Pluripotency-associated stem cell marker expression in proliferative cell cultures derived from adult human pancreas


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*(M G White and H R Al-Turaifi contributed equally to experimental data and manuscript preparation)

Abstract

The source of new β-cells in adult human pancreas remains incompletely elucidated with recent studies on rodents providing evidence for neogenesis from progenitor cells in addition to self-replication. The aim of this study was to investigate the expression of pluripotency-associated stem cell markers in proliferative cultures derived from adult human pancreas. Human pancreatic tissue was obtained from deceased donors following ethical approval and relative consent. Islet-enriched fraction was separated from the retrieved organ by digestion and density gradient centrifugation. Dissociated cells were seeded in adherent culture forming proliferative ‘islet survivor cells’ (ISCs). These were characterised at fifth passage by RT-PCR, immunofluorescence staining, FACS, western blot and transfection studies with an OCT4 promoter-driven reporter. Nuclear expression of the pluripotency-associated stem cell marker complex OCT4/SOX2/NANOG was confirmed in ISCs. The phenotype constituted ~8% of the overall population. OCT4 biosynthesis was confirmed by western blot and activation of an exogenous OCT4 promoter. Co-expression of pluripotency-associated markers has been confirmed in proliferative primary cells derived from adult human pancreas. Further studies are required to elucidate whether these cells possess functional stem cell characteristics and assess potential for differentiation into pancreatic cell lineages including new β-cells.

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Introduction

There is considerable evidence to support self-replication of existing β-cells as the predominant source of new β-cells in young growing rodents (Dor et al. 2004) and humans (Meier et al. 2008). In established adults (Mannesso et al. 2009) and in response to injury (Dor et al. 2004, Xu et al. 2008), however, the potential for non-β-cell sources including facultative endocrine progenitor cells has been proposed in convincing studies. Several groups have identified and isolated putative stem cells from adult pancreas (Seaberg et al. 2004, Suzuki et al. 2004, Eberhardt et al. 2006), but the expression of the pluripotency-associated embryonic stem (ES) cell markers has only been reported in exploratory studies on mice (Zuba-Surma et al. 2009) and unmanipulated human pancreas (Zhao et al. 2007, Al-Turaifi et al. 2008).

In humans, maintenance of the pluripotent circuitry within ES cells is coordinated by three transcription factors: OCT4, SOX2 and NANOG (Boyer et al. 2005). These proteins are essential for generation and maintenance of the ES cell identity by forming an auto-regulatory network that controls the expression and repression of pluripotency- and differentiation-associated genes respectively (Boyer et al. 2005). Although a key pluripotency mediator in ES cells, several studies have reported the expression of OCT4 in unmanipulated and cultured somatic tissues (Zhao et al. 2007, Kristensen et al. 2010, Pacini et al. 2010).

In common with many important human developmental genes, multiple isoforms of OCT4 are generated by alternative mRNA splicing. The two main isoforms, OCT4A and OCT4B, have been reported as having differential expression patterns with OCT4A localising to the nuclei and OCT4B detected within the cytoplasm (Atlasi et al. 2008). Whilst OCT4A has been confirmed as the isoform responsible for maintaining the ES cell identity, OCT4B fails to confer the self-renewal and pluripotency of ES cell and is thought to be involved in cell stress responses (Lee et al. 2006). In addition to OCT4 splice variant expression, the presence of transcribed pseudogenes that demonstrate significant homology to the OCT4A sequence has been reported (Liedtke et al. 2007). Collectively, potential pseudogene and splice variant expression generate the potential for false-positive results and subsequently the detection and role of OCT4 within the soma has come under scrutiny.
By using the necessary controls (ES cells (positive) and human skin fibroblasts (negative)), primers that only amplify the OCT4A transcript, and demonstration of nuclear protein expression with both western blotting and immunocytochemistry, we here provide robust evidence for OCT4A expression in proliferative cells derived from adult human pancreas. This is supported by transfection studies in which we identified a population of cells within the proliferative culture that were capable of activating the transgenic OCT4 promoter as demonstrated by GFP expression. Importantly, we have demonstrated that OCT4A-positive cells co-express SOX2 and NANOG, a phenotype that has not been previously reported in proliferative cultures derived from adult human pancreas.

