

# Phenotypic analysis of c-Kit expression in epithelial monolayers derived from postnatal rat pancreatic islets

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## Abstract

The limitation of available islets for transplantation is a major obstacle for the treatment of diabetes through islet therapy. However, islet monolayers expanded *ex vivo* may provide a source of progenitor cells and a model to help understand islet development from precursor cell types. The existence of progenitor cells within the islets is highly likely, yet, to date, no fully defined or characterized postnatal stem cell has been isolated, expanded or marked. Our study evaluates the expression of progenitor markers, including the haematopoietic stem cell marker c-Kit, in epithelial monolayers derived from postnatal rat islets through immunofluorescence and RT-PCR, and the ability of precursor-rich monolayers to reform islet-like structures. Islets formed confluent monolayers when cultured on a type I collagen gel which lacked endocrine phenotypes but were positive for cytokeratin 20 and contained an increased proportion of proliferating c-Kit-

expressing cells, with the proportion reaching a maximum of  $45 \pm 6\%$  at 8 weeks of culture. Evaluation of transcription factors at the mRNA level revealed constant PDX-1, ngn3 and Pax4 expression, while undifferentiated cell markers, such as Oct4 and  $\alpha$ -fetoprotein, were also detected frequently after 4 weeks of culture. Changing the extracellular matrix protein to laminin-rich Matrigel, the monolayers re-formed islet-like clusters that secreted insulin in a glucose-responsive fashion. Our data show that islets can be expanded *ex vivo* to form epithelial monolayers with rich undifferentiating cell populations that are characterized by cells expressing the progenitor markers. These monolayers are capable of extensive proliferation and retain plasticity to form new islet cells, and c-Kit-expressing cells may play an important role in new islet cluster formation.

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## Introduction

A major obstacle of islet transplantation is the limitation of available insulin-producing tissue (Serup *et al.* 2001). Stem cell biology provides an alternative to whole organ transplantation, as it offers the possibility of repopulating damaged or functionally inadequate tissues with specific cell types expanded *in vitro* that can restore normal biological activity (Ramiya *et al.* 2000). However, it is important to find a progenitor cell source for the application of stem cell biology to the treatment of diabetes.

Beta cell neogenesis has been shown to follow streptozotocin treatment in the neonatal rat (Wang *et al.* 1994), pancreatic duct ligation (Wang *et al.* 1995) and partial pancreatectomy (Bonner-Weir *et al.* 1993). These regeneration studies provide evidence of the existence of progenitor cells within the ducts, and they also suggest that intra-islet precursors may exist. However, an important question remains – how do we identify precursor cell types? Our

*in vivo* study identified subpopulations of cells within the islets immunopositive for the haematopoietic stem cell marker, c-Kit, and for a neural stem cell marker, nestin, in the pre- and postnatal rat pancreas which decreased in proportion with age, coincident with the appearance and development of endocrine cell types that normally accompany postnatal restructuring of the pancreas (Yashpal *et al.* 2004). These two candidate precursor markers have been previously described by others to be expressed in the pancreas in a fashion that suggests their role as putative progenitors (Rachdi *et al.* 2001, Zulewski *et al.* 2001, Huang & Teng 2003).

Of these precursor markers, c-Kit, a transmembrane receptor protein whose ligand is stem cell factor (SCF), is of critical importance for early epithelial stem cell differentiation in haematopoiesis and gametogenesis. c-Kit expression in the pancreas has been previously described in the pancreatic ducts (Obergh-Welsh & Welsh 1996), and it is implicated in the development, function and survival of the islets of Langerhans and, more importantly, in  $\beta$ -cell

survival (Welsh *et al.* 2000). Rachdi *et al.* reported that Kit mRNA is present in fetal and adult rat islets, and is specifically transcribed in  $\beta$  cells (Rachdi *et al.* 2001), as confirmed by our *in vivo* study on the developing rat pancreas. It is interesting that only a subpopulation of  $\beta$  cells express *c-Kit* at specific stages in their development (Rachdi *et al.* 2001, Yashpal *et al.* 2004). Furthermore, our previous *ex vivo* examination of the developing rat pancreas (Yashpal *et al.* 2004) demonstrated that islet development is accompanied by a decrease in the proportion of cells immunopositive for *c-Kit* during the prenatal to postnatal period of development, the highest expression occurring during the first postnatal week. We also demonstrated that *c-Kit* positive-cells lack islet hormone coexpression in the postnatal period, revealing their immature morphology.

The shortage of  $\beta$ -cells has prompted a search for a source of putative progenitors or stem cells and new mechanisms to increase donor  $\beta$ -cell mass. When islet monolayers form on extracellular matrix from the rat bladder carcinoma cell line 804 G, there is an increase in cell numbers but a decrease in islet-cell-specific gene expression (Beattie *et al.* 1997). Furthermore, islet expansion in monolayer cultures leads to a loss of  $\beta$ -cell function and senescence (Beattie *et al.* 2002). Our previous study (Wang *et al.* 2001) revealed that pancreatic islet isolation interrupts the islet–matrix relationship, induces some apoptosis, and leads to a loss of stability, resulting in transdifferentiation of an islet to a ductal phenotype on type I collagen through cAMP-pathway-dependent mechanisms. These two studies suggest that islet monolayers may be characterized by the expression of undifferentiated cell markers, which, once identified, will ease purification and manipulation of progenitor cell populations under the influence of appropriate environmental cues to re-form islet clusters or islet cell types through differentiation protocols.

