



Murine CD133⁺CD49^{flow/+} Cells Derived from ESCs Differentiate into Insulin Producing Cells *in vivo*

Jesús Ciriza^{1,3*}, Christa Caneda¹, Bryce McLelland¹ and Jennifer O. Manilay^{1,2}

¹Molecular and Cell Biology Unit, University of California, Merced, School of Natural Sciences, USA

²Quantitative and Systems Biology Graduate Group, School of Natural Sciences, University of California, USA

³Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, Spain

*Corresponding author: Jesús Ciriza, NanoBioCel Group, Laboratory of Pharmacy and Pharmaceutical Technology, Faculty of Pharmacy, University of the Basque Country UPV/EHU, Vitoria-Gasteiz, 01006, Spain, Tel: +34945014518, E-mail: jeciases@yahoo.es

Abstract

Pancreatic progenitors have been identified and isolated during embryonic development showing their potential to differentiate into insulin producing cells after transplantation. Subsequently, they have been proposed as an alternative to the treatment of Type I diabetes. However, fetal pancreata represent a limited donor supply and we proposed that ESC-derived pancreatic progenitors are another possible option. We isolated committed pancreatic CD133⁺CD49^{flow/+} progenitors, expressing neurogenin 3, from insulin producing cells derived from mouse embryonic stem cells *in vitro*. Here, we demonstrate their potential to differentiate into insulin producing cells *in vivo* while not forming teratomas. Our results can pave the way for future studies to ascertain the ability of *in vitro* ESC-derived pancreatic progenitors to be translated for clinical application for Type I diabetes treatments.

Keywords

Murine embryonic stem cells; Pancreatic progenitors; Type I Diabetes; Teratoma; Kidney capsule transplantation; Insulin producing cells

Introduction

Worldwide, 347 million people suffer from diabetes [1] and the World Health Organization projects that diabetes will be the 7th leading cause of death by 2030. In the United States, 25.8 million children and adults had diabetes in 2011, representing 8.3% of the total population [2], and costing \$116B for treatment and diagnosis. Type 1 diabetes (T1D) is an autoimmune disease that aggressively attacks the insulin producing cells (IPCs) in the pancreas, reducing the number of these cells to levels that force the patient to be dependent on daily insulin injections. Islet cell transplantation can be used for treatment, but sources of autologous and allogeneic donor insulin producing cells for transplantation are limited.

Embryonic stem cells (ESCs) have been proposed as an alternative source of insulin producing cells [3]. Major concerns about the use

of ESC-derived cells for therapy include their teratoma formation ability and their possible immune rejection after transplantation. Teratoma formation prevented successful treatment of diabetic mice with ESC-derived cells [4] and immune rejection of insulin producing cells derived from ESCs was observed as early as 3 days post-transplantation into immunocompetent mice [5,6]. To improve this outcome, the enrichment of definitive endodermal progenitors by flow cytometric sorting [7,8] could be used to deplete cultures of undifferentiated cells (which are the main sources of teratomas), while the prediction of the immunogenicity of insulin producing cells derived from ESCs (ESC-IPCs) could indicate possible preconditioning regimens for patients that received ESC-IPC transplants.

Several cell culture protocols have been described for the differentiation of murine nontransgenic ESCs into IPCs, [3] but three protocols appear to be the most highly utilized [9-11]. The three protocols share the common initial step of embryoid body (EB) formation, but differ in the use of selection of nestin-expressing cells. When compared side by side, ESC-IPC differentiated using the Blyszczuk et al. [11] protocol were able to produce and release more insulin *in vitro* than those differentiated by the other two methods. However, they only rescued 33% of diabetic mice within 2 weeks and ultimately, these mice developed teratomas [12], likely due to the remaining undifferentiated ESCs in the cultures at the time of transplantation. Exhaustive microarray studies studying gene expression profiles of ESC-IPC at different stages of *in vitro* differentiation following the Blyszczuk et al. [11] protocol have shown that murine ESC-derived pancreatic cells appear to be blocked at the embryonic/fetal stage of development [13].

In contrast to fully mature IPCs, pancreatic progenitors generated from ESCs (ESC-PPs) might be more suitable for cellular replacement therapy in T1D. A homogenous population of ESC-PPs identified by cell surface markers may not self-renew as stem cells do, but could differentiate into endocrine cells after transplantation [14]. For example, *Pdx1*⁺*Cxcr4*⁺ ESC-derived cells spontaneously differentiated

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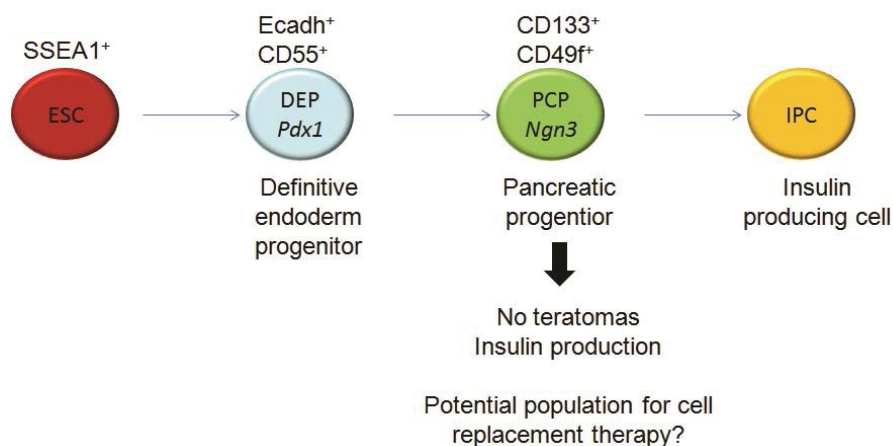