Materials and Methods

**Human islets, fibroblasts and ES cells**

Pancreases were retrieved from deceased donors following formal ethical approval and written informed relative consent. Organs were handled exactly as for a whole pancreas transplant including cooling of the lesser sac and were transferred into University of Wisconsin medium to the King’s College Hospital islet isolation facility.

Islets were separated from exocrine tissue by Liberase HI digestion in a Ricordi chamber and a continuous density gradient according to the published methods (Huang et al. 2004). Purified islets were established in adherent culture in CMRL-1066 medium (Gibco, Invitrogen) including 10% foetal bovine serum and 1% insulin–transferrin–selenium supplement (Gibco). The medium was changed every 48–72 h and cells passaged at 80% confluence.

The MIN6 mouse β-cell line was available within the group. Primary human skin fibroblasts were generously provided by Dr Matthew Wright and undifferentiated human ES cells (H9) from Dr Linda Lako (both Newcastle University UK).

**RNA analysis**

Total RNA was extracted using the RNase kit 50 (Qiagen) and treated with RQ1 RNase-Free DNase (Promega) to remove contaminating genomic DNA. cDNA was synthesised using reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The PCR contained 1 μl cDNA (500 ng/μl), 1.25 μl RedTaq polymerase (Sigma–Aldrich), 2.5 μl PCR buffer, 1 μl forward primer (10 pmol/l), 1 μl reverse primer (10 pmol/l), 1 μl dNTPs (10 mM) (Promega) and 17.75 μl nanopure water. Parallel PCRs with RNA alone served as negative controls. PCR primer sequences and cycle number are given in Table 1. RT-PCR for OCT4 was validated in serial dilutions of the H9 human ES cell line, confirming semi-quantification at 35 cycles. Semi-quantitative PCR was also validated for GAPDH as a reference gene at 32 cycles.

**Immunofluorescence**

Frozen sections cut from human pancreas blocks; accutase-dissociated single cells obtained from isolated islet fractions (IF) and cytospun onto slides; and cells grown on sterile coverslips were fixed with 4% paraformaldehyde for 20 min and permeabilised with 1% t-octylphenoxy-polyethoxyethanol (Triton, Sigma–Aldrich) for 45 min. To block non-specific binding, the slides were treated with blocking buffer (Roche Applied Science) for 1 h at room temperature.

Primary antibodies diluted in blocking buffer, at concentrations given in Table 2, were added and incubated for 1 h at room temperature or overnight in a humid chamber at 4°C. In all studies, parallel no primary antibody negative controls were undertaken. Secondary antibodies (Table 3) were applied for 1 h at room temperature in the dark. Quantification of OCT4-expressing cells was done by capturing 12 random fields at 200× magnification and expressing proportion of OCT4-positive cells as a percentage of total (DAPI-positive) cells per field. Images were captured using either a Nikon Eclipse 400 inverted fluorescence, Nikon Eclipse TE2000-5 transmission fluorescence or Leica SP2 AOBS UV confocal microscope.

<table>
<thead>
<tr>
<th>Table 1 PCR primers. All genes shown are human genes</th>
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<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
<tr>
<td>Insulin</td>
</tr>
<tr>
<td>Glucagon</td>
</tr>
<tr>
<td>Somatostatin</td>
</tr>
<tr>
<td>PAPP</td>
</tr>
<tr>
<td>CK19</td>
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<tr>
<td>Amylase</td>
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<tr>
<td>OCT4</td>
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<tr>
<td>SOX2</td>
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<tr>
<td>NANOG</td>
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**Materials and Methods**

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Fluorescence-activated cell sorting

Cells were fixed with 4% paraformaldehyde for 20 min, permeabilised by 1% saponin (Sigma–Aldrich) for 20 min and treated with blocking buffer for 30 min (all at room temperature). Primary antibody (Table 2), at a concentration of 1:50, diluted in blocking buffer was then added with incubation for 60 min. Following washing with 1% saponin in PBS, secondary antibody (Table 3) was added at a concentration of 1:50 in blocking buffer with incubation in the dark for 30 min. Following further washes, cells were resuspended in PBS for FACS analysis.