The following study examines a mechanism for increasing precursor cell populations by culturing rat pancreatic islets on a matrix of collagen type I and subsequently changing the extracellular environment to promote islet cell reaggregation. Here we have reproduced our previously described transdifferentiation model (Wang *et al.* 2001) and reveal that during monolayer expansion, the proportion of insulin- and glucagon-expressing cells diminishes – accompanied by an increase in the proportion of *c-Kit*-expressing cells which can be immunomagnetically sorted and cultured. These monolayers also express signature transcription factors and other progenitor markers revealing the multitude of heterogeneous undifferentiated cell types in culture. This transdifferentiation model may serve to establish future strategies aimed at the subsequent differentiation of islet-like structures from clonally isolated progenitors or reformation of islets directly from expanded heterogeneous cell populations under controlled *in vitro* conditions.

## Materials and Methods

### *Islet isolation and culture*

Due to the increasing evidence that islets harbour intra-islet progenitors, we isolated 7-day-old pancreata to examine the development and changes in phenotype and morphology of islet cells when cultured on type I collagen matrix. Timed-pregnant Wistar rats were obtained for the isolation of islets on postnatal day 7 (Charles River, Quebec, Canada). All protocols were approved by the Animal Care Committee at the University of Western Ontario, in accordance with the guidelines of the Canadian Council on Animal Care. Rat pancreata were dissected and subjected to collagenase digestion (Sigma), as described previously (Ilieva *et al.* 1999). 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). The final preparation consisted of 90% dithizone-positive structures.

Freshly isolated islets (500 islets per 25 cm<sup>2</sup> flask) (Corning, Fisher, Toronto, Ontario, Canada) were placed on type I collagen gel (Wang & Rosenberg 1999) and cultured in DMEM/F12 (Gibco, Burlington, ON, Canada) serum-free medium supplemented with transferrin (10  $\mu$ g/ml), insulin (1  $\mu$ g/ml) and EGF (10 ng/ml) (Sigma) to promote epithelial monolayer formation (Bonner-Weir *et al.* 2000, Wang *et al.* 2001). Cultures were maintained in 95% air/5% CO<sub>2</sub> at 37 °C, and the medium was renewed on alternate days. 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, 4 and 8 weeks of the culture period in the following investigations.

### *Immunocytochemistry and morphometric analysis*

Freshly isolated islets embedded in 2% agarose were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin (Wang & Rosenberg 1999). Islet-derived epithelial monolayers were fixed *in situ* by 4% PFA for 10 min at room temperature. Islet sections, the monolayers and islet-like clusters were immunostained with the primary antibodies as follows: rabbit anti-*c-Kit* (C-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-cytokeratin 20 (CK20, Dako, Mississauga, ON, Canada), mouse anti-human insulin and glucagon, and mouse anti-BrdU (Sigma), rabbit anti-PDX-1 (gift from Dr Wright, University of Vanderbilt, Nashville, TN, USA), rabbit anti-neurogenin 3 (*ngn3*, gift from Dr German, University of California, San Francisco, CA, USA) and rabbit anti- $\alpha$ -1-fetoprotein (Biocare Medical, Walnut Creek, CA, USA), by either the avidin–biotin complex method (Histostain Plus Kit, Zymed, San Francisco, CA,

**Table 1** Characteristics of primers and conditions for RT-PCR

	Accession number <sup>a</sup>	Primer pair sequence (sense/antisense)	Location (nt)	Fragment size (bp)	Number of cycles
<b>Primer (name)</b>					
c-Kit (rat)	NM022264	5'-AGCAAGAGTTAACGATTCCGGAG-3' 5'-CCAGAAAGGTGTAAGTGCCCTCCT-3'	887–909 1208–1230	344	33
PDX-1	X74342	5'-CCACCCAGTTTACAAGCTC-3' 5'-TGTAGGCAGTACGGGTCCTC-3'	418–437 723–742	325	33
ngn3	U76208	5'-TGGCGCTCATCCCTTGGATG-3' 5'-CAGTCACCCACTTCTGCTTCG-3'	161–181 300–320	160	33
Pax4	AF053103	5'-TGGCTTTCTGCTTCTGTGAGG-3' 5'-TCCAAGACTCCTGTGCGGTAGTAG-3'	10–32 228–251	242	30
Insulin	NM000207	5'-TCACACCTGGTGGAGGCTC-3' 5'-ACAATGCCACGCTTCTGC-3'	141–159 302–319	179	28
Insulin I	NM019129	5'-ATGGCCCTGTGGATGCGCTT-3' 5'-TAGTTGCAGTAGTTCTCCAG-3'	1–20 313–331	331	30
Insulin II	NM019130	5'-ATGGCCCTGTGGATGCGCTT-3' 5'-TGCCAAGGTCTGAAGGTCAC-3'	1–20 224–243	243	30
Glucagon	Z46845	5'-CAGAGGAGAACCCAGATCA-3' 5'-TCATGACGTTTGCAAGTT-3'	106–125 292–307	202	33
β-actin	X00351	5'-GACGGGGTCACCCACACTGTGCCATCTA-3' 5'-CTAGAAGCATTGCGGTGGACGATGGAGG-3'	510–538 1141–1169	660	28

<sup>a</sup>Genebank accession number for the sequences used in designing the primers.