Figure 1: Schematic representation of IPC differentiation: During differentiation from ESC to IPC, definitive endoderm and pancreatic progenitors can be isolated by means of different extracellular markers

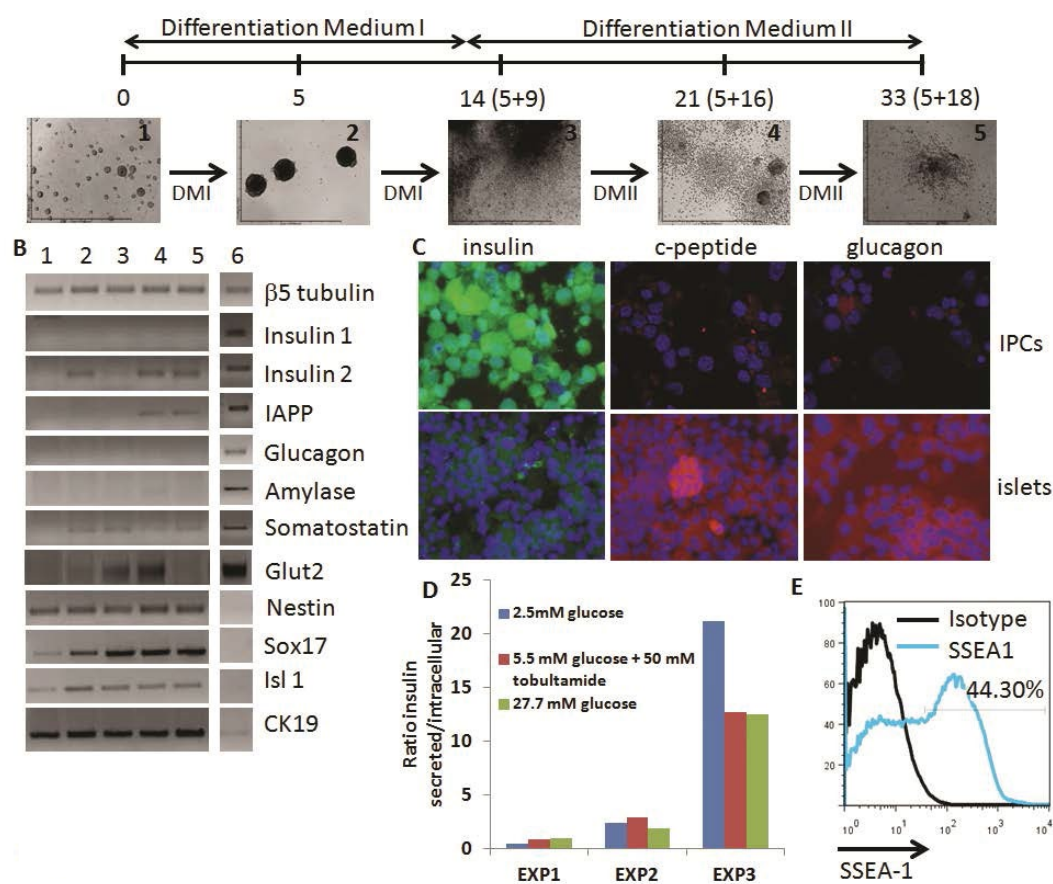


Figure 2: Analysis of ESC-IPCs. A) Scheme of ESC-IPC differentiation protocol. Stages of IPC differentiation: 1: D3-ESC on gelatin, 2: EBs, 3: Day 5+9, 4: Day 5+16, 5: Day 5+28 DMI: Differentiation Medium I and DMII: Differentiation Medium II. B) RT-PCR analysis of stages of IPC differentiation: 1: D3-ESCs, 2: 5+9, 3: 5+16, 4: 5+28, 5: adult whole pancreas, 6: isolated pancreatic islet. C) Immunostaining of ESC-IPCs (top row) and islets (bottom row): insulin (green), c-peptide (red), glucagon (red) and nuclei (blue) (20x magnification). D) Heterogeneous insulin release by IPCs after glucose challenge. E) IPC-ESCs express SSEA-1 (specific marker staining in blue, isotype control in black)

into IPCs *in vivo*, and led to a sustained correction of hyperglycemia in diabetic mice for a month with survival rates longer than three months [15]. Late definitive endoderm cells can be identified by the combination of E-cadherin and Decay Accelerating Factor (DAF1/CD55) cell surface markers on cells that co-express also *Pdx1* [16] (Figure 1). *Ngn3*⁺ cells have been suggested to be another possible murine pancreatic progenitor population [17,18]. CD133⁺CD49f^{low/+} cells isolated from murine fetal pancreas have been described to express *Ngn3* (Figure 1) as well as *insulin-1*, *glucagon*, *somatostatin* and *pancreatic polypeptide* after 2 days of co-culture with mitomycin C-treated mouse embryonic fibroblast (MEFs) [8]. Moreover,

transplanted CD133⁺ PDFGR β cells isolated during murine pancreas development induced the formation of IPCs that expressed Cpeptide (a definitive feature of functional insulin production) within 7 days of culture and showed self-renewal properties after serial transplantation, suggesting they might be true pancreatic stem cells [19]. *Ngn3*⁺ cells have been also detected in EBs from murine *Ngn3*-GFP ESC lines expressing *insulin*, *glucagon* and *Pdx1*, among other pancreatic markers, after sorting and differentiation *in vitro* [20]. However, the identification with cell surface markers of non-transgenic pancreatic progenitor cells expressing *Ngn3* from ESC-differentiation cultures and their application *in vivo* has not yet been reported.

Here, we present the identification of CD133⁺CD49f^{low/+} and E-cadherin⁺ CD55⁺pancreatic progenitor cells from a slightly modified ESC-IPC differentiation protocol. We show that CD133⁺CD49f^{low/+} ESC-PPs can be transplanted into the kidney capsule of immunodeficient mice, produce insulin, and do not form teratomas.

