

# Evidence for the presence of stem cell-like progenitor cells in human adult pancreas

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## Abstract

The origin of cells replacing ageing  $\beta$ -cells in adult life is unknown. This study assessed the expression of classic stem cell markers: Oct4, Sox2 and CD34 in islet-enriched fractions versus exocrine cell-enriched fractions from 25 adult human pancreases following human islet isolation. Expression of Oct4, Sox2 and CD34 mRNAs was found in all cell samples, with no significant differences between endocrine and exocrine cell fractions. Immunohistochemical staining for Oct4, Sox2, CD133, CD34, CK19, insulin and nestin on human pancreas sections showed that the majority of Oct4<sup>+</sup>ve

cells were found in the walls of small ducts. Similar localisations were observed for Sox2<sup>+</sup>ve cells. The majority of Sox2<sup>+</sup>ve cells were found to co-express Oct4 proteins, but not vice versa. Cells positive for Oct4 and Sox2 appeared to be a unique cell population in the adult human pancreases without co-expression for CK19, CD34, CD133, insulin and nestin proteins. The numbers of Oct4<sup>+</sup>ve and Sox2<sup>+</sup>ve cells varied among donors and were  $\sim$ 1–200 and 1–30 per 100 000 pancreatic cells respectively.

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## Introduction

Islet cell death leads to insulin deficiency and diabetes – both the insidious loss of insulin secretion over time in the evolution of Type 2 diabetes and the immunological loss of all insulin-secreting cells in Type 1. The ability, at least in the short term, to restore  $\beta$ -cells in Type 1 diabetes by islet transplantation (Shapiro *et al.* 2000) has renewed interest in attempts to find  $\beta$ -cell surrogates. There are far fewer organ donors than people with diabetes and, at least in its present form, islet transplantation remains inefficient (Close *et al.* 2005, Shapiro *et al.* 2006) and more cells to transplant are needed. At the same time, understanding the origins of new islet  $\beta$ -cells in life may help devise ways of slowing down the progression of Type 2 diabetes. The presence of adult stem cells or pancreatic-specific progenitor cells in human pancreas is controversial. In animals, the  $\beta$ -cell population does increase following pancreas duct ligation (Wang *et al.* 1995) or pancreatectomy (Starich *et al.* 1991), in Type 2 diabetes, in obesity (Parsons *et al.* 1995, Chua *et al.* 2002) and during pregnancy (Hayashi *et al.* 2003) when demand for insulin is increased, showing some capacity for regeneration of  $\beta$ -cells during adulthood. However, the origin of the cells that replace the ageing  $\beta$ -cells is not clear. Lineage-tracing experiments in rodents indicate that mature  $\beta$ -cells are able to divide to generate new  $\beta$ -cells (Dor *et al.* 2004). However, more recent data suggest that there are other sources

for new  $\beta$ -cells in the pancreas (Bonner-Weir *et al.* 2004, Paris *et al.* 2004). The *ex vivo* expansion of islet cells is thought to occur through epithelial-to-mesenchymal transition (Gershengorn *et al.* 2004). It has been shown that some pancreatic cells can redifferentiate into insulin-producing cells but the origin of these new cells is not known. In a previous study following *ex vivo* expansion of human pancreatic non-endocrine cells transplanted into a diabetic severe combined immunodeficiency (SCID) mouse model, we showed that a small portion of the expanded cells expressed insulin following differentiation induction *in vitro*, with further development of insulin-producing cells following transplantation. *IPF1* gene transfection increased the number of insulin-expressing cells, possibly by inducing differentiation of those cells with pluripotency (Zhao *et al.* 2005). New islet cells can also be generated from adult pancreatic ductal cells of mice (Ramiya *et al.* 2000) and humans (Bonner-Weir *et al.* 2000, Gao *et al.* 2003, Yatoh *et al.* 2007), again arguing for the presence of stem cell-like progenitor cells in adult pancreas. Identification of these progenitor cells, particularly from human adult pancreas, would be of great interest for the generation of insulin-producing cells *ex vivo* to treat diabetes or to develop means to expand these cells in Type 2 patients to increase their  $\beta$ -cell mass. The use of adult progenitor cells reduces the risk of tumorigenicity, in comparison with cells derived from embryonic stem (ES) cells (Fujikawa *et al.* 2005).