Western blotting

Nuclear or whole cell extracts were prepared by performing western blotting as described previously (Macfarlane et al. 2000). Protein concentration was determined by Bradford protein assay and 30 μg of each sample were fractionated by SDS–PAGE. Following electrotransfer to a nitrocellulose membrane and blocking with 10% dried skimmed milk, the membrane was incubated overnight with primary antibody (GAPDH or OCT4 (Table 2) prepared in 1:200 dilutions) at 4 °C and then with secondary antibody (HRP-conjugated anti-rabbit 1:2000 dilution, Sigma–Aldrich) for 1 h at room temperature. Immunoreactivity was detected using a chemiluminescence kit (ECL, Amersham).

Transgene expression studies

Transfections were performed using 10 μl Lipofectamine 2000 (Invitrogen) and 3 μg plasmid DNA (pIRES-eGFP (available within the group) and OCT4–eGFP (provided by Dr Wei Cui, Imperial College London)) following the manufacturer’s instructions.

Results

Expression of OCT4 was initially demonstrated in unmanipulated human pancreas by semi-quantitative RT-PCR in comparison to human ES cells and primary human skin fibroblasts (Fig. 1A). Following isolation and purification, expression of the pluripotency-associated markers OCT4, SOX2 and NANOG (Fig. 2A) could be detected by RT-PCR in the IF in addition to the classical differentiated pancreatic phenotypic markers (insulin, glucagon, somatostatin, pancreatic polypeptide, CK19 (ductal) and amylase (exocrine)) (Fig. 2A and B).

Isolated human islet preparations of >70% purity were established in proliferative monolayer culture conditions. Islets and single cells with a range of morphologies adhered to the flask over the first 48–72 h, with proliferation over subsequent days including outgrowth of cells from the islets as described previously (Hardikar et al. 2002). Following passage, proliferative cells with a fibroblast-like morphology became the predominant phenotype. These cells were termed islet survivor cells (ISCs).

Characterisation by RT-PCR of ISCs in culture demonstrated loss of differentiated endocrine phenotypic marker expression including insulin, glucagon, somatostatin and pancreatic polypeptide over time with all being consistently undetectable at fifth passage (Fig. 2A and B). Whilst expression of the exocrine marker amylase was also lost, the specific ductal marker CK19 was maintained (Fig. 2A).

Expression of the classical pluripotent stem cell markers OCT4, SOX2 and NANOG appeared to diminish over time with increased expression in ISCs at fifth passage (Fig. 2A). H9 human ES cells and human skin fibroblasts were used as positive and negative controls respectively. Consistent OCT4 expression in ISCs was confirmed by semi-quantitative RT-PCR in three repeated studies, expressed at ~30% of the level in ES cells at fifth passage (Fig. 2C).

Table 2 Primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species raised in</th>
<th>Clonality</th>
<th>Specificity</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Ki67</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>H/M/R</td>
<td>1:100</td>
<td>Novocastra Laboratories (Milton Keynes, UK)</td>
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<tr>
<td>Anti-Oct4</td>
<td>Rabbit</td>
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<tr>
<td>Anti-Nanog</td>
<td>Goat</td>
<td>Polyclonal</td>
<td>H</td>
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<tr>
<td>Anti-Sox2</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>H/M</td>
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<td>Abcam Ab15830</td>
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<tr>
<td>Anti-CK19</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>H/M</td>
<td>1:200</td>
<td>DAKO M088801 (Cambridge, UK)</td>
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H, human; M, mouse; R, rat; Cy3, cyanine 3; TR, Texas Red.