Results

Heterogeneous and immature ESC-IPCs are generated using current protocols

We initiated our studies with the ESC-IPC differentiation protocol described by Blyszczuk et al. [11] since it seemed to be the most efficient in terms of insulin production and release [12]. This protocol includes the formation and growth of EBs for 5 days, their expansion for 9 days on gelatin-coated plates and their specific differentiation into IPCs for 18 days on collagen I coated plates with a defined differentiation medium (Figure 2A). Healthy groups of cells could be observed *in vitro* after Day 5+16, with as low as 5% FCS in the media (Figure 2A). Gene expression analysis showed similarities between ESC-IPCs and murine pancreatic β islet samples such as expression of *insulin 2* and *IAPP* with very low expression of *somatostatin* and *amylase* (Figure 2B). Genes described to be expressed during the development of insulin producing cells (*Glut2*, *Nestin*, *Sox17*, *Isl1* and *cytokeratin19* (CK19)) were also detected at different stages of the differentiation protocol including the final stage at Day 5+28 (Figure 2B). However, in contrast to β islet samples, *insulin 1* was not detected at any time of differentiation (Figure 2B).

Immunohistochemistry analysis showed a high concentration of insulin accumulated inside the IPCs with low expression of c-peptide, in contrast to the β islet samples (Figure 2C). These data suggested

that incomplete insulin processing at the protein level was occurring, and indicated that IPCs were not fully functional. Similarly, glucagon expression was much lower in IPCs than β islets (Figure 2C). The insulin release response in IPCs to glucose stimulation *in vitro* was low and heterogeneous, with higher ratios of release at lower glucose concentrations in some experiments (Figure 2D). Finally, we detected that almost half of the IPCs at Day 5+28 of differentiation expressed SSEA-1 (Figure 2E), which suggested that they were teratoma-forming cells [12]. Based on these results, we next considered a different population of cells for further investigation.

Pancreatic progenitors can be detected in ESC-IPC cultures

We investigated if putative pancreatic progenitors could be identified *in vitro* during differentiation of ESCs into IPCs (Figure 1). First, using flow cytometry, we analyzed the presence of definitive endoderm progenitors with the cell surface marker phenotype. Ecadh⁺CD55⁺, as previously described *in vitro* with other ES cell lines [16]. Low percentages of Ecadh⁺CD55⁺ cells were detected at Day 5+16 of differentiation (mean \pm SD: 0.93 \pm 0.18 %, n=3) (Figure 3A). Almost all Ecadh⁺CD55⁺ cells were SSEA-1⁺, suggesting a high teratoma formation ability potential if transplanted (Figure 3B).

We then searched for pancreatic progenitors that were more differentiated than definitive endoderm progenitors and with less potential of forming teratomas (Figure 1). *In vivo*, murine and human committed pancreatic progenitors have been identified by the CD133⁺CD49f^{low/+} cell surface phenotype, and their potential to differentiate *in vitro* into insulin producing cells has been verified [8,19]. We were able to quantify an average of 4.07 \pm 1.29 % of CD133⁺CD49f^{low/+} pancreatic progenitors at Day 5+16 in our IPC differentiation cultures (Figure 3C). This higher frequency of cells, compared to Ecadh⁺CD55⁺ cells, allowed for the collection of