Large quantities of adult human pancreatic non-endocrine cells are readily available and are currently discarded from islet isolation programmes. Identification of adult pancreatic tissue-specific progenitor cells from this source of tissues would potentially provide a valuable resource for the generation of insulin-producing cells for human transplantation. In this study, we sought evidence for the presence of the classical stem cell marker proteins, Oct4, Sox2 and CD34 in fractions of tissues from human islet isolation. Oct4 and Sox2 are transcription factors essential for maintaining the pluripotency of stem cells (Scholer *et al.* 1990, Ellis *et al.* 2004) and CD34 is the marker of haematopoietic stem cells (Lu *et al.* 2007). In order to determine the localisation of these potential stem cell-like cells, we examined sections of human pancreas for the presence of Oct4<sup>+</sup> and Sox2<sup>+</sup> cells, and their co-localisation with the duct cell marker CK19, the  $\beta$ -cell marker insulin, the haematopoietic progenitor cell markers CD34, CD133, and the neural stem cell marker, nestin.

## Materials and Methods

Human pancreases were obtained for research with informed consent from the families of organ donors and this study was approved by the Ethics Committee of King's College Hospital, London. Twenty-five pancreas organs (10 males and 15 females) from conventional (brain death) organ donors, without diabetes symptoms, were used in this study. The donor age ranges were 22–62 years with a mean age of  $42.3 \pm 0.93$  years. The cold ischaemia time was  $5.91 \pm 2.15$  h.

Human pancreases were retrieved by experienced surgeons, using standard techniques for organ retrieval for whole organ transplantation. In brief, after cross-clamping, pancreases were kept cold with ice pads *in situ*. The pancreas was perfused with 1 l ice-cold Marshall's solution in the body and 1 l University of Wisconsin Solution (UWS) on the bench after removal, before being delivered to our Human Cell Isolation Unit, in pre-oxygenised perfluorocarbon (PFC)/(UWS) two layer solutions on ice, for islet isolation.

Human islet isolation was carried out as previously described (Huang *et al.* 2004). In brief, each pancreas was weighed and

dissected from surrounding fat and digested with collagenase (Liberase, Roche Diagnostics) injected via the main pancreatic duct and gentle shaking. The digestion was stopped by cooling and diluting with cold MEM medium when the islets were perceived as being free of exocrine tissue but still intact. The islets in the digests were then enriched by continuous gradient centrifugation on a cooled Cobe centrifuge (Gambro BCT, Gloucester, UK). Exocrine cell-enriched fractions were taken from the bottom of Cobe centrifuge bag following the separation of human islets. Portions of islet- and exocrine-enriched fractions were lysed in the RNA lysis buffer (Promega) and stored at  $-80^{\circ}\text{C}$  until total RNA isolation.

## Semi-quantitative RT-PCR

Total RNA was isolated using an RNA Miniprep Kit (Promega) and quantified using a spectrometer (Gene Quant II, Pharmacia Biotech) at 260 nm wavelength. The messenger RNA (mRNA) in 100 ng total RNA was converted into first-strand cDNA using reverse transcriptase (Invitrogen) in a volume of 20  $\mu\text{l}$ . The hot-start (Qiagen), RT-PCR used the following parameters:  $95^{\circ}\text{C}$  for 15 min for 1 cycle and then  $94^{\circ}\text{C}$  for 45 s,  $50\text{--}62^{\circ}\text{C}$  for 1 min and  $72^{\circ}\text{C}$  1–3 min for 20–35 cycles. RT-PCR primers for the target genes (human preproinsulin gene (435 bp), Oct4 (429 bp), Sox2 (341 bp), CD34 (474 bp) and the reference gene human  $\beta$ -actin (664 bp)) are listed in Table 1. Human  $\beta$ -actin gene PCR was done in parallel as an internal control. DNA relative quantity was analysed using a gel program (version 3.0 computer analyzer of Media Cybernetics, LP, Silver, Spring, MD, USA) and expressed relative to the density of  $\beta$ -actin gene PCR product as described previously (Zhao *et al.* 2005).

## Quantitative PCR

Q-PCR was performed with the LightCycler-2 (Roche Diagnostics) using SYBR Green I fluorescence Kit (Roche Diagnostics). The genes were amplified in duplicate and at least three donors' cDNAs were tested for each set of experiments, according to the manufacturer's instructions. Briefly, the master reaction mix with the solutions from the

**Table 1** PCR primer sets

Name of primer	Primer sequences	Product length (bp)
H $\beta$ -actin F	5' CCCAGATCATGTTTGAGACC 3'	664
H $\beta$ -actin R	5' CCAACAGGAGTACTTGCGCTCAG 3'	
H Sox2 F	5' CCGGCGGCAACCAGAAAAACAG 3'	341
H Sox2 R	5' ACCCCGCTCGCCATGCTATTG 3'	
H Oct4 F	5' GCTTGGGGCGCCTTCCTCC 3'	429
H Oct4 R	5' GACTCCTCCGGGTTTTGCTCCAG 3'	
H CD34 F	5' GCCTGGCCCGAGTGCTGTGT 3'	474
H CD34 R	5' CCTGGCCGGTCCCGTTTTTCT 3'	
INS F	5' CATCAAGCACATTGTCC 3'	435
INS R	5' CTGGTTCAAGGGCTTATTC 3'	

kit was prepared and the appropriate amounts of template DNA and 0.25  $\mu$ M primers were added in a total volume of 20  $\mu$ l. PCR grade water was used as a negative control as described previously (Zhao *et al.* 2005).