USA) or immunofluorescence, as described previously (Wang & Rosenberg 1999). The sections were incubated overnight at 4 °C with the appropriate dilution of primary antibody. To evaluate cell proliferation, freshly isolated islets and islet-derived epithelial monolayers were incubated with 10 μM 5-bromo-2'-deoxyuridine (BrdU, Sigma), followed by immunostaining for BrdU (Wang *et al.* 1994). Double immunofluorescence staining was performed to identify coexpression of phenotypes in islet-derived epithelial monolayers and islet-like clusters. Fluorescent secondary antibodies obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) were fluorescein (FITC) antimouse or antirabbit antibodies (1:100), or rhodamine (TRITC) antirabbit or antimouse antibodies (1:100). For identification of alkaline phosphatase expression within the islet-derived epithelial monolayers, histological staining was performed with NAMP/fast blue, and the nuclei were counterstained with nuclear fast red (Sigma). Double-labelled images were recorded by a Lecia DMIRE2 fluorescence microscope with the Openlab image software (Improvision, Lexington, MA, USA). Negative controls included the omission of the primary antibodies and preabsorption of the antiserum with excess peptides where available.

The number of cells expressing c-Kit and BrdU labelling index was determined with a Carl Zeiss transmitted light microscope connected to computer-assisted image analysis software (Northern Eclipse version 2.0, Empix Imaging Co., Mississauga, ON, Canada). For each time point, at least 1000 cells were counted, and each time point was repeated six times. The data were expressed as a percentage of the total number of cells counted and

compared by Student's *t*-test, with differences considered to be statistically significant when  $P < 0.05$ .

#### Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from freshly isolated islets, islet-derived epithelial monolayers and reformed islets with the RNeasy-4 PCR kit (Ambion, Austin, TX, USA). cDNAs were synthesized from 2 μg total RNA, using oligo(dT) as a primer with the SuperScript First Strand Synthesis System (Invitrogen). Subsequent PCR assays were performed, and the PCR oligonucleotide primers used to amplify specific cDNA fragments are listed in Table 1. The amplified products were analyzed on 1.5% agarose gels and visualized by ethidium bromide staining. Sequencing of PCR products for verification was done in the Robarts Research Institute Core Molecular Biology Facility (London, ON, Canada).

#### Differentiation culture

To determine whether islet-like structures could be re-formed *in vitro* from progenitor cell-enriched monolayers, cells were replaced on Matrigel-coated chamber slides (Lab-Tek Chamber Slides, four-well; Nunc, Naperville, IL, USA) and cultured with DMEM:F12 (Sigma) medium containing 10% fetal bovine serum (FBS), keratinocyte growth factor (KGF, 50 ng/ml; R&D System Inc., Minneapolis, MN, USA) to induce the islet cell differentiation (Bonner-Weir *et al.* 2000). Islet-derived epithelial monolayers grew as islet-like clusters,

adherent on Matrigel and were examined after 7 days of differentiation in culture by immunofluorescent staining and RT-PCR analysis.

#### *Purification of c-Kit-positive cells*

To purify a population of c-Kit-expressing cells from the islet-derived epithelial monolayers, immunomagnetic c-Kit/FITC-microbeads (Chemicon, Temecula, CA, USA) were used to isolate a population of c-Kit-positive cells through a magnetic column (Miltenyl Biotec., Auburn, CA, USA). A purity of 70% c-Kit-expressing cells was achieved following purification, and the differentiation culture as described above was followed to determine the potential of c-Kit-expressing cells to re-form islet-like clusters.

#### *Measurement of insulin secretion*

Insulin content and basal insulin release from islet-derived epithelial monolayers or reformed islet-like clusters were measured using a rat insulin ELISA kit (DRG Diagnostic Inc., Mountainside, NJ, USA) with a sensitivity of 0.15 ng/ml. The DNA content of the samples was determined alongside the insulin content, which was calculated as ng/μg DNA. Glucose responsiveness of re-formed islet-like clusters was examined with an acute glucose challenge, as described previously (Wang & Rosenberg 1999), on day 7 of the differentiation culture. Islet-like clusters were incubated in RPMI1640 medium (Sigma) containing 22 mM glucose plus 50 μM IBMX, and insulin secretion was expressed as ng/well. A static glucose stimulation index was calculated by dividing the insulin output from the high glucose plus IBMX incubation by the insulin output during low glucose (2.2 mM) incubation (Wang & Rosenberg 1999).

## Results

#### *Patterns of c-Kit expression and proliferation in islet-derived epithelial monolayers*

Islets cultured on a type I collagen gel spread to form epithelial monolayers, as demonstrated by their immunoreactivity for the epithelial cell marker, cytokeratin 20 (Fig. 1A), and expression of insulin and glucagon was diminished during the transdifferentiation process (Fig. 1C, D), as observed previously (Wang *et al.* 2001). Interestingly, staining for the progenitor cell marker c-Kit increased progressively over the culture period (Fig. 1B). Quantitative analysis demonstrated that the percentage of c-Kit-positive cells ( $9 \pm 3\%$  at day 0) reached a maximum at 45% after 8 weeks of culture ( $P < 0.001$ , Fig. 2A). A constant proportion of c-Kit-expressing cells could be maintained in long-term culture as well. The islet-derived

epithelial monolayers were highly proliferative, demonstrating up to a  $41 \pm 5\%$  BrdU labelling index after 4 weeks of culture (Fig. 2B), with no subsequent significant change in the labelling index after 4 weeks *in vitro*. Double immunostaining for BrdU and c-Kit at 4 weeks of culture showed that  $21 \pm 3\%$  of c-Kit-positive cells were BrdU labelled.