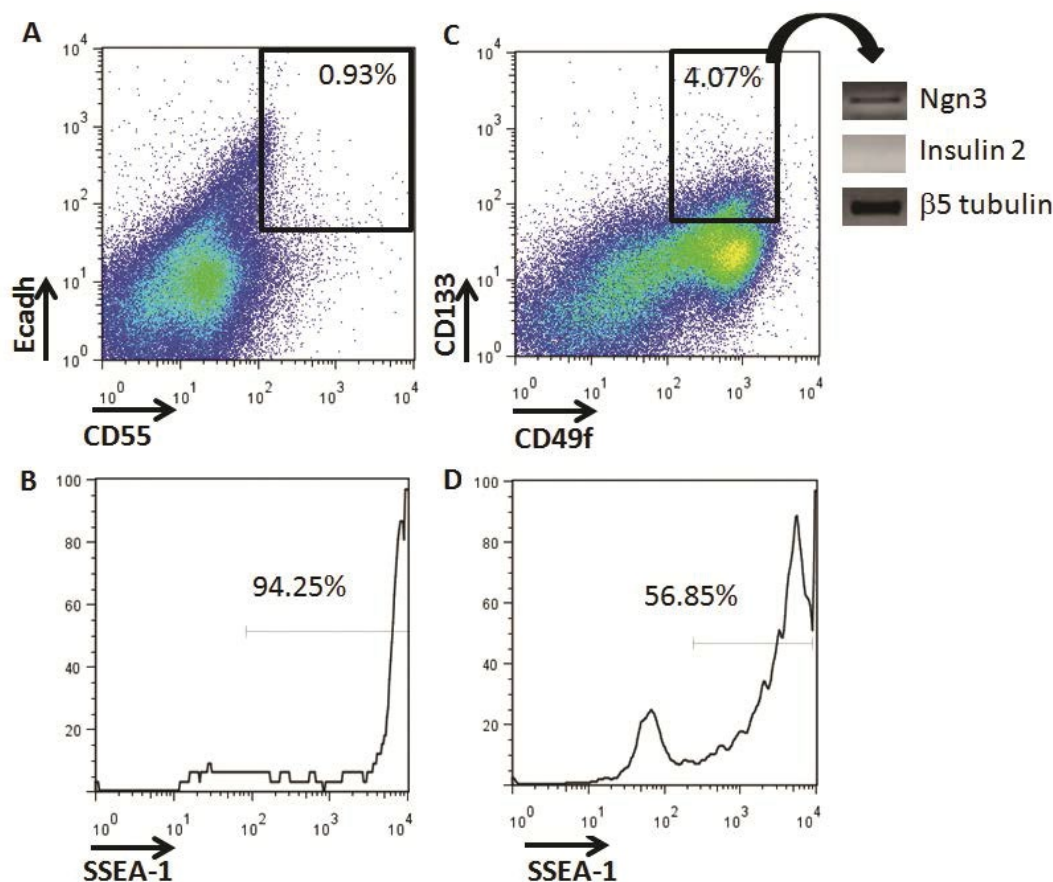


Figure 3: Identification of definitive endoderm and committed pancreatic progenitors from *in vitro* IPC-ESC cultures by flow cytometry. A) Live cells at Day 5+16 were analyzed for the expression of E-cadherin and CD55. The gate shows the Ecadherin⁺CD55⁺ definitive endoderm progenitors. The data shown are representative of three independent experiments. B) Histogram analysis of SSEA1 expression on Ecadherin⁺CD55⁺ definitive endoderm cells. C) Live cells at Day 5+16 were analyzed for the expression of CD133 and CD49f. The gate shows the CD133⁺CD49f^{low/+} committed pancreatic progenitors. Data shown are representative of three independent experiments. D) Histogram analysis of SSEA1 expression on CD133⁺CD49f^{low/+} committed pancreatic progenitors

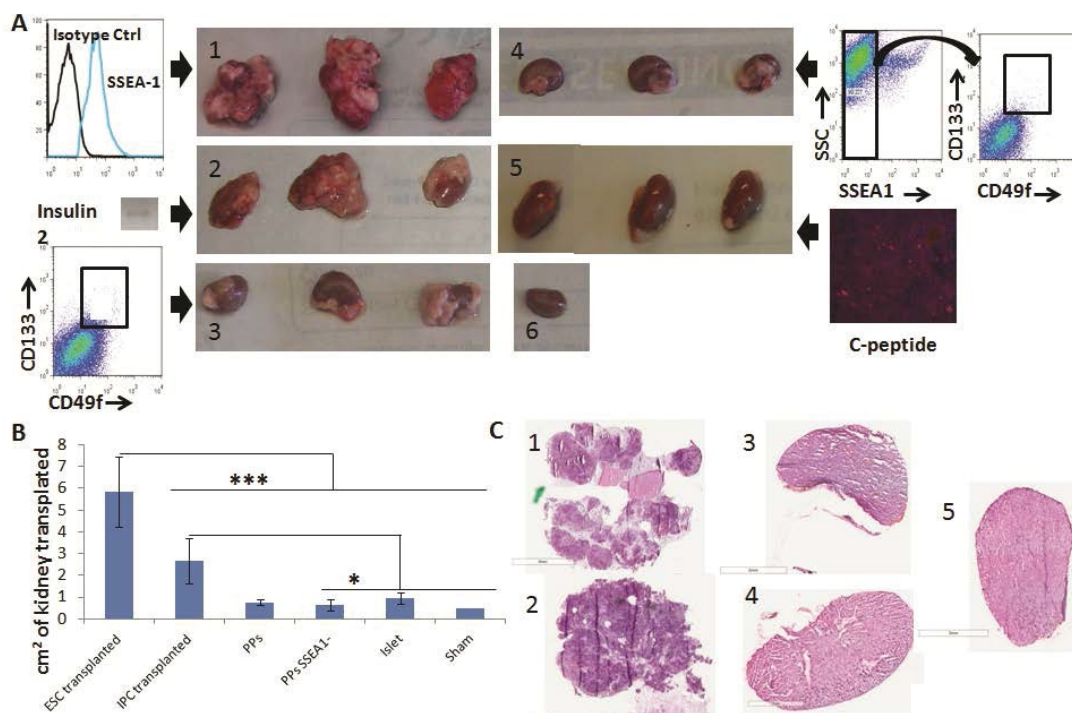


Figure 4: Engraftment and teratoma formation analysis of ESC-IPCs and ESC-PPs *in vivo*. A) Macroscopic observations of kidneys on Day 28 after transplantation of: 1: 10^6 ESCs; 2: 6×10^6 IPC-ESCs (expressing *insulin 2*, inset); 3: 5×10^5 CD133⁺CD49f^{low/+} committed pancreatic progenitors; 4: 5×10^5 CD133⁺CD49f^{low/+}SSEA1- committed pancreatic progenitors; 5: 300 islets (positive for c-peptide); and 6: no cells (sham control). B) Comparison of kidney sizes after transplantation (*: $p < 0.05$ and ***: $p < 0.001$). C) Hematoxylin/eosin staining from kidney sections transplanted with: 1: 10^6 ESCs; 2: 6×10^6 IPC-ESCs; 3: 5×10^4 CD133⁺CD49f^{low/+} committed pancreatic progenitors; 4: 5×10^4 CD133⁺CD49f^{low/+}SSEA1- committed pancreatic progenitors and 5: 300 islets

enough cells by flow cytometry to analyze their gene expression. CD133⁺CD49f^{low/+} pancreatic progenitors did not express *insulin-2*, but expressed *Ngn3*, indicating that they belong to an earlier developmental stage, but were committed to differentiate into IPCs (Figure 3C). These progenitors did not express either pancreatic marker such as *Pdx1* or *Pax4* by RT-PCR (data not shown). CD133⁺CD49f^{low/+} pancreatic progenitors also expressed SSEA-1 (Figure 3D). The expression of SSEA-1 indicated their potential to form teratomas, which we then tested *in vivo*.