#### Immunocytochemical staining

Sections of pancreas were removed before the cannulation for collagenase injection and snap frozen in liquid N<sub>2</sub> and stored at  $-80^{\circ}\text{C}$  until processing. Cryosections at 5  $\mu$ m intervals were prepared and were stained for Oct4 and Sox2 respectively. The sections were then blocked with biotin-avidin system (Vector Laboratories, Peterborough, UK) to prevent cross-staining to the previous antigen, followed by a second staining for insulin, CK19, nestin, CD34, CD133 or staining for Sox2 to Oct4 staining sections and vice versa respectively as described previously (Zhao *et al.* 2005). Negative controls were performed by replacing the first antibodies with same origin and same isotype antibodies against other non-related antigens. Oct4 and Sox2 positive cells and total cells in the same field were counted. The cells in 10 consecutive fields per section, 5 sections per donor pancreas and 5 donor pancreases were counted to assess the percentage of Oct4<sup>+</sup> and Sox2<sup>+</sup> cells.

#### Antibodies

The monoclonal antibody to human insulin (clone K36aC10) and the antibody to Sox2 (rabbit anti-human Sox2) were purchased from Sigma. The antibodies against Oct4 (goat polyclone, sc-8629) and CK19 (monoclonal, sc-6278) were purchased from Santa Cruz, Biotechnology (Santa Cruz, CA, USA), while the nestin antibody (monoclonal, MAB5326) was from Chemicon International (Temecula, CA, USA). The antibody to human CD34 (monoclonal QBEND/10) was from AbD Serotec (MorphoSys, UK) and the anti-human CD133/2 pure (monoclonal, # 293C3) was from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

#### Data analysis

All data are expressed as mean  $\pm$  S.D. and analysed using the ANOVA computer program. Student's *t*-test was used for comparison between two groups. The significance level was set at  $P=0.05$ .

## Results

#### Expression of Oct4, Sox2 and CD34 mRNAs in pancreatic tissues and their relationship with donor age

All of the cell samples from the 25 human pancreases showed the expression of mRNAs of Oct4, Sox2 and CD34 by RT-PCR. The expression level of these genes was variable amongst the donors and was not particularly associated with islet cell fractions, nor with exocrine cell fractions (Fig. 1A). The PCR

products of Oct4 and Sox2 were partially sequenced and confirmed to be authentic according to the published sequences (Oct4: NM\_002701, Sox2: NM\_003106).