#### *Patterns of mRNA expression of c-Kit, endocrine cell markers and transcription factors in islet-derived epithelial monolayers*

The formation of islet-derived epithelial monolayer was preceded by decreased insulin and glucagon mRNA expression (Fig. 3), paralleling their loss at the protein level (Fig. 1). On the other hand, the expression of c-Kit mRNA as determined by RT-PCR paralleled the increase in their protein levels. The progression in morphology from progenitor epithelial cell to functional endocrine cell is defined and controlled intrinsically by the expression of a series of transcription factors such as PDX-1, ngn3 and Pax4. These factors provide a distinct signature for the differentiation of each endocrine cell type and were also detected throughout the culture period (Fig. 3) (Sander & German 1997, Dohrmann *et al.* 2000, Schwitzgebel *et al.* 2000).

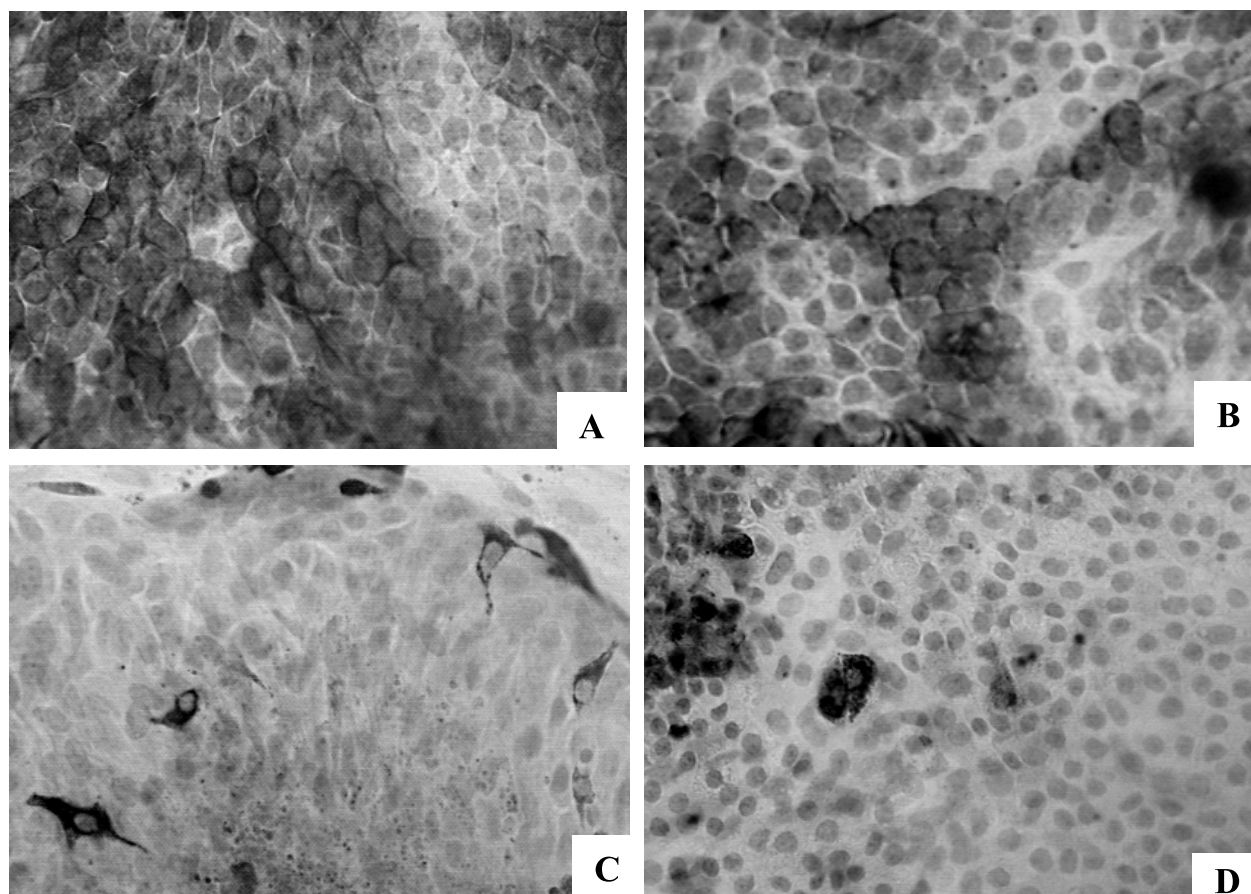
#### *Expression of undifferentiated cell markers and coexpression patterns in islet-derived epithelial monolayers*

After 4 weeks of culture, the monolayers frequently stained for α-fetoprotein (Fig. 4A) and alkaline phosphatase (Fig. 4B). This increase was associated with the mRNA expression of the transcription factor Oct4 (Fig. 4C). All of these markers have been previously identified as undifferentiated cell markers (Lumelsky *et al.* 2001).

To identify coexpression of immunophenotypes in islet-derived epithelial cells, dual immunofluorescent staining was performed for the epithelial cell marker CK20 with c-Kit or transcription factors. Coexpression of CK20 with either PDX-1 ( $65.9 \pm 5.6\%$ ), or c-Kit ( $33.2 \pm 9.2\%$ ) was frequently observed in monolayers during the expansion of c-Kit-expressing cells after 8 weeks in culture (Fig. 5). Although the number of ngn3-positive cells was quite low, the coexpression of ngn3 with CK20 was found throughout the culture period ( $1.6 \pm 0.2\%$ ) (Fig. 5).