CD133⁺CD49f^{low/+} pancreatic progenitors did not form teratomas and expressed insulin

First, we transplanted 10^6 SSEA1⁺ D3-ESCs under the kidney capsule of NSG mice and all mice developed teratomas by 28 days post-transplantation, as expected (Figure 4A, panel 1). Conversely, as a negative control for teratoma formation, we transplanted 300 islets isolated from adult pancreas and they did not form teratomas (Figure 4A, panel 5). We then tested the ability of our ESC-IPCs to form teratomas after transplantation. First, we confirmed that IPCs at Day 5+28 of differentiation expressed *insulin 2*, and then we transplanted $4-6 \times 10^6$ IPCs. All of the IPC-recipient mice developed teratomas by 28 days after transplantation (Figure 4A, panel 2). This result confirmed previous results [11] and justified the exploration of an alternative ESC-derived cell population for the replacement of insulin.

We hypothesized that more immature pancreatic progenitors could be generated, isolated and transplanted *in vivo*, and that after transplantation, they would not form teratomas and would produce insulin. As mentioned above, CD133⁺CD49f^{low/+} cells have been demonstrated to be pancreatic progenitors (PPs) in mouse embryos [8] and we confirmed that CD133⁺CD49f^{low/+} cells were generated in our *in vitro* cultures. We tested the function and potential of these CD133⁺CD49f^{low/+} ESC-PPs to form teratomas *in vivo*. Since SSEA-1 expression is often equated with pluripotency and teratoma-forming ability in ESCs, we also compared the behavior of sorted SSEA-1- CD133⁺CD49f^{low/+} ESC-PPs. We transplanted 5×10^4 pancreatic progenitors (SSEA1⁺ depleted or not) as previously described [8]. No teratomas were observed in mice transplanted with either

population (Figure 4A, panels 3,4). No significant differences were found amongst the sizes of the kidneys transplanted with ESC-PPs, islets or sham controls (Figure 4B), a result that correlated with a lack of teratoma forming ability in ESC-derived PPs. The absence of teratoma formation in recipients of CD133⁺CD49f^{low/+} or SSEA-1- CD133⁺CD49f^{low/+} ESC-PP cells was confirmed by histological analysis of kidney sections from the transplanted mice (Figure 4C, panels 3,4). In contrast, teratomas were clearly observed in kidneys transplanted with ESCs and ESC-IPCs (Figure 4C, panels 1,2). ESC-IPCs, CD133⁺CD49f^{low/+} ESC-PPs, SSEA-1- CD133⁺CD49f^{low/+} ESC-PPs and islets exhibited positive staining for insulin (Figure 5A, panels 2-5), whereas sections of kidney capsules transplanted with ESCs did not (Figure 5A, panel 1). Insulin production was confirmed via the observation of strong c-peptide expression in the kidneys that were transplanted with CD133⁺CD49f^{low/+} ESC-PPs and control islets (Figure 5B, panels 1,3). SSEA-1-CD133⁺CD49f^{low/+} ESC-PPs showed positive, but lower expression of c-peptide (Figure 5B, panel 2).

Discussion

We generated ESC-IPCs that express endocrine genes such as *insulin 2*, *glucagon* and *somatostatin*, and our data showed that ESC-IPC cultures heterogeneously secrete insulin protein, corroborating the findings of other groups [12]. Based on the lack of peptide signal by immunohistochemistry, we posit that the insulin is not completely processed intracellularly within ESC-IPCs and consequently, is not released properly outside the cell. One possible reason for this non-functional insulin release is incomplete differentiation of the ESC-IPCs, resulting in a cell that is devoid of fully developed mechanisms for insulin processing. Other groups have suggested that the detection of insulin from ESC-IPCs is an artifact from apoptotic or necrotic cells [21] or a mixture of *de novo* insulin production mixed with insulin uptake from the medium [22] and this controversy is still unresolved. In our hands, insulin release from ESC-IPCs after glucose challenge varied widely from experiment to experiment, perhaps reflecting the presence of a heterogeneous mixture of cells that are generated in each independent ESC-IPC culture [12]. The immature nature of ESC-IPCs, their inconsistent release of insulin, and their propensity to