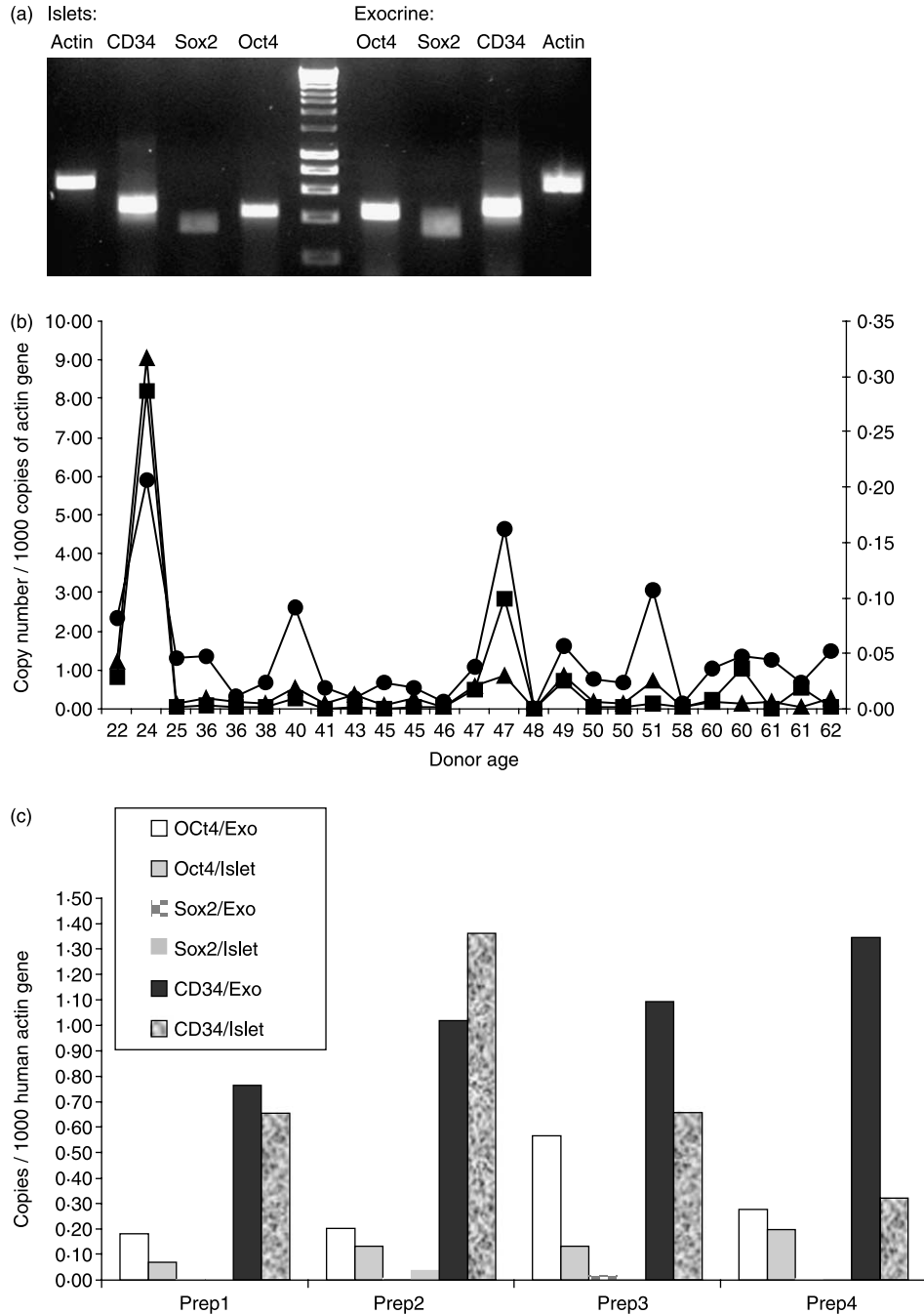
Quantitative PCR was performed on Oct4, Sox2 and CD34 using human  $\beta$ -actin as a control. The relevant expression levels were expressed as copies per 1000 copies of  $\beta$ -actin mRNA. The expression levels for the markers were variable among the preparations, ranging from 0.02 to 9 copies for Oct4 and 0.0004 to 0.2 copies for the Sox2 per 1000 copies of human  $\beta$ -actin mRNA. There was a positive correlation between the expressions of the two markers (a coefficient factor  $R=0.613$ ,  $P=0.01$ ). Although there was a trend for higher expression from younger donors, this did not achieve significance across the range of donor ages (Fig. 1B). The expression levels were not significantly different between islet and exocrine cell fractions (Fig. 1C). The expressions of Oct4 and Sox2 were not correlated with body mass indexes of their donors (data not shown).

#### Expression of Oct4 and Sox2 protein in pancreatic tissues

The tissue sections from five donors were first stained for Oct4 (Fig. 2A) or Sox2 (Fig. 2D) proteins and then restained for CK19 (Fig. 2B and E), insulin and nestin respectively. The estimated frequency of the Oct4<sup>+</sup> cells ranged 1–200 cells/100 000 pancreatic cells, varying by donor, while numbers of Sox2<sup>+</sup> cells, also varied between donors, were estimated at 1–30 cells/100 000 pancreatic cells. The majority of Oct4<sup>+</sup> and Sox2<sup>+</sup> cells were located either within small pancreatic ductal structures or close to them; the majority of them were cytosolic staining (Fig. 2A and D), with small numbers of Oct4<sup>+</sup> cells showing nuclear staining (Fig. 2A,  $1.61 \pm 0.04\%$ ,  $P<0.05$ ). The majority of Sox2<sup>+</sup> cells co-expressed Oct4 proteins (Fig. 2G and I), but not all Oct4<sup>+</sup> cells co-expressed Sox2 proteins. Oct4<sup>+</sup> and Sox2<sup>+</sup> cells did not co-express ductal cell markers CK19 (Fig. 2C and F), and neither did insulin nor nestin (data not shown).