#### *Differentiation of islet-derived epithelial monolayers and c-Kit<sup>+</sup> cells into hormone-secreting islet-like clusters*

For examination of the potential of islet-like structures to be re-formed from expanded epithelial monolayers *in vitro* based on the extensive appearance of heterogeneous populations of undifferentiated cells types, a laminin-rich extracellular matrix, Matrigel, was utilized to monitor islet cell differentiation (Bonner-Weir *et al.* 2000). Cells adhered and reformed islet-like clusters on Matrigel within

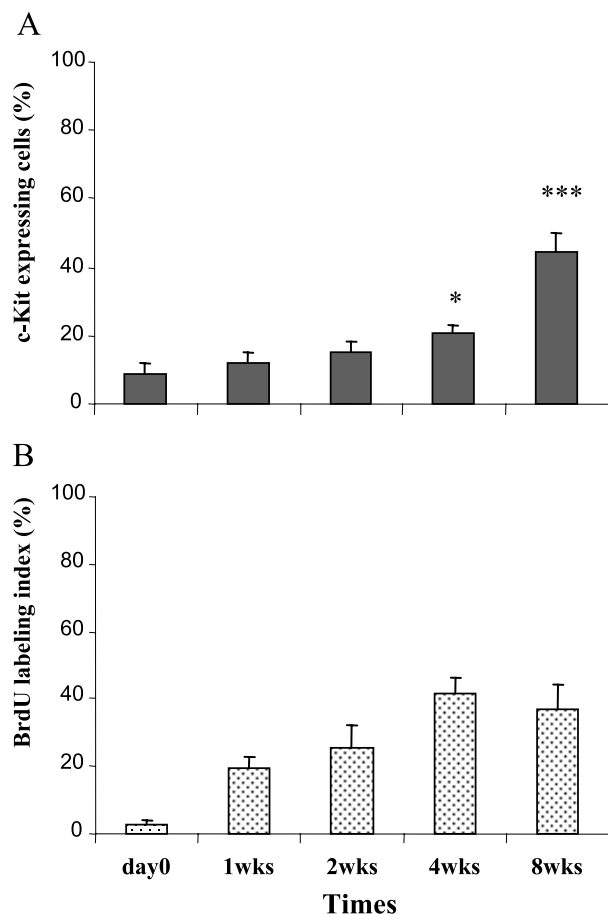


**Figure 1** Phenotypic characterization of islet-derived epithelial monolayers cultured on type I collagen gel for 4 weeks. Immunostaining for the epithelial cell marker, cytokeratin 20 (A), the putative endocrine progenitor cell marker c-Kit (B), and the endocrine hormones insulin (C) and glucagon (D). Original magnification  $\times 400$ .

a week, with 17-fold and 2-fold significant increases in the proportion of insulin- and glucagon-immunopositive cells respectively in comparison to undifferentiated monolayers (Table 2). Their mRNA levels detected by RT-PCR were also observed to increase (Fig. 6A). In contrast, the presence of c-Kit immunoreactivity and mRNA decreased over the 1-week differentiation culture period, with a 40% decrease in c-Kit-expressing cells (Table 2, Fig. 6A). Interestingly, coexpression of c-Kit with insulin, which is frequently observed in fetal rat islet cell development (Yashpal *et al.* 2004), was also detected after 1 week (Fig. 6B). Comparison of total cellular insulin content and insulin release before and after islet-like cluster formation showed that the clusters undergo progressive maturation until day 7 in the differentiation culture. The total insulin content of islet-like cluster cells increased 8-fold over that of islet-derived epithelial monolayers (Table 3), and basal insulin release rate was also detectable after 7 days in culture (Table 3). Moreover, in

response to a glucose challenge, the newly formed islet-like clusters showed a 2.5-fold increase in insulin secretion after cells were exposed to 22 mM glucose plus 50  $\mu$ M IBMX.

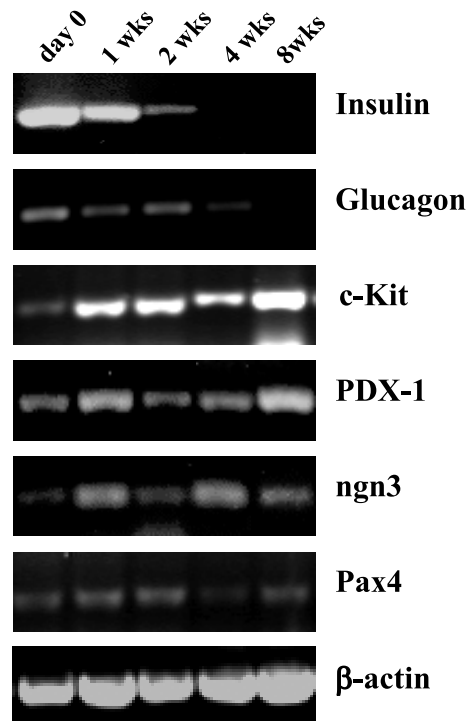
Given that a high proportion of c-Kit-expressing cells were present in the islet-derived epithelial monolayers, we next examined the potential of c-Kit expression cells to re-express insulin and form islet-like clusters. c-Kit-positive-cells were immunomagnetically sorted and expanded on a type I collagen gel (Fig. 6C). Subsequently, after expansion, the extracellular matrix was replaced by Matrigel, and the mRNA expression of insulin and glucagon was examined. RT-PCR analysis of c-Kit<sup>+</sup> cells cultured on type I collagen revealed that no detectable insulin or glucagon signals were present; however, c-Kit positive-cells later cultured on Matrigel expressed insulin and glucagon mRNA, although the expression was weak. Furthermore, c-Kit mRNA expression was greatly diminished when Matrigel was used (Fig. 6D).



**Figure 2** Quantitative analysis of *c-Kit*-expressing cells within islet-derived epithelial monolayers (A), and the BrdU labelling index (B) of the monolayers. \* $P < 0.05$ , \*\*\* $P < 0.001$  vs day 0 control.