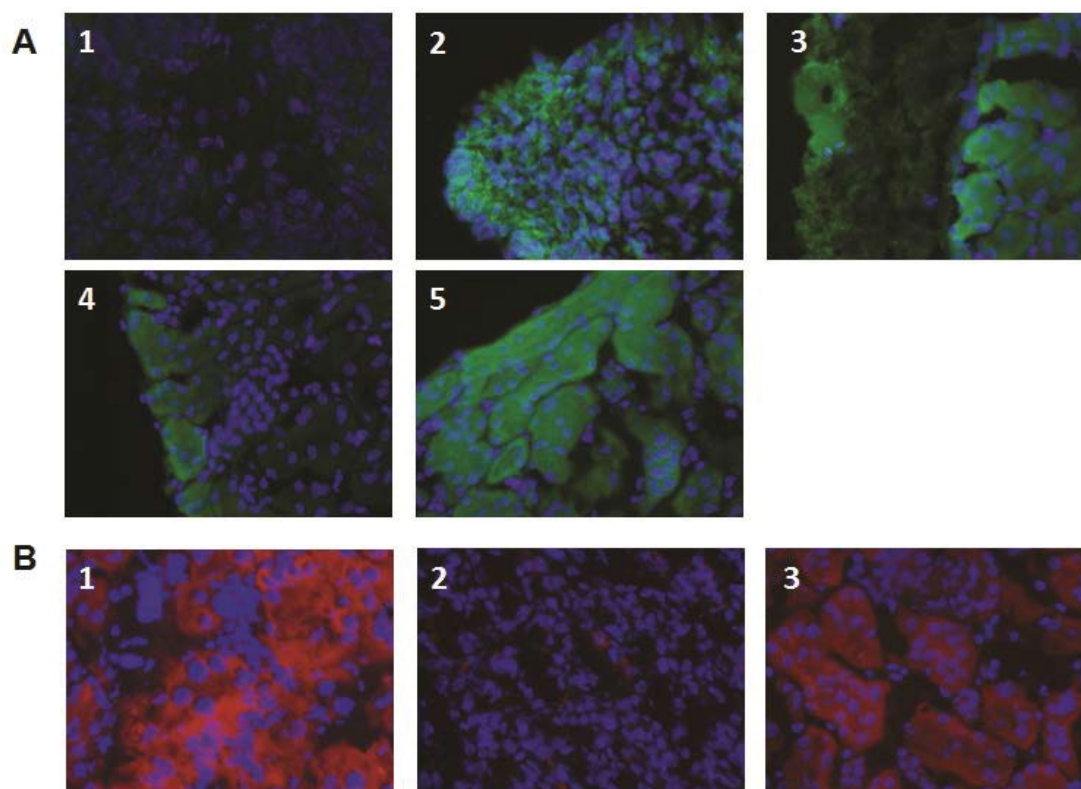


Figure 5: Insulin and c-peptide expression in kidneys transplanted with ESC-IPCs and ESC-PPs. (A) Kidney sections were stained for insulin from mice transplanted with: 1: 10^6 ESCs; 2: 6×10^6 IPC-ESCs; 3: 5×10^5 CD133⁺CD49f^{low/+} committed pancreatic progenitors; 4: 5×10^4 CD133⁺CD49f^{low/+}SSEA1⁻ committed pancreatic progenitors; and 5: 300 islets. (B) Kidney sections were stained for c-peptide from mice transplanted with: 1: 5×10^4 CD133⁺CD49f^{low/+} committed pancreatic progenitors; 2: 5×10^4 CD133⁺CD49f^{low/+}SSEA1⁻

form teratomas precludes their clinical translation for the treatment of hyperglycemia in diabetic patients.

In contrast, we confirmed that ESC-derived CD133⁺CD49f^{low/+} pancreatic progenitor cells did not form teratomas after 28 days of *in vivo* transplantation. This was surprising, as 50% of CD133⁺CD49f^{low/+} cells expressed SSEA1, which is often associated with pluripotency. It has been previously hypothesized that human pancreas cells that are positive for stage-specific embryonic antigen 4 (SSEA4) co-express ductal pancreatic progenitor markers and can be differentiated *in vitro* to pancreatic hormone-expressing cells [23]. We hypothesize that SSEA1 could be a murine marker for pancreatic progenitors, sharing similarities to human SSEA4 as a murine ductal pancreatic progenitor marker. However, after transplantation *in vivo*, SSEA1⁻CD133⁺CD49f^{low/+} ESC-PPs expressed lower c-peptide levels than CD133⁺CD49f^{low/+} ESC-PPs, a result which suggests that some pancreatic progenitors may be depleted if the SSEA1 marker is used for isolation of PPs. Regardless of the expression of SSEA1, both unfractionated CD133⁺CD49f^{low/+} and sorted CD133⁺CD49f^{low/+}SSEA1⁺ populations did not form teratomas *in vivo*.

It is possible that the lack of teratoma-formation we observed is simply due to the transplantation of insufficient numbers of cells required to form teratomas *in vivo*. We do not feel this is the case, as we selected our cell dosage based on previous studies in which the same minimal cell dose resulted in 100% teratoma-forming ability after transplantation into immunodeficient mice [8,24,25]. The lack of teratoma-forming ability and the clear expression of insulin and C-peptide in both unfractionated CD133⁺CD49f^{low/+} and sorted CD133⁺CD49f^{low/+}SSEA1⁺ populations lead us to conclude that PPs are a potentially feasible cellular therapy.

One challenge to the use of ESC-derived cells is that *in vitro* differentiation of ESCs to specific cell lineages may not necessarily produce cells that express the same cell surface markers as their *in vivo*-derived counterparts [26]. To our knowledge, our study is the first to identify murine pancreatic progenitor populations

derived from ESCs in culture, and we have confirmed that ESC-derived CD133⁺CD49f^{low/+} cells are functionally similar to their *in vivo* counterparts in mouse embryos. Clearly, improvements in the generation of higher yields of pancreatic progenitor cells are required for practical application, and this could be achieved by discovery of methods to block unwanted lineage differentiation, as well as scaled up production and isolation by means of bioreactors and other stem cell separation technologies [27].

In conclusion, we have identified mouse ESC-derived pancreatic progenitor cells *in vitro* and discovered that they can survive and produce insulin *in vivo*. We envision that our phenotypic and functional data could be further utilized to study and improve the long-term survival of *in vitro* derived pancreatic progenitor cells and their ability to produce insulin in sufficient quantities to alleviate hyperglycemia in humans.

Materials and Methods

Mice

NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ(NSG) and 129S2/SvPasCrl (129) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and Charles River (Wilmington, MA, USA) respectively, or bred in house and housed in sterile microisolator cages with sterile feed and autoclaved water. Mice were euthanized by CO₂ asphyxiation or by cervical dislocation in the case of pancreatic islet isolation. All procedures were approved by the UC Merced Institutional Animal Care and Use Committee (IACUC).