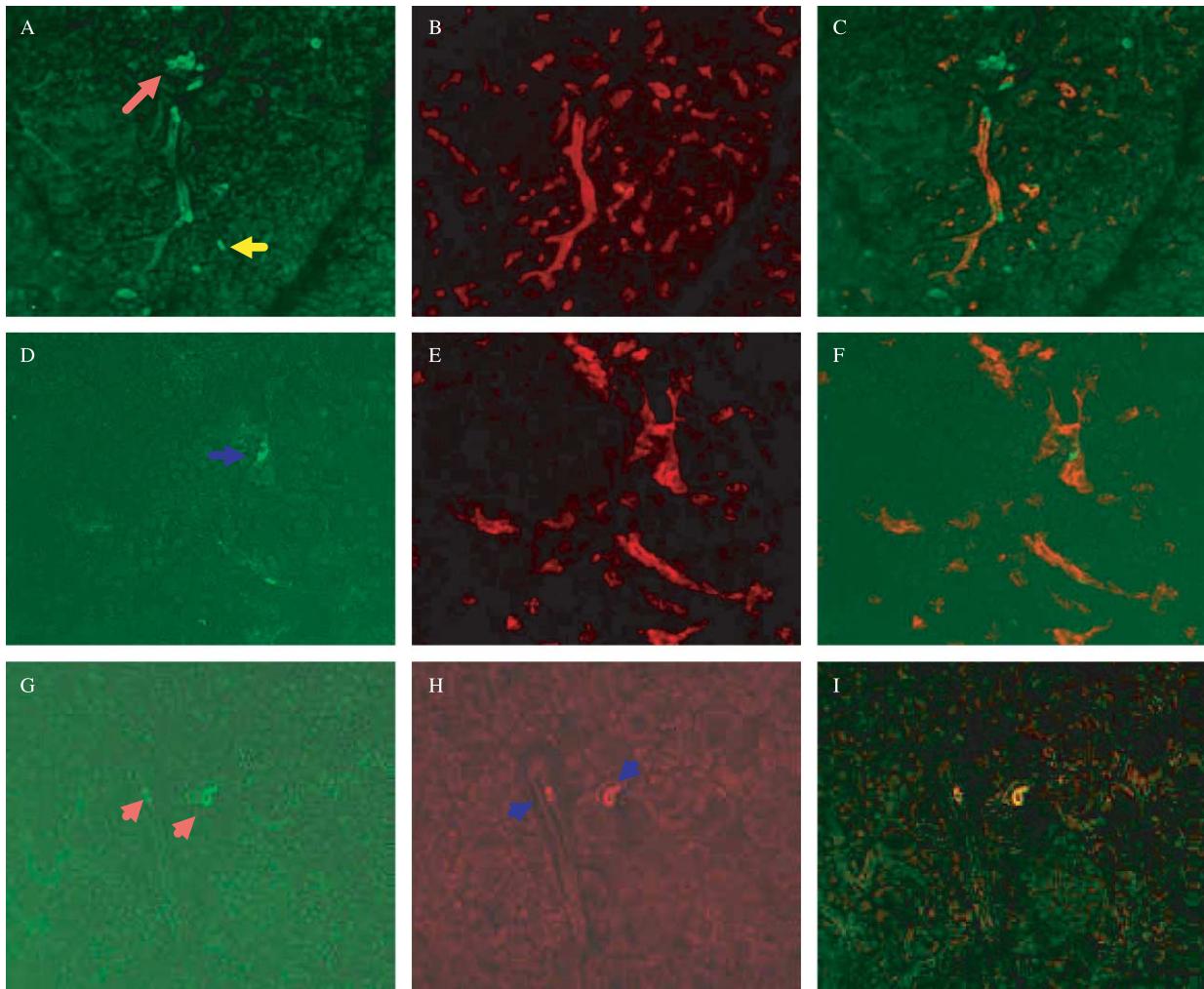
#### Oct4<sup>+</sup> and Sox2<sup>+</sup> cells did not co-express haematopoietic stem/endothelial progenitor cell marker CD34 and CD133 in human pancreatic tissues

There were no close relationships between the cells expressing CD34 (Fig. 3A and D) and CD133 proteins (data not shown) and cells staining positive for Oct4 (Fig. 3B and C) and Sox2 (Fig. 3E and F) in the adult human pancreases as assessed by immunohistochemical staining ( $n=5$ ). Human pancreatic consecutive sections were used to analyse the co-localisation of CD34 and CD133 antigens using CK19 as a comparing marker. The double staining for CD34 and CD133 proved very difficult as both monoclonal antibodies were of mouse origin. CD34<sup>+</sup> cells did not co-express CD133 protein, neither with CK19 (Fig. 4) nor with insulin and nestin (data not shown).



