Nestin expression and clonal analysis of islet-derived epithelial monolayers: insight into nestin-expressing cell heterogeneity and differentiation potential

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Abstract

There has over the last several years been renewed interest in developing a system for generating new islets and a search for a self-renewing population in the pancreas. In particular, the neural stem cell marker nestin has been implicated as an islet precursor marker and its immunoreactivity has been localized in the islets of Langerhans. This study examines islet-derived epithelial monolayers expanded *ex vivo* to provide a source of nestin-expressing progenitor cells – a model that will help us understand the role of nestin-expressing cells in islet cell development. When cultured on a type I collagen gel, islets formed confluent monolayers which lacked endocrine phenotypes but were positive for cytokeratin 20 and contained an increased proportion of proliferating nestin-expressing cells, reaching a maximum of $54 \pm 10\%$. Co-expression

studies demonstrated that the nestin-positive cells are heterogeneous, with some nestin-expressing cells co-localizing with the transcription factor PDX-1 and glucose transporter type 2 or lack of co-expression with vimentin. When clonal populations of nestin-positive cells were expanded and subjected to a differentiation protocol, only a population that expressed the transcription factor PDX-1 at the mRNA level was capable of re-expressing insulin at the mRNA and protein level. In conclusion, these studies demonstrate that expanded nestin-expressing cells *in vitro* from islet-derived epithelial monolayers are heterogeneous; clonal analysis of nestin-positive cells reveals that a distinct subpopulation of nestin/PDX-1-expressing cells is capable of forming insulin-producing cells.

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Introduction

β -Cell precursors could theoretically act as a self-renewing and unlimited source of cells; however, their precise phenotypic nature and identifying markers need to be further examined. In recent years, cell populations expressing specific markers of undifferentiated cells such as the protein nestin have been implicated in participation in the neogenesis of newly forming islets. Nestin is a neuronal stem cell marker (Cattaneo & McKay 1990, Lendahl *et al.* 1990) which has been shown to generate a wide spectrum of cell types in the developing nervous system, mesenchyme and blood vessels throughout the embryo, and bone marrow cells (Kachinsky *et al.* 1994, Dahlstrand *et al.* 1995, Mokry & Nemecek 1998, 1999, Shih *et al.* 2001). It is an intermediate filament protein that is involved in cytoskeletal formation as well as cellular rearrangements to facilitate processes characteristic of undifferentiated cells such as migration and mitosis (Palm *et al.* 2000, Rietze *et al.* 2001). Its role in differentiation has been well documented in the central nervous system (CNS), where nestin is abundantly expressed in neuro-

epithelial stem cells during embryogenesis, but it is nearly absent from all mature CNS cells (Dahlstrand *et al.* 1995). Furthermore, freshly isolated neural stem cells (nestin-positive), when co-cultured with a muscle line, rapidly differentiate *in vitro* into myocytes, demonstrating that there is even more plasticity than originally envisaged in the nestin-positive population and that it can contribute to a wide range of cells lineages (Rietze *et al.* 2001).

Within the pancreas, there has been much evidence of nestin precursor activity, where nestin-positive cells in the pancreas of humans and rats have been described previously. Through the examination of nestin expression patterns, nestin localization has been demonstrated in the pancreatic islets and ducts (Hunziker & Stein 2000, Street *et al.* 2004) and nestin expression has been shown during embryogenesis to precede the appearance of β -cells (Abraham *et al.* 2002, Huang & Tang 2003). Street *et al.* (2004) revealed that in human neonatal pancreatic sections, there are an increased number of islet-associated positive nestin cells as compared with adult islets, suggesting a role for this marker in early pancreatic development. Our own *in vivo* rat study shows that during embryonic

development, nestin and insulin frequently co-localize, suggesting that mature β -cells may derive from a population of cells expressing nestin (Yashpal *et al.* 2004). Further evidence of the precursor function of nestin has been provided by the ability of islet-derived nestin-positive cells from both fetal and adult pancreas to differentiate *ex vivo* into pancreatic endocrine, exocrine and hepatic cell phenotypes (Zulewski *et al.* 2001, Huang & Tang 2003), that nestin expression was an intermediate step in the differentiation of insulin-producing cells from embryonic stem cells (Lumelsky *et al.* 2001) and when cultured under certain conditions, selectively expanded nestin-positive cells are capable of generating three dimensional clusters that resemble islet-like clusters with endocrine cell phenotypes (Lumelsky *et al.* 2001). Furthermore, human-islet-derived cultures enriched for nestin-positive cells express the transporters ABCG2 and MDR-1, showing that these cells have properties that are consistent with a pluripotent stem-cell like population (Lechner *et al.* 2002).

