by RT-PCR), we evaluated insulin accumulation in the culture medium derived from 7- and 21-day cultures maintained on Matrigel for 4 days in comparison to cells maintained on plastic dishes. As shown in Fig. 12, accumulation of conditioned media of cells maintained in Matrigel was around 10-fold higher than that observed in cells maintained on an uncoated plastic surface.

Discussion

Controversial findings regarding the behavior of nestin-positive cells derived from islet cultures in vitro and the role of this cell during pancreas regeneration and embryogenesis in vivo have been reported. Some authors were able to demonstrate some level of differentiation of nestin-positive pancreatic cells toward endocrine and/or ductal pancreatic phenotypes in vitro upon specific stimulation (Zulewski et al. 2001, Huang & Tang 2003, Esmi et al. 2004) while, in vivo, nestin staining is absent from progenitor cells during pancreatic development (Piper et al. 2002, Selander & Edlund 2002) and is present in stellate and endothelial cells of normal and regenerating adult pancreas (Abraham et al. 2002, Lardon et al. 2002, Humphrey et al. 2003, Klien et al. 2003).
Figure 8 Fractionation in agarose gels (A) and densitometric data (B) of insulin, nestin, PDX-1 and the GAPDH housekeeping gene amplification products at the exponential range of RT-PCR. Long-term cultures of human pancreatic islets, obtained from three different donors, cultured for different periods of time onto uncoated plastic, showing that nestin-positive cells are present for long culture periods, but insulin is detectable only in the first month of culture. Insulin and PDX-1 were detected for up to 28 to 42 days in culture, with very low levels of transcripts being present after this period. Insulinoma and bladder carcinoma RNA were used as, respectively, positive and negative controls, in addition to the PCR control (no template).
Here we describe that both freshly isolated and cultured human islet preparations display co-localization of nestin and insulin as a rare event, suggesting that these cells could be undergoing early stages of differentiation to a β cell phenotype. This finding is in agreement with evidence recently described by Street et al. (2004), who showed co-expression of insulin and nestin in cells of some small islets in one pancreatic biopsy. In spite of the rarity of this co-expression in their study, these authors do not rule out an involvement of nestin in islet neogenesis. Moreover, Yashpal et al. (2004) have also demonstrated co-expression of nestin and insulin in a large proportion of cells in the prenatal rat pancreas and in rare cells in the postnatal pancreas. These authors suggest that mature endocrine cells derive from nestin-positive cells and lose their progenitor marker expression as they fully differentiate into the postnatal life.

We rarely observed co-localization of nestin and insulin in our long-term cultures. The scarcity of this co-localization probably reflects the culture conditions we used, which favored proliferation of the nestin-positive cells (as shown by the BrdU assay) instead of differentiation, since cells were maintained in 10% FCS with no differentiation factor added to the medium. In our long-term cultures, ductal cells and fibroblasts were poorly represented, insulin-positive cells decreased with time, while nestin-positive cells predominated and formed islet-like structures, as previously described (Selander & Edlund 2002, Esni et al. 2004).

During subculturing in the presence of 10% serum, low levels of insulin expression were detected and gradually decreased to become undetectable. However, after prolonged subculturing, low levels of insulin expression could be observed in two out of three monolayer cultures grown to confluency. In addition, long-term subculturing of these cells under low (1%) serum conditions led to detection of low levels of insulin mRNA, somatostatin and glucagon, at the limit of sensitivity of the RT-PCR technique, whereas no PDX-1 expression could be detected. We hypothesize that culture conditions that induce growth arrest or replicative senescence, and thus cell cycle exit, might favor entry of these cells into the early stages of pancreatic islet cell differentiation pathway. It is known, for other cell types, that cell senescence alters the expression of growth and differentiation-specific genes, probably due to an altered profile of transcription factors (Dimri & Campisi 1994). It has been demonstrated in Schwann cells that the signaling events associated with the expression of myelin-associated proteins are closely related with cessation of mitosis. Besides, growth arrest induction in an immortalized Schwann cell line initiates a sequence of changes that ultimately leads to terminal differentiation and the appearance of several myelin-related markers (Rushton et al. 1999). In fact, promoting in vitro terminal differentiation of progenitors of diverse tissues such as neuronal (Galderisi et al. 2003), hematopoietic (Taniguchi et al. 1999) and myogenic (Walsh & Perman 1997) tissues into fully differentiated and functional cells invariably requires induction of cell cycle exit, mostly by deprivation of growth factors and serum, and/or concomitant addition of differentiation-inducing factors. Presumably, pancreatic islet cell progenitors would be no exception and, therefore, growth arrest may be essential, although not sufficient, to promote differentiation toward a pancreatic islet cell.
phenotype that, at the early stages, expresses low levels of three islet hormones (insulin, glucagon and somatostatin). The lack of PDX-1 expression concomitantly with insulin expression in vitro has been previously reported by Huang & Tang (2003) in fetal nestin-positive cells treated with differentiation factors and could be explained by a complex post-transcriptional control of PDX-1 expression, as suggested by transgenic mice expressing the *E. coli* LacZ gene under the control of the PDX-1 promoter (Stoffers et al. 1999).