**Figure 1** (a) A typical semi-quantitative RT-PCR amplification of Oct4, Sox2 and CD34 from both islet-enriched fractions (left 4 lanes) and exocrine cell-enriched fractions (right 4 lanes) following the human islet preparations shows the presence of these markers in both cell fractions. Actin is used as an internal control for the cDNA used in the RT-PCR. The central lane is the molecular marker. Pancreas donor was a female, age 48 years. (b) Quantitative RT-PCR analyses on the expression of Oct4, Sox2 and CD34 mRNA in cell samples from 25 donors, plotting against donor age. RT-PCR was performed on cDNA templates, synthesised from 100 ng total RNA each of both islet and exocrine cells (per donor). Solid circle represents CD34, solid square represents Sox2 and solid triangle represents Oct4. Please note that CD34 and Oct4 were against the left Y-axis while Sox2 was against the right Y-axis. (c) Quantitative RT-PCR analyses show the expression levels of *Oct4*, *Sox2* and *CD34* gene in islet cell- and exocrine cell-enriched samples from four human islet preparations.





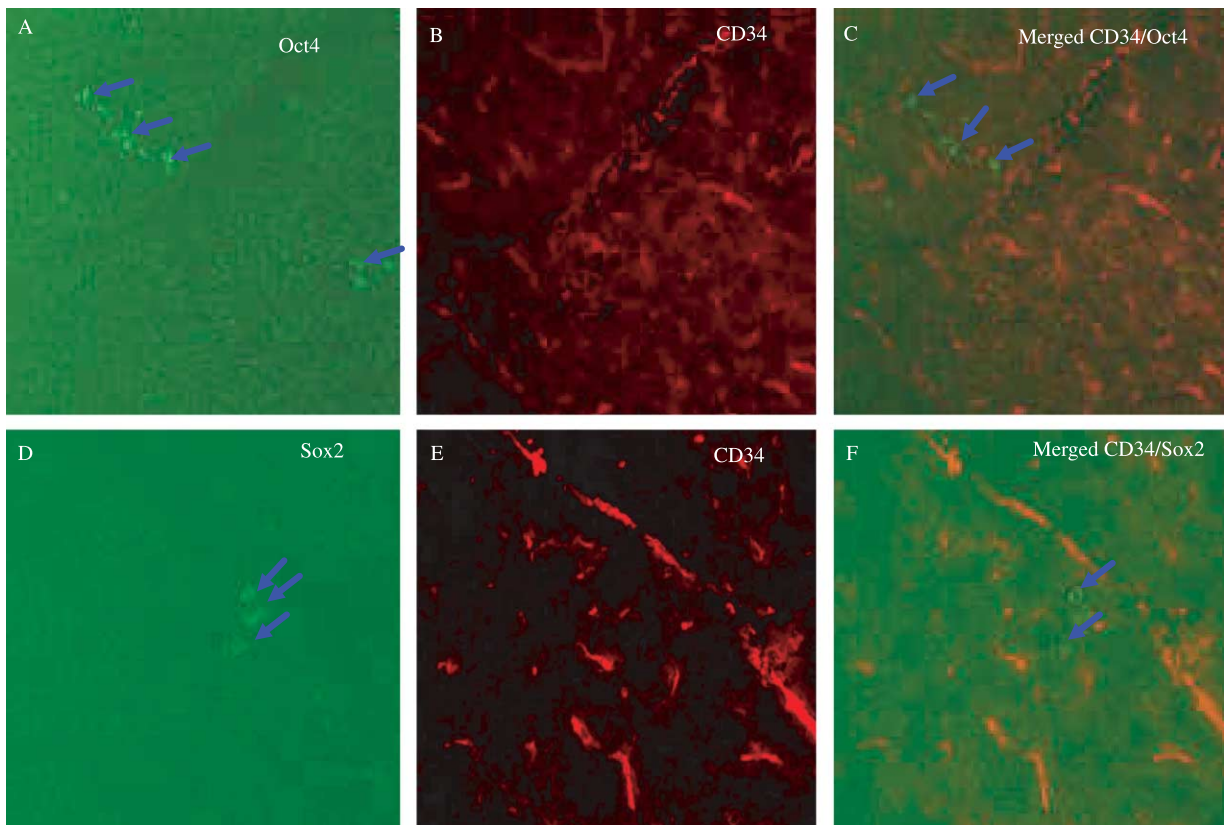
**Figure 2** Typical immunohistochemical staining images of human pancreas sections show the Oct4- and Sox2-expressing cells. Panel A shows Oct4<sup>+</sup> cells (green) in a scattered distribution around and within ductal structures. The majority of the Oct4<sup>+</sup> cells show cytoplasmic staining (red arrow shows an example), with a small number of cells showing nuclear staining (yellow arrow shows an example). Panel 2B shows the CK19<sup>+</sup> duct cells (red) and 2C is the merged image of 2A and 2B. Panel 2D shows the Sox2 positive cells (green, arrowed cell is an example), 2E the CK19 positive cells (red). 2F is the merged image of 2D and 2E. Panel 2G shows the Oct4<sup>+</sup> cells (green) and 2F shows the Sox2-expressing cells (red), while 2I is the merged image of 2G and 2F, showing the co-localisation of the two proteins in the same cells.