Antibodies

The following monoclonal antibodies were purchased from BioLegend (San Diego, CA) and used in flow cytometry: PE anti-mouse CD55 (DAF) (RIKO-3), PE Armenian Hamster IgG Isotype Ctrl (HTK888), APC anti-human/mouse CD49f Antibody (GoH3), APC Rat IgG2a, κ Isotype Ctrl Antibody (RTK2758), biotin anti-mouse/human SSEA-1 (MC-480) and biotin mouse IgM, κ Isotype

Ctrl (MM-30). Anti-Mouse CD133 (Prominin-1) PE (13A4), Rat IgG1 K Isotype Control PE (eBRG1), anti-CD324 (E-Cadherin) Alexa Fluor® 647 (DECMA-1) and Rat IgG1 K Isotype Control Alexa Fluor® 647 (eBRG1) were purchased from eBioscience (San Diego, CA). Fc receptors were blocked with anti-CD16/32 (2.4G2) purchased from eBioscience (San Diego, CA). Biotin labeled antibodies were stained with streptavidin (SA)-PE, SA-PE-Cy7 or SA-PE-Cy5 purchased from Biologend (San Diego, CA). The following polyclonal antibodies were used for immunohistochemistry: guinea pig polyclonal to insulin (Abcam, Cambridge, MA, USA), anti-c-peptide and anti-glucagon (Cell Signaling Technology, Danvers, MA, USA), donkey anti guinea pig IgG Fluorescein conjugated (Millipore, Billerica, MA, USA) and goat anti-rabbit IgG-Atto 633 (Sigma-Aldrich, St. Louis, MO). Samples were previously blocked with normal goat serum purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Embryonic stem cell differentiation and culture

The D3 embryonic stem cell line (ES-D3) from ATCC (Manassas, VA, USA) was cultured in undifferentiated state by subculture every 2-3 days on confluent feeder layers (STO cells) treated with 10 μ g/ml of mitomycin C (Fisher, Pittsburg, PA, USA) in a medium containing 10³ units/ml LIF (Millipore, Billerica, MA, USA), 0.15mM monothioglycerol (MTG), 100 units/ml penicillin 100 μ g/ml streptomycin (Invitrogen, Grand Island, NY, USA), 15% FCS (lot # A50A01X, Gemini Bio Products, West Sacramento, CA, USA) and DMEM (Invitrogen, Grand Island, NY, USA). STO cells were removed by plating ES-D3 and STO cells cocultures on gelatin coated plates for 2 days in medium containing 103 units/ml LIF, 0.15mM MTG, 100 units/ml penicillin 100 μ g/ml streptomycin, 15% FCS and Iscove's modified Dulbecco's medium (IMDM, Invitrogen, Grand Island, NY, USA).

Cells were differentiated in embryoid bodies (EBs, 600 ESCs/EB) by hanging drops for 2 days, further growing EBs for 3 days on suspension in IMDM supplemented with supplemented with 20% FCS (lot # A0039, Atlanta Biologicals, Lawrenceville, GA, USA), 2mM L-glutamine (Invitrogen, Grand Island, NY, USA), 0.1mM essential amino acids (Invitrogen, Grand Island, NY, USA), 0.15mM MTG, 100 units/ml penicillin and 100 μ g/ml streptomycin. Next, EBs were cultured on gelatin coated plates with the same medium for 9 days changing it every 2-3 days. At day 5+9 of gelatin-attached EBs and their outgrowths were dissociated with 0.25 % trypsin-EDTA (Invitrogen) and re-plated onto collagen I-coated tissue culture dishes. Differentiation medium consisted of DMEM/F12 (Invitrogen) supplemented by 20nM progesterone, 80 μ M putrescine, 1 μ g/ml laminin, 10mM nicotinamide, 25 μ g/ml insulin, 30nM sodium selenite, 50 μ g/ml transferrin, B27 media supplement (dilution 1:50) (Invitrogen), 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% FCS (lot # A0039, Atlanta Biologicals, Lawrenceville, GA, USA). All the reagents were ordered from Sigma-Aldrich (St. Louis, MO) unless specified differently. At Day 5+10, medium was changed and cells were cultivated in the same differentiation medium but 5% FCS, changing medium every 2 days until they were used. Cultures were stopped at Day 5+28.

Gene expression analysis

RNA from ESCs, EBs at Day 5+9 and differentiated cells at Days 5+16 and 5+28 was extracted using RNeasy® Protect Mini Kits (Qiagen, Valencia, CA) and from sorted pancreatic progenitors using Trizol (Invitrogen, NY), following the manufacturer's instructions. RNA samples were treated with DNaseI (Roche, San Francisco, CA) and the concentration of RNA was determined using a Nanodrop spectrophotometer (ThermoScientific, Waltham, MA). Next, cDNA was synthesized with SuperScriptIII First-Strand Synthesis System for RT-PCR Kit (Invitrogen, Grand Island, NY, USA). The gene expression of 17 genes was analyzed by PCR as previously described with β 5-tubulin as the housekeeping gene [28]. For *Ngn3*, *Pdx1* and *Pax4* gene expression analysis, clones containing the respective gene were purchased from Thermo Scientific (Waltham, MA, USA) and used as positive controls. PCR products were visualized after

electrophoresis in ethidium bromide stained 2% agarose gels with a BioRadChemiDoc imaging system (BioRad, Hercules, CA, USA). Sizes of PCR products were compared with a 100 bp Low Scale DNA ladder (Fisher, Pittsburg, PA, USA).

Immunohistochemistry

Immunohistochemistry was performed on IPC-ESC grown on six well-plates at Days 5+16 or 5+28 of differentiation. Cells were collected by trypsinization and cytospun on slides (3x10⁵ cells/slide) with a Shandon Cytospin III cytocentrifuge at 1000 rpm for 8 minutes. INS-1 832/13 insulinoma cell line cultures (a gift from Dr. Chris Newgard, Duke University) were also prepared as controls for insulin expression. Samples were fixed with 4% paraformaldehyde (PFA) for 15 minutes at room temperature, rinsed 3 times with phosphate buffered saline (PBS) and blocked for 30 minutes at room temperature with blocking buffer consisting of 10% normal goat serum and 0.1 % Triton®-X-100 in PBS. After blocking, samples and controls were incubated at 4°C overnight with guinea pig anti-insulin, rabbit anti-c-peptide or rabbit antiglucagon. Samples stained with secondary antibodies alone were prepared as negative controls. The next day; samples were rinsed three times with PBS, incubated for 45 minutes at 37°C with anti-guinea pig IgG FITC or anti-rabbit IgG Atto 633, and stained with 0.5 μ g/ml DAPI. Finally, samples were sealed with Vectashield mounting medium (Vector, Burlingame, CA, USA) and visualized by fluorescence microscopy, using the Olympus BX51 Microscope, the 10x/0.30 and 40x/0.75 U Plan FLN lenses and the Image-Pro Plus 5.0 software.