Table 3 Secondary antibodies (all supplied by Jackson Immunoresearch, Newmarket, UK). Specificity of all IgG. Dilution of all 1:300

<table>
<thead>
<tr>
<th>Antibody against</th>
<th>Species raised in</th>
<th>Conjugation</th>
<th>Supplier code number</th>
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<tr>
<td>Goat</td>
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<td>TR</td>
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</tr>
<tr>
<td>Rabbit</td>
<td>Donkey</td>
<td>FITC</td>
<td>711-095-152</td>
</tr>
<tr>
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<td>Donkey</td>
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<td>712-095-153</td>
</tr>
<tr>
<td>Mouse</td>
<td>Donkey</td>
<td>Cy3</td>
<td>715-165-151</td>
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</table>

H, human; M, mouse; R, rat; Cy3, cyanine 3; TR, Texas Red.
Immunofluorescence studies demonstrated OCT4 staining in a small number of cells throughout the pancreas (Fig. 1B) with confocal microscopy confirming that stained cells were very small (1.5–3 µm) and nucleated with cytoplasmic OCT4 localisation (Fig. 1C). Cytoplasmic localisation was also demonstrated in IF cells with no evidence of co-localisation with insulin (Fig. 3A) or CK19 (Fig. 3B).

Following culture, the expression pattern of OCT4 was altered with nuclear localisation of the transcription factor evident in 8.6 ± 2.2% (mean ± s.d. of > 500 cells from each of the three donors) of ISCs at fifth passage (Fig. 3C). There was no co-localisation of OCT4 and CK19 (Fig. 3D), with protein expression of insulin and all other end-differentiated endocrine markers lost (data not shown).

Nuclear co-localisation of NANOG (Fig. 4A) and SOX2 (Fig. 4B) in OCT4-positive cells was confirmed by confocal microscopy. H9 human ES cells were used as a positive OCT4, SOX2 and NANOG control and human skin fibroblasts as negative control showing no positive staining (data not shown).

OCT4 protein expression in ISCs was quantified by flow cytometry (Fig. 5B), with no OCT4 positivity detected in the absence of primary antibody (Fig. 5A) or in control human skin fibroblasts (data not shown).

Approximately 8% of ISCs at fifth passage expressed OCT4 (Fig. 5C). Nuclear OCT4 protein localisation in ISCs was further demonstrated by western blot with a band corresponding to that in H9 human ES cells (Fig. 5D).

No OCT4 protein expression was seen in human skin fibroblasts. Western blot densitometry (corrected for GAPDH band intensity) demonstrated nuclear expression in ISCs at 48% of that in human ES cells.

ISCs were transfected with a plasmid designed to label undifferentiated pluripotent ES cells in which the GFP reporter gene is expressed under the control of the human OCT4 promoter. Control transfections with the pIRE-S-GFP plasmid in which GFP is driven by the constitutive cytomegalovirus demonstrated the capability of ISCs to express exogenous GFP with a transfection efficiency of ~5% (Fig. 6A1). Expression of eGFP under the control of the OCT4 promoter in ISCs was confirmed in ~0.5% of cells (Fig. 6A2) with expression of nuclear OCT4 (Fig. 6B) and NANOG (Fig. 6C) in all GFP-positive cells indicating the accurate designation of putative stem cells in these reporter gene studies. Furthermore, the absence of Ki67 staining in all GFP-positive ISCs demonstrated that OCT4-expressing cells showed no evidence of active proliferation.