## Discussion

Our study examines the development and expression of the putative islet progenitor marker, *c-Kit*, in epithelial monolayers derived from isolated postnatal rat islets and intra-islet progenitor plasticity. Using an islet transdifferentiation model, our data show that highly enriched proliferating populations of cells expressing *c-Kit* and signature pancreatic transcription factors can be expanded on type I collagen *in vitro*, and these monolayers retain the ability to give rise to new islets. Furthermore, *c-Kit*-expressing cells could be maintained in long-term culture, and the appearance of other undifferentiated markers highlights the heterogeneous populations of intra-islet progenitors that are present in the monolayers. This experimental model and phenotypic analysis of putative intra-islet progenitors is a fundamental starting point for experimental approaches that utilize islets as a source for progenitor cells and for a novel approach to the expansion,

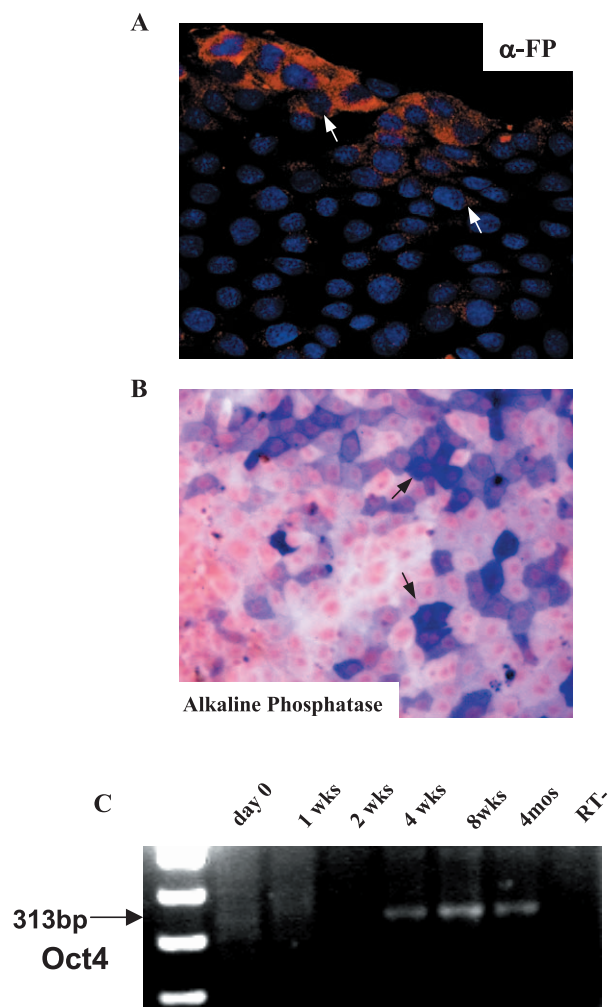


**Figure 3** RT-PCR analysis of *c-Kit* mRNA, endocrine signals and developmental transcription factors in islet-derived epithelial monolayers during the culture period.

clonal isolation and manipulation of precursor cells into islet cell types.

Our previous findings, in agreement with those of others, confirm that the early postnatal rat pancreas retains a population of *c-Kit*-positive precursor cells within the islets (Oberge-Welsh & Welsh 1996, Welsh *et al.* 2000, Rachdi *et al.* 2001, Yashpal *et al.* 2004). Islets cultured on type I collagen matrix rapidly adhered to the matrix, and spread to form a monolayer of cytokeratin-positive epithelial cells. Epithelial monolayer formation was preceded by two events, the loss of insulin and glucagon phenotypes, and an increase in cells expressing *c-Kit*. The populations of *c-Kit*-expressing cells were maintained at relatively high and constant levels throughout 8-week culture, as determined by immunocytochemistry and RT-PCR.

Examining gene expression of transcription factors related to endocrine cell development, we found constant expression of PDX-1, *ngn3* and Pax4, further indicating that the cells present in the monolayers are fetal-like. *Ngn3* is a developmental transcription factor and pro-endocrine gene that operates downstream of PDX-1 specifically to control embryonic pancreas and endocrine cell differentiation (Gu *et al.* 2002) by activating the NeuroD/ $\beta$ 2 promoter. The expression of these two pancreatic transcription factors reveals additional immaturity of phenotypes. Additionally, Pax4, which acts downstream



**Figure 4** (A) Immunofluorescent analysis for  $\alpha$ -fetoprotein (TRITC), (B) histochemical staining for alkaline phosphatase in islet-derived epithelial monolayers at 4 weeks of culture and (C) RT-PCR analysis of Oct4 expression in the monolayers with time *in vitro*. (A) Nuclei were stained by DAPI (blue colour; arrows indicate the positive cells), and (B) cytoplasm stained as blue for alkaline phosphatase and nuclei was counterstained by nuclear fast red. Original magnification  $\times 400$ .

of NeuroD/ $\beta$ 2 to drive  $\beta$ -cell differentiation (Grapin-Botton *A et al.* 2001), is also expressed constantly throughout the culture period, suggesting that expression of these genes throughout the culture period may indicate the potential of monolayer cells under appropriate conditions also to be driven down a  $\beta$ -cell developmental pathway. Other markers of undifferentiated cells, such as Oct4,  $\alpha$ -fetoprotein and alkaline phosphatase (Lumelsky *et al.* 2001), in the culture period were also detected. Taken together, our data demonstrate that islet-derived epithelial monolayers are enriched with immature cell types, which express not only immunoreactive stem cell markers, including c-Kit,  $\alpha$ -fetoprotein and alkaline phosphatase,