Controversy exists, however, over the use of nestin as a progenitor marker and its significance in islet neogenesis. Some studies report that nestin immunoreactivity is present in ductal epithelium, a pancreatic compartment that is known to harbor islet progenitors. At another extreme, others have reported that nestin only marks a population of mesenchymal or endothelial cell types, thereby excluding nestin's role in islet cell development (Lardon *et al.* 2002, Selander & Edlund 2002, Klein *et al.* 2003). Such differences in observations may be due to species-specific differences in nestin expression or choice of nestin antibodies used. In light of these observations, our own previous observations in the developing rat pancreas and that of Street *et al.* (2004) on the human pancreas demonstrate that nestin-positive cells are observed in small clusters of β -cells randomly distributed through the pancreas – indicating that nestin expression in these extra-islet cells is indicative of newly forming islets. Therefore, nestin cannot be completely excluded as a precursor marker and it is important to find a source of nestin-positive cells and to subsequently further evaluate the role of nestin in islet cell neogenesis and development.

Islet cell neogenesis is known to occur through the differentiation of an islet precursor which is present either in the ducts or within the islets. This study aims at expanding a source of nestin-positive cells from the islets of Langerhans, clonally isolating them, and subsequently using a protocol for the differentiation of this population in order to understand the role of nestin-expressing cells in the islet cell development. In view of the recent controversy over nestin-expressing cells, we demonstrate an added dimension in the complexity of this cell type and show that two separate clonally isolated populations can be derived, one which can re-express insulin, and another that cannot, reiterating the heterogeneity of this undifferentiated cell type in culture.

Materials and Methods

Islet isolation and culture

Timed pregnant Wistar rats were obtained from Charles River (Quebec, QC, Canada), housed individually under standard conditions and allowed to deliver naturally. All protocols were approved by the Animal Care Committee at the University of Western Ontario, and were in accordance with the guidelines of the Canadian Council on Animal Care. Isolation of the islets was carried on postnatal day 7. Rat pancreata were dissected and subjected to collagenase XI digestion (Sigma), as described previously (Wang *et al.* 2004). Eight postnatal pancreata were used per isolation and six different islet isolations were performed for each time point in the study. Purification was achieved by a modified Ficoll density gradient (McDaniel *et al.* 1984). Freshly isolated islets were placed on type I collagen gel and cultured in DMEM/F12 (GIBCO) serum-free medium, as described previously (Wang *et al.* 2004). The epithelial monolayers were subcultured when the cells had grown to near confluence. Representative islet cultures were examined immediately after isolation (day 0), and the derived epithelial monolayers were examined at 1, 2 and 4 weeks, and 2 and 4 months of the culture period using the following investigations.

Clonal nestin-positive cell purification

The islet-derived epithelial monolayers cultured between 4 and 8 weeks, a culture period during which there was an increased proportion of nestin-positive cells, were dissociated by incubation with 0.025% trypsin–EDTA. Clonal nestin-positive cells were achieved by hand-picking. Serial hand-picked cells which contained either single cells or two to five clustered cells were placed into 96-well plates and grown on type I collagen with the medium as described above. Once cloned cells grew near confluence, they were subcultured and separated into two groups – one group of cells was continually expanded and the other was examined for nestin immunoreactivity using immunofluorescence staining. In all processes, clonal cells which stained positively for nestin were selected for further islet differentiation experimental studies. To date, we have grown six pure nestin-expressing clones.