## Discussion

The mechanisms by which  $\beta$ -cell mass is maintained in adulthood are not yet known and the origin of new  $\beta$ -cells to replace the ageing  $\beta$ -cells is the focus of intensive research (Lipsett *et al.* 2006, Xu *et al.* 2006). During pancreas development, endocrine cells are thought to bud off the pancreatic ducts (Slack 1995). Whether some stem cell-like cells may remain in the ductal structures is still unknown. It is well established that duct cell neogenesis takes place in adult animal models in settings where  $\beta$ -cell mass does expand, e.g. in pancreatic duct ligation (Wang *et al.* 1995) or following >90% pancreatectomy (Starich *et al.* 1991), but again the source of the new cells is not known. Whether stem cells are

retained in the adult pancreatic ducts, and are then responsible for the neogenesis, remains unanswered and uncertain. Our data are compatible with the hypothesis that stem cells are to be found in the adult pancreas. We found mRNA for the classic stem cell markers Oct4 and Sox2 in cells derived from both islet and exocrine cell-enriched fractions following human islet preparations.

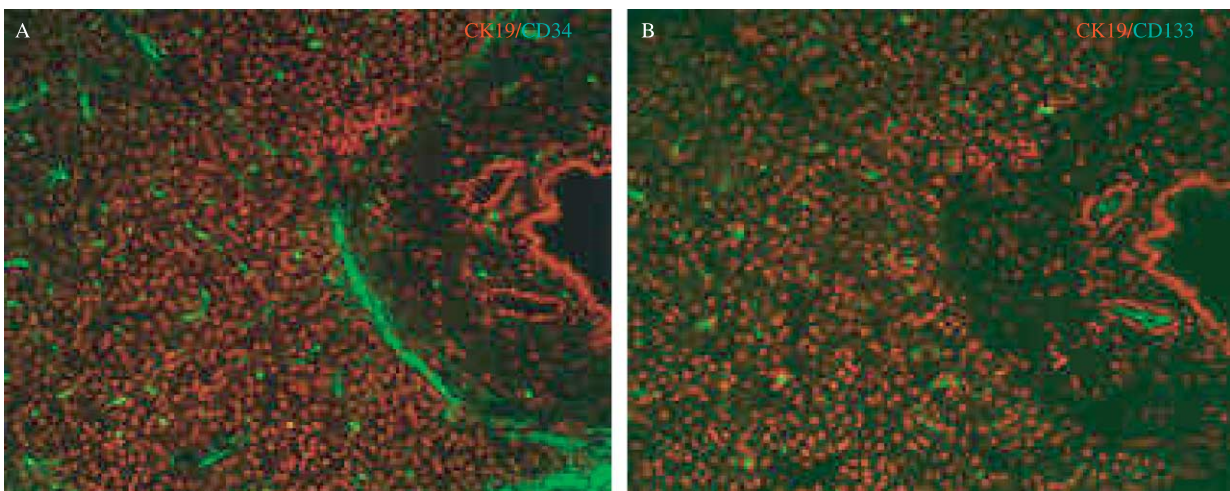
Oct4 and Sox2 are both expressed in inner cell mass stem cells and are critical in maintaining the pluripotency of stem cells (Scholer *et al.* 1990, Ellis *et al.* 2004, Ferri *et al.* 2004). In the mouse, Oct4 is highly expressed in the totipotent inner cell mass, germ cells, undifferentiated ES cells and undifferentiated embryonal carcinoma cells (Hong *et al.* 2004). Oct4 and Sox2 have been shown to jointly regulate the Nanog



**Figure 3** Co-localisation analysis of Oct4, Sox2 and CD34 proteins. Panel A and D show the Oct4 and Sox2 positive cells (green and typical cells were arrowed) in the human pancreatic sections. The sections were re-stained for CD34 protein (red) shown in panel B and E. Panel C is the merged image of A and B, and F, the merged image of D and E respectively.