Figure 1 OCT4 expression in unmanipulated adult human pancreas. (A) RT-PCR analysis of OCT4 expression in human pancreas (HP) compared to human embryonic stem cells (hESCs) and human skin fibroblasts (HSF). (B) Immunofluorescence staining of OCT4 (red) in human pancreas sections. Size bar=100 µm. (C and D) Confocal images of small OCT4 (red)-positive cells (~2–3 µm). Size bars=3-07 µm (C) and 2-23 µm (D). Nuclei are stained with DAPI blue. Data are representative of results from four separate human pancreases.

Figure 2 Expression of differentiated and pluripotency-associated genes in ISCs. (A) Analysis of insulin, CK19 and pluripotency-associated gene expression in islet-enriched fraction (IF) and cultured ISCs over ten passages. Human embryonic stem cells (hESCs) and human skin fibroblasts (HSFs) were included as positive and negative controls respectively. (B) Expression of endocrine and exocrine genes in ISCs at P5 in comparison to IF and primary HSFs. GAPDH was used as a reference gene control in all RT-PCR experiments. (C) Percentage expression of OCT4 mRNA relative to GAPDH expression in HSFs, ISCs (P5) and hESCs determined by densitometry. Data are mean ± s.d.; *P<0.05; **P<0.01 (one-way ANOVA). Results are representative of those from three separate human pancreases (all of which are included in the data comprising the graph in panel C).
Stem cell specificity was evidenced by OCT4–GFP expression in transfected H9 human ES cells but absence of expression in transfected MIN6 mouse β-cells and primary human skin fibroblasts (data not shown).

**Discussion**

Several groups have previously published evidence of proliferative cultures derived from purified human islet preparations, with lineage tracing studies confirming the contribution of de-differentiated β-cells (Gershengorn et al. 2004, Kayali et al. 2007, Russ et al. 2008, 2009). However, these islet-enriched fractions are not entirely pure as evidenced by initial expression of ductal and exocrine markers in these studies. Following culturing to the fifth passage, exocrine and differentiated endocrine marker expression was lost, although the ductal marker CK19 was maintained with evidence of proliferation by Ki67 positivity.

Collectively, OCT4, SOX2 and NANOG form the core transcriptional network responsible for maintaining pluripotency in ES cells (Boyer et al. 2005). Although a key ES cell regulator, OCT4 expression has been detected in multiple somatic tissues including the liver, kidney, bone marrow and umbilical cord blood (Tai et al. 2005, Seo et al. 2009, Wer & Shen 2011). Cells expressing OCT4 termed ‘very small embryonic-like stem cells’ have recently been isolated from murine pancreas (Zuba-Surma et al. 2009), and it has been proposed that these may provide a quiescent pool of stem cells for replenishing tissue-specific progenitors in times of extensive tissue injury. The presence of cells expressing OCT4 and SOX2 in unmanipulated human pancreas has previously been reported (Zhao et al. 2007). In this study, we have confirmed the size of OCT4-positive cells as only ~1.5–3 µm, in keeping with murine ‘very small embryonic-like stem cells’. Furthermore, we have demonstrated the presence of larger OCT4-expressing cells in purified islet-enriched fractions, with no co-localisation with insulin or CK19-positive cells.

Expression of OCT4 in human pancreas-derived primary cell cultures has been previously reported (Tai et al. 2005, Gorjup et al. 2009). However, here we have demonstrated for the first time evidence for the nuclear co-localisation of OCT4, SOX2 and NANOG, a phenotype that constitutes ~8% of the overall population following five passages. Critically, through specific experimental design, we have provided novel evidence for expression in human pancreatic derived proliferative cells of OCT4A, the only isoform capable of maintaining pluripotency.

Two main OCT4 isoforms have been described, OCT4A and OCT4B (Lee et al. 2006, Atlasi et al. 2008). Since OCT4A is the only isoform capable of conferring pluripotency, considerable concerns have been expressed regarding the potential for falsely designating expression of OCT4B as OCT4A in cells derived from adult tissues. Given that...
the OCT4B mRNA transcript is truncated without exon 1 of the original OCT4A isoform (Atlasi et al. 2008), we were able to eliminate false-positive RT-PCR results by designing the 5' primer to recognise a sequence within the first exon. Additionally, the potential for pseudogene expression was eliminated by treating all RNA samples with DNase and conducting parallel RNA only PCR-negative control reactions.