Immunofluorescence and morphometric analysis

Freshly isolated islets embedded in 2% agarose were fixed in 4% paraformaldehyde, and islet-derived epithelial monolayers and cloned nestin-positive cells were fixed *in situ*. Cell sections were immunostained with the primary antibodies as follows: mouse anti-rat nestin (Pharmingen, Mississauga, ON, Canada), mouse anti-cytokeratin 20 (CK20; Dako, Mississauga, ON, Canada), guinea pig

anti-human insulin and rabbit anti-human glucagon (Zymed, Markham, ON, Canada), mouse anti-5-bromo-2'-deoxyuridine (BrdU) (Sigma), rabbit anti-telomerase (CalBiochem, San Diego, CA, USA), rabbit anti-glucose transporter type 2 (Glut-2) (Chemicon, Temecula, CA, USA), rabbit anti-PDX-1 (gift from Dr Wright, University of Vanderbilt, Nashville, TN, USA) and rabbit anti-neurogenin 3 (ngn3), a gift from Dr German, University of California, San Francisco, CA, USA), using immunofluorescence, as described previously (Wang *et al.* 2004, Yashpal *et al.* 2004). The sections were incubated overnight at 4 °C with the appropriate dilution of primary antibody. To evaluate cell proliferation, freshly isolated islets and epithelial monolayers were incubated with 10 µM BrdU (Sigma), followed by immunostaining for BrdU (Wang *et al.* 2004). To identify co-expression of phenotypes in the nestin-positive cell populations, double immunofluorescence staining was performed. Fluorescent secondary antibodies labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Negative controls included the omission of the primary antibodies.

The number of cells expressing nestin and the BrdU labeling index, as well as the co-expression of phenotypes, was determined using a Lecia DMIRE2 inverted microscope connected to computer-assisted Openlab image software (Improvision, Lexington, MA, USA). For each time point, at least 1000 cells were counted, and each time point was repeated six times. The data are expressed as a percentage of the total number of cells counted and compared using a Student's *t*-test, with differences considered to be statistically significant when $P < 0.05$.

RT-PCR

Total RNA was extracted from freshly isolated islets, islet-derived epithelial monolayers and cloned nestin-positive cells using the RNeasy-4 PCR kit (Ambion, Austin, TX, USA). cDNAs were synthesized and PCR assays were performed (Wang *et al.* 2004). The PCR oligonucleotide primers used to amplify specific cDNA fragments are listed as follows: (i) nestin (rat): fw-5'-TTCCCTTCCCCCTTGCCATAATACC-3'; rv-5'-TGGGCTGAGCTGTTTTCTACTTTT-3' (464 bp), (ii) preproinsulin (human): fw-5'-TCACACCTGGTGGAAGCTC-3'; rv-5'-ACAATGCCACGCTTCTGC-3' (179 bp), (iii) glucagon: fw-5'-CAGAGGAGAACCCCAGATCA-3'; rv-5'-TCATGACGTTTGGCAAGTT-3' (202 bp), (iv) PDX-1: fw-5'-CCACCCCAGTTTACAA GCTC-3'; rv-5'-TGTAGGCAGTACGGGTCTC-3' (325 bp), (v) Glut-2: fw-5'-TTAGCAACTGGGTCTGCAAT-3'; rv-5'-GGTGTAGTCCTACACTCATG-3' (343 bp), (vi) β -actin: fw-5'-GACGGGGTCACCCACACTGTGCCCATCTA-3'; rv-5'-CTAGAAGCATTTGCGGTGGACGATGGAGG-3' (660 bp). The amplified

products were analyzed on 1% agarose gels and visualized by ethidium bromide staining. The controls involved omitting reverse transcriptase, cDNA and DNA polymerase and showed no reaction band. Sequencing of preproinsulin PCR products for verification was performed in the Robarts Research Institute Core Molecular Biology Facility (London, ON, Canada).