The evidence presented here, showing an increased insulin accumulation in the presence of Matrigel, is in good agreement with previous data showing the effect of this product on the maintenance of normal β cell function (Perfetti et al. 1996, Edamura et al. 2001, Oberg-Welsh 2001). However, in contrast with the transdifferentiation/differentiation effect that has been described for islet (Kerr-Conte et al. 1996), acinar (Arias & Bendayan 1993) and ductal cells (Bonner-Weir et al. 2000), our attempt to promote differentiation of nestin-positive cells by subculturing onto a Matrigel-coated surface only induced cell migration and formation of islet-like clusters after 24 h, with unaltered insulin and nestin expression levels. These findings suggest that culturing nestin-positive cells in Matrigel three-dimensional matrix alone may not be sufficient to induce differentiation, at least for the time point tested.

In summary, our report of co-localization of nestin and insulin and expression of islet cell markers in long-term human pancreatic nestin-positive cell cultures corroborates previous findings that, at least in vitro, nestin-positive cells can undergo early stages of differentiation to an islet cell phenotype. In spite of the fact that several *in vivo* models do not support a role for nestin-positive cells as an islet progenitor (Piper et al. 2002, Selander & Edlund 2002), they are able to express insulin, GLUT-2 (Huang & Tang 2003) and transcription factors normally expressed in insulin-producing cells such as PDX-1 (Zulewski et al. 2001, Huang & Tang 2003, Esni et al. 2004) and NKX6·1 and ISL1 (Huang & Tang 2003). Therefore, it cannot be ruled out that, under specific stimulation in culture, nestin-positive cells are fully able to differentiate into a mature, functional β cell phenotype, confirming the high level of plasticity which has previously been attributed to

**Figure 11** Analysis of insulin and nestin expression by semi-quantitative RT-PCR in long-term cultures 1 and 2 of nestin-positive islet cells maintained on plastic or Matrigel substrates for 44 and 95 days respectively. Nestin and insulin transcripts were amplified at the exponential phase of the PCR. The amount of different templates was normalized relative to GAPDH. Very low levels of insulin transcripts are occasionally present in long-term cultures and Matrigel does not seem to modulate its expression.

**Figure 12** Basal insulin secretion by islet cells growing in the presence or in the absence of Matrigel. Basal insulin secretion was measured, by an electrochemiluminescence assay, in conditioned media obtained from cells plated in plastic or Matrigel coated dishes which were incubated with CMRL supplemented with 0·5% FCS for 4 days. Data represent the mean ± S.E.M. (*n* = 3), *a* vs *b* *P*<0·05.
these cells (Blyszczuk et al. 2003), since under the influence of various factors, they were able to assume features of neural (Cattaneo & McKay 1990), hepatic and pancreatic cells (Lumelsky et al. 2001, Ensi et al. 2004).

Recently, Seaberg et al. (2004) described that multipotent precursor cells (named PMP) from adult mouse pancreas were able to differentiate into neurons, endocrine and exocrine cells and stellate cells. Nestin expression did not seem to predict the identity of these cells, but all of the colonies derived from these cells expressed nestin and did not co-express endothelial and epithelial cell markers. Based on these findings, the authors suggested the existence of a different nestin-positive progenitor cell other than those pancreatic epithelial cell progenitors previously described in vivo (Ensi et al. 2004, Klein et al. 2003, Selander & Edlund 2002). It still remains to be elucidated whether different populations of nestin-positive cells committed to different cell fates exist in different stages of pancreatic development.

Since the strategies used so far were not able to promote complete differentiation to a β cell phenotype of ductal, acinar or embryonic stem cells, further studies are necessary to establish the actual potential of nestin-positive cells as an alternative source of β cells for transplantation.

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