gene, another transcription factor critical to the pluripotentiality of stem cells (Kuroda *et al.* 2005). Reduction of the expression of Oct4 and Nanog leads to differentiation (Hough *et al.* 2006). Mouse embryos lacking Oct4 develop to the

blastocyst stage and die after implantation, because of the lack of pluripotent embryonic cells (Bortvin *et al.* 2003). The ablation of Sox2 expression also causes early embryonic lethality for similar reasons (Avilion *et al.* 2003)



**Figure 4** Co-localisation analysis of CD34 and CD133 proteins. Human pancreatic consecutive sections were stained for CK19 (red) and then re-stained for CD34 and CD133 proteins (green) respectively, showing no co-localisation of the two markers.



The mRNA of *Oct4* and *Sox2* genes was found in cells from all the 25 human adult pancreases examined, with no obvious segregation to the endocrine or exocrine fractions. Immunohistochemical staining showed that Oct4- and Sox2-expressing cells were scattered within or very close to small ductal structures. They did not co-express CK19, insulin or nestin. Their failure to co-express CK19, the ductal cell marker, despite the fact that they were located in the ductal structures, suggests that the Oct4, Sox2 positive cells are pre-existing cells, although the possibility that Oct4 and/or Sox2 are expressed by duct cells after decreasing expression of CK19 cannot be ruled out. The majority of Sox2<sup>+ve</sup> cells co-expressed Oct4 protein, but not all Oct4<sup>+ve</sup> cells co-expressed Sox2 protein, possibly due to the low expression level of Sox2 protein; this was consistent with the observation that there was higher expression of Oct4 mRNA than Sox2 (Fig. 1b) and also indicates that they may belong to the same cell population in the adult human pancreas. Expression levels of Oct4 and Sox2 varied between pancreases but their expression was positively correlated with each other. We did not find any predictors of this expression associated with donor criteria, as a trend to an association between higher expression and younger donor age did not reach significance. We may not have included donors young enough to show this correlation.

The localisation of Oct4, Sox2-expressing cells within small duct structures in the adult human pancreas is compatible with observations of endocrine cells budding from duct structures during development and the neogenesis of duct cells that takes place after pancreatic duct ligation or >90% pancreatectomy. The plausible explanation is that Oct4 and/or Sox2 positive cells are stem cells retained in adult pancreases and may be one of the origins of new  $\beta$ -cells.

The majority of Oct4<sup>+ve</sup> cells had cytosolic staining, while a small number (~1.6%) of cells showed nuclear positivity. As Oct4 is a transcription factor, theoretically it should be located in the nuclei. The immunohistochemical staining was specific, as replacing it with other goat polyclonal antibodies did not yield any positive cells. We speculate that when Oct4<sup>+ve</sup> protein, and possibly also Sox2 protein, was present in cytosol, the cells are not in the active proliferative state. The localisation of Oct4 may reflect the biological activity of the cells (Cauffman *et al.* 2005) and nuclear staining for Oct4 may indicate cells I in a proliferation active phase. A similar scenario is seen with PDX-1 protein, which is located in the cytosol when it is dephosphorylated and only translocated into nuclei and functioning as transcription factor, when it is phosphorylated (Elrick & Docherty 2001). Another piece of evidence supporting this notion is that majority of Oct4 positive cells in pancreatic cancers are nuclear staining (Iki & Pour 2006).

Human pancreas tissues contain CD34<sup>+ve</sup> cells as confirmed by the presence of mRNA and protein, although the antibody against human CD34 can stain other cell types such as endothelial cells. It is unlikely that mRNA for Oct4, Sox2 and CD34 were derived from blood cells retained in the pancreas as the pancreas was extensively perfused in the body and on the bench by artificial media. In addition, most loose cells will be lost

during human islet isolation procedures due to the low speed centrifugation and extensive washing steps in the recovery of digested pancreatic cells, which appear as cell clumps.

In summary, this study demonstrates the presence of stem cells in adult human pancreases. Further investigation of these cells and understanding their behaviour may help us in the preservation and expansion of pancreatic islet cells in the treatment of diabetes. The presence of stem cells in the islet-enriched pancreatic fractions may be explored to improve the outcome of human islet transplantation. Equally the ready availability of these cells in the large exocrine cell-enriched fractions obtained during human isolation suggests that these may be exploitable as a new source of adult human stem cells to generate  $\beta$ -cell surrogates for cell replacement therapy in diabetes and is certainly worthy of further exploration.

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