In all of these studies, H9 ES cells were used as the gold standard positive control. Moreover, no OCT4 gene or protein expression was detected in human skin fibroblasts used as a negative control. OCT4 protein expression was confirmed by immunostaining, flow cytometry and western blotting. Nuclear localisation provided further evidence for functional OCT4A, as opposed to OCT4B expression, which has been reported as being expressed exclusively within the cytoplasm (Atlasi et al. 2008). In addition, activation of the OCT4 promoter in a reporter gene construct was seen in H9 ES cells and ISCs but not in control differentiated β-cells or human skin fibroblasts.

Recently, expression of OCT4B in peripheral blood mononuclear cells has been reported (Papamichos et al. 2009). This study also found the regulatory sequences of the human OCT4 promoter sequence within our reporter construct comprised the full-length promoter sequence upstream of the transcription start site (Okazawa et al. 1991, Minucci et al. 1996, Yeom et al. 1996). This reporter construct has been extensively characterised with several reports demonstrating its stem cell-specific expression (Gerrard et al. 2005). In particular, GFP expression has been shown to closely correlate with endogenous OCT4 expression in human ES cells (Gerrard et al. 2005). Furthermore, utilisation of this reporter construct has enabled groups to identify false-positive detection of OCT4 in adult tissues. For example, by using several published primers, OCT4 expression was detected in a number of adult tissues; however, the only cells capable of activating GFP expression under the control of the OCT4 promoter were embryonic carcinoma cells (positive control) (Redshaw & Strain 2010). Sequencing of each mRNA transcript identified OCT4A expression in EC cells only, with all adult cells demonstrating OCT4 pseudogene or splice variant expression.

OCT4 promoter sequence within our reporter construct comprised the full-length promoter sequence upstream of the transcription start site (Okazawa et al. 1991, Minucci et al. 1996, Yeom et al. 1996). This reporter construct has been extensively characterised with several reports demonstrating its stem cell-specific expression (Gerrard et al. 2005). In particular, GFP expression has been shown to closely correlate with endogenous OCT4 expression in human ES cells (Gerrard et al. 2005). Furthermore, utilisation of this reporter construct has enabled groups to identify false-positive detection of OCT4 in adult tissues. For example, by using several published primers, OCT4 expression was detected in a number of adult tissues; however, the only cells capable of activating GFP expression under the control of the OCT4 promoter were embryonic carcinoma cells (positive control) (Redshaw & Strain 2010). Sequencing of each mRNA transcript identified OCT4A expression in EC cells only, with all adult cells demonstrating OCT4 pseudogene or splice variant expression.
Demonstration of OCT4–GFP-expressing ISC with nuclear co-localisation of OCT4, NANOAG and SOX2 and absence of co-localisation with any differentiated markers provides robust evidence for the existence of a pancreatic phenotype, which has not previously been adequately characterised. Although the origin of these cells remains unknown, migratory mesenchymal stem cells (MSCs) are a possible candidate with studies indicating the presence of cells within pancreatic primary cultures expressing markers characteristic of MSCs, including CD90 and CD105 (Carlotti et al. 2010). Indeed, expression of OCT4, SOX2 and NANOAG has been previously demonstrated in MSCs derived from adult tissues (Rieckstina et al. 2009) with OCT4 and NANOAG RNA expression in pancreas-derived cells (Gorjup et al. 2009). Further studies are planned to explore the origin of these cells and to undertake in vitro lineage tracing studies to determine whether these are derived from cells in unmanipulated pancreas expressing OCT4 in the cytoplasm, whether they possess functional stem cell characteristics and potential for differentiation into mature pancreatic cell types.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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