Differentiation culture and measurement of insulin content

To determine the potential of nestin-positive cells to differentiate into insulin-expressing cells, clonal nestin-positive cells were subcultured with a defined differentiation medium which contained DMEM:F12 (1:1) (Sigma) medium supplemented with 10% fetal bovine serum treated with or without 50 ng/ml keratinocyte growth factor (KGF) (ID Labs, London, ON, Canada) for 1 week (Wang *et al.* 2004). The epithelial monolayers cultured in a defined differentiation medium served as a control group. After 7 days of differentiation in culture, cells were collected for RT-PCR and morphological analysis. Insulin content and basal insulin release from cloned nestin-positive cells and islet-derived epithelial monolayers after 1 week in differentiation culture were measured using a rat insulin ELISA kit (DRG Diagnostic, Inc., Mountainside, NJ, USA). The insulin content within the cells from each experimental group was determined and normalized to the DNA content and expressed as ng per µg DNA (Wang *et al.* 2004).

Results

Nestin expression and proliferation in islet-derived epithelial monolayers

Our previous *ex vivo* examination of the developing rat pancreas (Yashpal *et al.* 2004) demonstrated that nestin-expressing cells were present during islet development in the fetal and postnatal pancreas, with frequent co-expression with insulin during embryonic life and a high expression level in the islets within the first week of life. We therefore isolated 7-day-old pancreata to examine and promote the expansion of nestin-expressing cells cultured on type I collagen matrix, and found that the staining for the neural stem cell marker nestin increased progressively over the first 4 weeks *in vitro* (Fig. 1A). Quantitative analysis demonstrated that the percentage of nestin-positive cells at the beginning of the culture period (day 0) was $15 \pm 2\%$ and increased significantly, reaching its peak value of 54% after 4 weeks of culture ($P < 0.01$, Fig. 1B). A constant expression of nestin signals at both protein and mRNA levels was maintained in long-term culture as well (Fig. 1C). A highly proliferative capacity was also observed in the epithelial monolayers, with $41 \pm 5\%$ of the monolayer cells labeling for BrdU after 4 weeks of culture with

Figure 1 Immunostaining for nestin in freshly isolated islet and islet-derived epithelial monolayers (A) and quantitative analysis of nestin-expressing cells (B) within islet-derived epithelial monolayers during the time of the culture. (C) The expression of nestin mRNA was determined by RT-PCR. ** $P < 0.01$, *** $P < 0.001$ vs day 0. Original magnification $\times 400$.

no subsequent significant change in the labeling index after 4 months *in vitro* (Wang *et al.* 2004). Double immunostaining for BrdU and nestin at 4 weeks of culture showed that $24 \pm 8\%$ of nestin-positive cells were BrdU labeled.

Telomerase is an enzyme that can re-lengthen telomeres, providing cells with the ability to proliferate continuously. Figure 2 shows single and double labeling of islet-derived epithelial monolayers using 4,6-diamidino-2-phenylindole (DAPI) to stain the nuclei and an antibody against the telomerase catalytic subunit to detect the presence of telomerase. The level of telomerase present in cells during the first 4 weeks of culture was almost undetectable, but frequent telomerase expression was found after 2 months with clear nuclear staining (Fig. 2A). The telomerase signal was located in cells at the edge of the monolayers, which is a region associated with proliferating

and migrating cells. Dual immunofluorescent staining for nestin with telomerase (Fig. 2B) indicated that the highly proliferative populations of nestin-positive cells co-expressed telomerase.

Co-expression patterns of nestin-expressing cells in islet-derived epithelial monolayers

To identify co-expression of nestin immunophenotypes in islet-derived epithelial cells, dual immunofluorescent staining was performed. There were two sets of co-expression pattern studies. First co-expression of nestin with transcription factors was examined. Co-staining of nestin with PDX-1 was observed at $15 \pm 4\%$ in the monolayers after 2 months of culture, while no co-expression of *ngn3* and nestin was detected (Fig. 3). Secondly, the co-expression of nestin with Glut-2, a

Figure 2 Expression of the telomerase catalytic subunit (A) in islet-derived epithelial monolayers at 4 weeks and 4 months of culture. Cells were stained with anti-telomerase antibody and with DAPI to visualize nuclei; arrows indicate the co-localization. (B) Dual immunofluorescent staining for nestin (FITC) and telomerase (TRITC) in islet-derived epithelial monolayers at 4 months of culture (arrows indicate the co-staining of nestin and telomerase; arrowheads show telomerase-positive nuclei only). Original magnification $\times 400$.

mature β -cell marker as well as a potential islet cell precursor marker (Pang *et al.* 1994), and with the mesenchymal marker vimentin was examined. There were $11 \pm 1\%$ of nestin-positive cells stained for Glut-2 and the majority of vimentin-positive cells expressed nestin with only $8 \pm 2\%$ of nestin-positive cells that negatively stained for vimentin in the monolayers after 2 months of culture (Fig. 3).