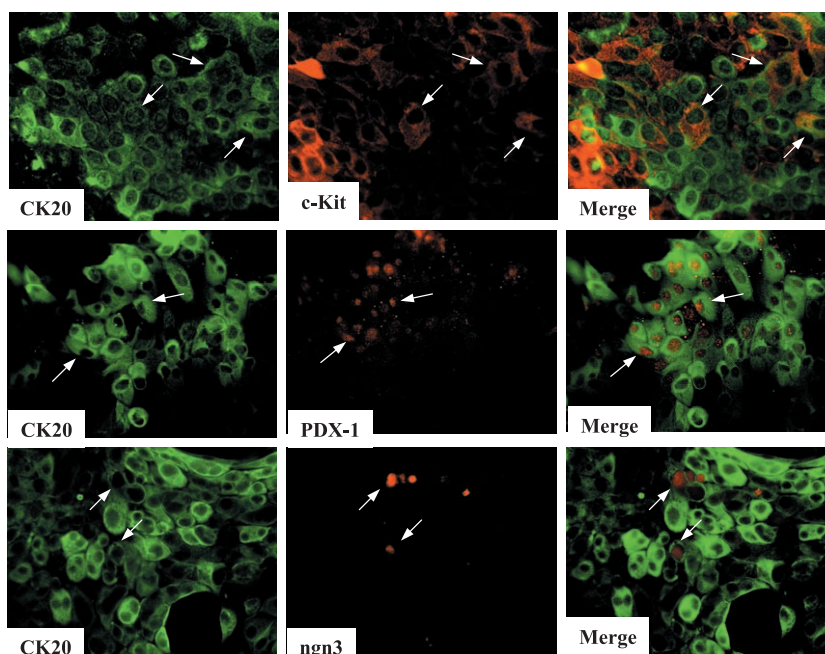
but also mRNA of transcription factors reminiscent of the fetal pancreas and important in islet development.

Postnatal islets have a low proliferative capacity; however, our expanded c-Kit-positive monolayers reveal that highly proliferative cell populations predominate *in vitro*. Beattie *et al.* have revealed that monolayers derived from islets increase in cell number, an effect attributed to an increased proportion of proliferative cells. Such a mitogenic effect is also characteristic of precursor cells.

Coexpression studies of the monolayer cells demonstrated frequent colocalization of c-Kit, PDX-1 or ngn3 with cytokeratin 20, suggesting that these particular cells may represent epithelial precursor cells that are essential for proper  $\beta$ -cell development. Unfortunately, we could not confirm or exclude the idea that the c-Kit cell population is the same as PDX-1- or ngn3-expressing cells, as the available antibodies were produced in the same animal species.

To address the potential of these islet-derived monolayers to differentiate and re-form islet clusters, monolayers were transferred to Matrigel. Transfer of monolayers was marked by the emergence of clusters immunoreactive for both insulin and glucagon; however, there was a marked decrease in the proportion of c-Kit-expressing cells. It has been documented that c-Kit expression is an intermediate step in the differentiation of  $\beta$ -cells (Rachdi *et al.* 2001, Yashpal *et al.* 2004). These results suggested that the disappearance of c-Kit expression may coincide with  $\beta$ -cell appearance such that mature  $\beta$ -cells may derive from these c-Kit-expressing cell populations. We therefore purified c-Kit<sup>+</sup> cells and demonstrated that c-Kit<sup>+</sup> cells potentially contribute to the process of new islet cluster formation because c-Kit<sup>+</sup> cells which lack insulin and glucagon mRNA expression can be induced to upregulate gene transcripts of both these hormones under differentiation protocols. Clustered cells, but not epithelial monolayers, were able to secrete insulin and respond to a glucose challenge. Although the insulin response to glucose stimulation suggests these are mature  $\beta$  cells, the amount of insulin content and the number of insulin-expressing cells in the re-formed islet-like clusters are lower than those of freshly isolated islets (Bonner-Weir *et al.* 2000). Further studies will be necessary to develop suitable micro-environments *in vitro* to optimize  $\beta$ -cell differentiation and proliferation, and enhance insulin production.

On a practical level, these studies offer a mechanism to search for and expand precursor cells from islets themselves. We have demonstrated that a combination of growth factor and extracellular cues promotes the expansion of precursor cell populations. The appropriate change in the extracellular environment subsequently promotes islet reaggregation, and enhances the expression of the insulin gene, insulin secretion and insulin-expressing cell proportions, while a decrease in the population of progenitor marker-expressing cells is observed. This model is more feasible since monolayers derived from whole islets



**Figure 5** Coexpression of CK20 (FITC) with c-Kit, PDX-1 and ngn3 (TRITC) in islet-derived epithelial monolayers at 2 months of culture determined by double-immunofluorescence staining (arrows indicate the colocalization; original magnification  $\times 400$ ).

may circumvent the need to elucidate necessary cell–cell interactions or paracrine signalling that can control differentiation of progenitor cell types. Our study, in which c-Kit-expressing cells have been purified through immunomagnetic cell sorting, reveals that pure cell populations can be selected from these monolayers, with a purity of 70%. The *ex vivo* expansion of c-Kit-expressing cells in our defined differentiation culture promoted insulin and glucagon mRNA expression, suggesting that c-Kit progenitor populations have the potential of forming new  $\beta$ - and  $\alpha$ -cells. However, the purified cells show weak glucagon and insulin mRNA expression, suggesting that alone

sorted cells may not form functionally adequate  $\beta$ -cells. Instead, the necessary trophic support, cell–cell interactions and paracrine factors that may be offered through expansion of the entire islet may be a more practical way of reforming the islet cell mass. All in all, this study shows that considerable islet cell plasticity is present in the postnatal rat pancreas, as demonstrated by the capacity to form epithelial cells alone, and presents an exciting new experimental model for the generation of islet clusters from whole islet populations, or for providing a source to isolate progenitor cell types and examine the cell lineage commitment and differentiation into islet cells.