Insulin release *in vitro*

At Day 5+28 of differentiation, IPC-ESC were cultured with insulin-free differentiation medium for 48 hours prior to challenge cells with different concentrations of glucose. Medium was changed again 3 hours before glucose challenge and cultures were washed five times with PBS. Cells were further incubated for 90 minutes with 2.5mM glucose in Krebs Ringer bicarbonate HEPES (KRBH) buffer consisting of 118mM NaCl, 4.7mM KCl, 1.1mM KH₂PO₄, 25mM NaHCO₃, 3.4mM CaCl₂, 2.5 MgSO₄, 10mM HEPES, and 2mg/ml BSA at 7.4 pH. ESIPCs were challenged with KRBH containing 2.5 mM glucose, 5.5mM glucose and 50 μ M tolbutamide, or 27.7mM glucose for 1 hour. The supernatants from the different cultures were collected and cells were dissociated by trypsinization and resuspended in acid ethanol for protein extraction. Upon resuspension, cells were sonicated and incubated overnight at 4°C. Next day, acid ethanol suspension was neutralized with 1 M Tris Buffer, pH 7.5. Total protein content was determined by Bradford assay with the Coomassie Protein Assay Kit (ThermoScientific, Waltham, MA). Released and intracellular insulin levels were measured by means of ELISA with the Mercodia Ultrasensitive Mouse Insulin Enzyme-Linked Immunosorbent Assay (Mercodia Developing Diagnostics, Uppsala, Sweden) according to manufacturer instructions.

Flow cytometry analysis and sorting

Samples containing 10⁶ single cells from ES or ES-IPCs cells at stages 5+16 or 5+28 were blocked with anti-CD16/32 for 5 minutes at 4°C. Next, they were stained for 15 minutes at 4°C with either an antibody cocktail containing anti CD133-PE, anti CD49f -APC and biotin anti SSEA-1, or the cocktail containing anti E-Cadherin-Alexa Fluor® 647, anti CD55- PE and biotin anti SSEA-1. For each antibody cocktail, "fluorescence minus-one" or fluorescently-labeled isotype antibody controls were also prepared. Finally, cells were resuspended in 0.1 μ g/ml DAPI before sorting to exclude dead cells. Cells were analyzed or isolated using a FACS Aria flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Single color samples for dye compensation were prepared for each experiment. For each study, three independent experiments were performed.

Pancreatic islet isolation

After cervical dislocation, pancreata from 129 mice were infused with 3 ml/mouse of 0.8 mg/ml collagenase P solution (Roche Applied Science, Indianapolis, IN, USA), removed and digested for at least 16 minutes at 37°C in the same collagenase P solution. Cell suspensions

were filtered through a 70 micron cell strainer and washed several times with a solution consisting of 10mM HEPES (Fisher, Pittsburg, PA, USA), 10 μ g/ μ l Dnase I (Roche Applied Science, Indianapolis, IN, USA), 100 units/ml penicillin, 100 μ g/ml streptomycin, 1.7mM CaCl₂ (Fisher, Pittsburg, PA, USA) and 1.2mM MgCl (Fisher, Pittsburg, PA, USA) in HBSS (Invitrogen), until clumps were not visible in the supernatant. Cells were resuspended in 5 ml of Histopaque-1119 (Sigma-Aldrich, St. Louis, MO) and slowly added 5 ml of culture medium consisting of 25mM HEPES, 0.1mM non-essential amino acids (Invitrogen), 100 units/ml penicillin, 100 μ g/ml streptomycin, 10% FBS in RPMI 1640 (Invitrogen) on top of the Histopaque solution. Islets in the Histopaque-culture medium interface were collected and 2000 rpm for 20 minutes without brake spinning, washed three times with washing medium, and resuspended in 5 ml of culture medium to further being collected by hand picking under a dissecting microscope [29].

In vivo engraftment

Eight to 10 week-old NSG mice were anesthetized with isoflurane and shaved. A vertical incision through dermal layers and peritoneum was made and the left kidney pulled out. After a small incision in the kidney capsule, 300 pancreatic islets, 4-5x10⁶ ESC-IPCs, 5x10⁴pancreatic progenitors (SSEA1⁺ depleted or not) or 10⁶ ESCs were inserted under the capsule. The kidney capsule was cauterized, the peritoneum sutured and skin stapled after transplantation. The post-operative analgesic buprenorphine was injected at doses of 0.05-0.1 mg/kg immediately after surgery and 18 hours later. Kidneys were collected 28 days post-transplantation and grafts were assessed by visualization and measurement of their diameter as well as histology. Sections between 5 to 6 microns from transplanted and non-transplanted kidneys were prepared at the UCSF Mouse Pathology Core for a fee. Immunohistochemistry was performed as described previously above.

Data analysis

Flow cytometry data was analyzed using FlowJo software, version 7.2.4 (TreeStar) or version 8.8.7 (TreeStar). Fluorescence microscopy analysis was performed with Image-Pro Plus5.0 software, choosing randomly the representative micrographs from all samples in each experiment. Tukey and Student t-test statistical analysis was performed using SPSS software, 20 Version 13.0. Data are expressed as means \pm standard deviation. $P \leq 0.05$ and $p \leq 0.001$ were considered significant for comparison of groups using Tukey test.

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