Cloned nestin-expressing cells and their potential to differentiate

To examine the potential for islet cells to re-form from nestin-positive monolayers *in vitro*, and more importantly to determine if nestin is a progenitor of insulin-producing cells, a defined differentiation culture medium was utilized to monitor islet cell differentiation. Six cloned

Figure 3 Co-expression of nestin (FITC) with PDX-1, ngn3, Glut-2 and vimentin (TRITC) in islet-derived epithelial monolayers at 2 months of culture determined by double immunofluorescence staining (arrows indicate the co-localization and arrowheads indicate the absent co-staining cells). Original magnification $\times 400$.

nestin-positive cells (Fig. 4A) were subjected to the differentiation treatment. Interestingly, only one (clone number 4017) of the clones showed co-expression of nestin with insulin and insulin with PDX-1 (Fig. 4B and C), while the rest of the clones were negative for either insulin or glucagon as well as PDX-1 staining (Fig. 4D).

RT-PCR analysis demonstrated that clone number 4017 clonally derived nestin-positive cells that co-expressed insulin, also expressed mRNA for the transcription factor PDX-1, as well as preproinsulin and glucagon mRNA (Fig. 5). Those insulin-negative nestin-cloned populations such as clone number 6018 did not express PDX-1 and

Figure 4 (A) Single nestin-positive cells cloned (clone number 4017) and expanded on type I collagen and immunostained for nestin to determine the purity of the nestin population. Double immunofluorescence for nestin (FITC) with insulin (TRITC) (B), and PDX-1 (FITC) with insulin (TRITC) (C) of clone 4017 was observed during the differentiation culture at 1 week (arrows indicate the co-expression phenotypes), but there is no co-staining of nestin with insulin observed in the rest of the nestin clones (D). Nuclei were counterstained with DAPI. Original magnification $\times 400$.

preproinsulin signals, with only a weak signal for glucagon. Both Glut-2 and nestin mRNA expression was observed in all cloned nestin-positive cells (Fig. 5). However, the signals of preproinsulin and glucagon from clone number 4017 nestin-positive cells and the islet-derived epithelial monolayers were weaker than that of freshly isolated islets,

and their cellular insulin content was detected at the lower levels as well (Table 1). In addition, we examined all clones for their expression of the exocrine cell marker amylase after being subjected to the differentiation culture. No amylase mRNA was detected in any of the clones (data not shown).

Figure 5 RT-PCR analysis of preproinsulin, glucagon, Glut-2, PDX-1 and nestin for cloned nestin-positive cells 4017 and 6018 after 1 week differentiation culture. Nestin +/KGF: differentiation medium contained KGF (50 ng/ml); Nestin +: only with differentiation medium; EPM: islet-derived epithelial monolayers contain nestin-positive cell populations cultured with differentiation medium; Islets: freshly isolated islets; RT – : omitting reverse transcriptase.

Discussion

Discovering a pancreatic stem cell is an important step towards creating an unlimited source of islets for transplantation to treat type 1 diabetes. Attention has shifted to nestin and we have characterized the nestin-expressing cells *in vitro* derived from postnatal rat pancreatic islets. Our study reveals firstly that islets expanded on type I

collagen are enriched with populations of nestin-expressing cells that are highly proliferative, can be maintained in long-term culture, and are characterized by their heterogeneous expression of protein markers such as PDX-1, Glut-2 and vimentin. Secondly, we demonstrate that nestin-positive populations can be isolated with distinct gene expression profiles and that only a particular nestin clone that expresses the transcription factor PDX-1

Table 1 Cellular insulin content and basal insulin release of cloned nestin-positive cells and islet-derived epithelial monolayer after 1 week in differentiation culture. Data presented are means ± s.d. The results were reproduced in triplicate wells