**Table 2** Comparison of endocrine and progenitor cell proportions in freshly isolated islets, islet-derived monolayers and differentiated cultures

	Total cells (%)		
	Insulin <sup>+</sup>	Glucagon <sup>+</sup>	c-Kit <sup>+</sup>
Fresh islets (day 0)	62.3 $\pm$ 8.5	28.9 $\pm$ 7.6	9.05 $\pm$ 2.9
Epithelial monolayers (8-week)	1.22 $\pm$ 0.9 (4wks) <sup>a</sup>	4.14 $\pm$ 2.6 (4wks)	44.2 $\pm$ 5.6
Differentiation (day 7)	21.2 $\pm$ 6.3 <sup>b</sup>	7.1 $\pm$ 2.9	25.3 $\pm$ 6.8 <sup>c</sup>

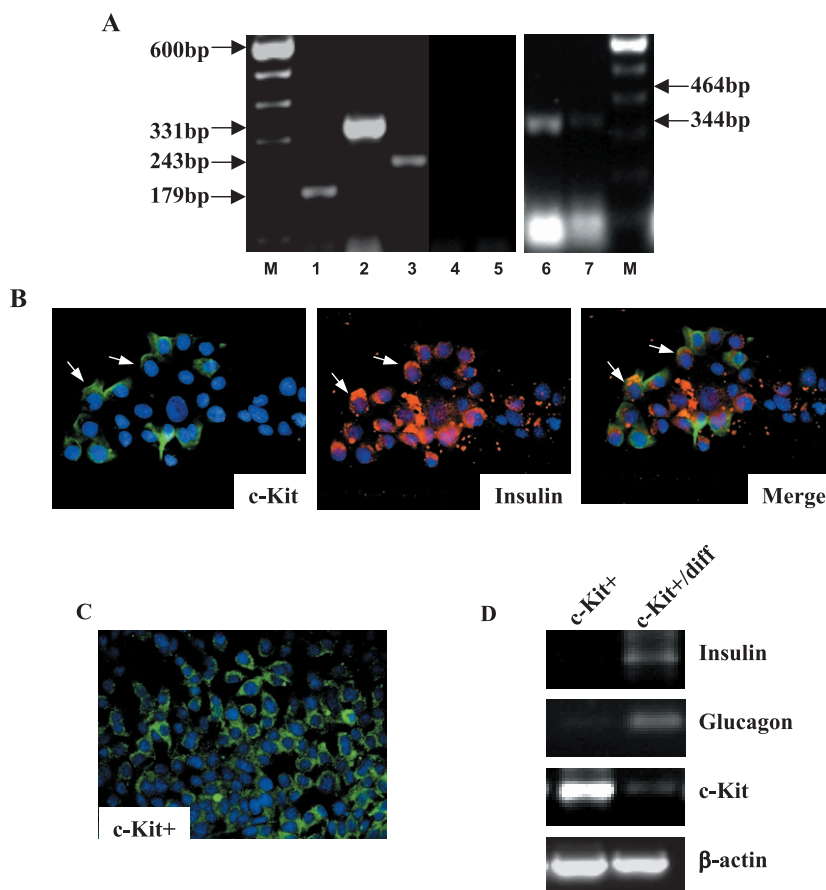
Data presented are means  $\pm$  s.d. The results were reproduced in six independent experiments.

<sup>a</sup>After 4 weeks of culture, both insulin- and glucagon-immunoreactive cells are undetectable in the monolayers. Statistical analysis showed significant differences between the cells in epithelial monolayers and after the differentiation culture.

<sup>b</sup> $P < 0.001$ .

<sup>c</sup> $P < 0.01$ .





**Figure 6** (A) RT-PCR analysis of preproinsulin (lanes 1 and 4), insulin I (lane 2), insulin II (lane 3) and c-Kit (lanes 6 and 7) mRNA expression before (lanes 4 and 6 monolayers) culture on type I collagen and after (lanes 1–3 and 7 islet-like clusters) culture on Matrigel (M: DNA ladder; lane 5: RT negative). (B) Double immunofluorescence for c-Kit (FITC) with insulin (TRITC) was observed in a reformed islet-like cluster; arrows indicate the coexpressing phenotypes. Nuclei were counterstained by DAPI ( $\times 400$ ). (C) Positively selected c-Kit<sup>+</sup> cells cultured on type I collagen gel for 1 week with c-Kit immunostaining ( $\times 400$ ). (D) RT-PCR analysis of preproinsulin and glucagon, as well as c-Kit mRNA in c-Kit<sup>+</sup> monolayers (c-Kit<sup>+</sup>) and c-Kit<sup>+</sup> differentiated islet-like clusters (c-Kit<sup>+</sup>/diff) on Matrigel.

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**Table 3** Islet-derived epithelial monolayers redifferentiate, forming islet-like clusters to store and release insulin

	Basal insulin release (ng/ml)	Insulin content (ng/ $\mu$ g DNA)	Static glucose-stimulation index
Epithelial monolayers	ND	0.04 $\pm$ 0.02	ND
7 days of differentiation	2.7 $\pm$ 1.5	0.32 $\pm$ 0.07	2.49 $\pm$ 0.22

Data presented are means  $\pm$  s.d. The results were reproduced in six independent experiments. ND, not detected.

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