Samples	ng/μg DNA	ng/well
Nestin-positive/KGF (4017)	0.21 ± 0.10	0.28 ± 0.05
Nestin-positive (4017)	0.19 ± 0.05	0.22 ± 0.11
Islet-derived epithelial monolayers	0.36 ± 0.09	0.38 ± 0.09

Note: there are no detectable insulin release and content levels in the rest of the cloned nestin-positive cells.

can be induced to differentiate and re-express insulin. Taken together, this study reveals that nestin-expressing cells *ex vivo* display heterogeneity in their protein and gene expression profiles, and that a distinct fraction of the nestin-positive cells, which are marked through their expression of PDX-1, display endocrine precursor cell activity.

Islets cultured on type I collagen matrix rapidly adhered to the matrix, and spread to form a monolayer of cytokeratin-positive epithelial cells, as described previously (Wang *et al.* 2001, 2004). Epithelial monolayer formation was preceded by two events, the loss of insulin and glucagon phenotypes, and an increase in cells expressing nestin at both the protein and mRNA level respectively. The population of nestin-expressing cells was maintained at relatively high and constant levels throughout a 4 month culture period as determined by immunocytochemistry and RT-PCR. Our observations also demonstrated that dividing cells persisted within our epithelial monolayers as shown through the incorporation of BrdU. In particular, nestin and BrdU co-localization was also revealed. The observation of nestin and telomerase co-localization is of particular interest. Telomerase is a specific reverse transcriptase that synthesizes new telomeric repeats on the chromosome end to compensate for telomeric loss due to an 'end-replication-problem', providing the basis for an unlimited proliferative capacity (Krupp 2000). It is present in normal immortal human embryonic stem cells (Amit *et al.* 2000) and in germ line cells, both of which proliferate continuously without signs of aberrant growth control. The identification of nestin and telomerase co-expression suggests further that these nestin-positive cells are undifferentiated, immature cell types.

Analysis of the co-expression patterns of nestin with pancreatic transcription factors and the marker vimentin demonstrated that cells expressing this marker may be heterogeneous. In our previous *in vivo* examination of nestin expression in the developing rat pancreas, we found that there was a developmental progression of nestin expression that coincides with the formation of newly forming islets. During embryonic to postnatal transition, nestin-positive cells sequentially were characterized by signature protein markers, such that during embryonic life nestin and insulin frequently co-localized, but this occurrence was seldom seen in postnatal life. Also, nestin immunoreactivity was localized to the ducts early in postnatal life but shifted to encircle the ducts, a location of mesenchyme, which was further demonstrated by a population of nestin-expressing cells that co-localized with vimentin-expressing cells. In the present study, such observational findings *ex vivo* are also observed such that a small proportion of nestin-positive cells were found which expressed the early pancreatic transcription factor PDX-1 and Glut-2. Interestingly, less than 10% of nestin-expressing cells lack vimentin expression. Thus we concluded that nestin-positive populations are hetero-

geneous and may mark not only endocrine progenitors, but also mesenchymal and exocrine cells as well. Furthermore, the population of cells that express PDX-1 and Glut-2 may represent a subpopulation of nestin-positive cells which may play a potential role in islet cell development and retain precursor activity *in vitro* based on their expression of these early pancreatic progenitor markers.

To address the potential of these nestin-positive cells to differentiate into insulin-expressing cells we isolated nestin-positive cells through a single clone method. Previous studies have documented that nestin expression is an intermediate step in the differentiation of insulin-producing cells (Lumelsky *et al.* 2001, Yashpal *et al.* 2004); thus under the appropriate differentiation cues that have been used previously, we examined the potential of our nestin-cloned cells to re-express insulin and glucagon. No islet clusters were formed; however, insulin immunoreactivity and mRNA expression were found in a population of nestin-cloned cells that also expressed the transcription factor PDX-1 at the mRNA level. On the other hand, the majority of the nestin-cloned cell populations did not respond to the differentiation medium and thus lacked insulin or PDX-1 expression. This *ex vivo* observation reiterates that only a subset of nestin-expressing cells may contribute to newly forming β -cells. However, it must be noted that the insulin content was lower than that of normal islets, which indicates that adequate β -cell mass does not form in these cloned nestin-positive/PDX-1 cells. In particular future studies may be conducted to characterize the gene expression profiles, isolate and optimize differentiation of these nestin/PDX-1-positive cells, as well as the necessary cell-cell interactions and paracrine factors.

Nestin as a marker of pancreatic stem cells has indeed been a pancreatic conundrum. Importantly, no consensus has been reached on the use of nestin as a precursor marker. Although the usefulness of nestin as a precursor marker has been challenged (Lardon *et al.* 2002, Selander & Edlund 2002), our own *in vivo* study, and the works of others, illustrate that nestin expression coincides with the appearance and formation of insulin-producing cells (Lumelsky *et al.* 2001, Yashpal *et al.* 2004). Furthermore, nestin-positive cells have been confirmed through *in vitro* studies to form pancreatic endocrine cell types from adult rat, human and fetal human pancreas (Zulewski *et al.* 2001, Huang & Tang 2003). Controversy exists, however, at two levels – from expression pattern studies of nestin and from controversies associated with transgenic studies. First, studies have suggested that nestin is limited to mesenchymal and/or endothelial cells (Lardon *et al.* 2002, Selander & Edlund 2002, Klein *et al.* 2003). Such discrepancies in nestin immunoreactivity, however, may be accounted for by species-specific differences in nestin expression or differences between antibodies used and the heterogeneity of the nestin population. Secondly, the

debate against nestin as a progenitor marker has been further intensified by many studies that have examined progeny of cells that transcribe the nestin gene by marking them genetically through a Cre/loxP approach (Treutelaar *et al.* 2003, Delacour *et al.* 2004, Esni *et al.* 2004). Despite these elegant transgenic studies the answer to whether or not nestin is a stem cell marker for β -cells remains a mystery since nestin regulatory elements that are used in several of these studies may be incomplete, Cre expression may not accurately recapitulate the entire pattern of endogenous nestin expression or because nestin-positive cells from which endocrine cells arise are from a fraction of nestin-positive cells in which expression results from cis-regulatory elements not present in the transgenes used. Through Cre-mediated labeling, Esni *et al.* (2004) demonstrated that pancreatic exocrine cells in the developing pancreas originate from nestin-positive cells and that PDX-1 co-expressed with nestin in pancreatic epithelium. However, they were unable to exclude the expression of nestin in an early endocrine precursor. They concluded that there may be a low frequency of endocrine differentiation within the broader population of nestin-positive and *ngn3*-positive cells. Interestingly, in our own study, a very small proportion of nestin-cloned cells co-expressed the transcription factor PDX-1 and could differentiate to re-express insulin, suggesting that there may very well be a small subpopulation of nestin-positive cells with the potential to give rise to new endocrine cells, while none of the clones expressed the exocrine marker amylase. More recently, explants of neogenic ductules isolated from rats after partial pancreatectomy were characterized by their expression of nestin-positive ductal stem cells, which were found adjacent to newly forming single endocrine cells or small clusters (Kim *et al.* 2004). Importantly, a subpopulation of nestin-positive cells expressed PDX-1, a finding that is interesting in the context of our own study. These findings and our own only further suggest that nestin-expressing PDX-1-positive cells may in fact represent a cell population capable of cytodifferentiation into insulin-secreting cells.

In conclusion we have shown the presence of a subset of nestin-positive cells *in vitro* that under the appropriate environmental cues can differentiate and re-express insulin. The data presented strongly suggest that nestin is definitely a heterogeneous cell marker and that although the majority of nestin-positive cells in adults may not be islet precursors, there still remains a population that requires further investigation and examination. In particular the analysis and comparison of nestin clones should be closely examined and our study highlights that nestin-positive populations that express PDX-1 with the ability to re-express insulin are a population that must be examined and exploited. The involvement of nestin in islet cell neogenesis cannot be ruled out without an exhaustive assessment of all the nestin-positive populations expressing the protein. Isolation and clonal analysis of nestin-positive

cells will provide further insight into the cellular role of nestin in islet cell development and differentiation.

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