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In Vitro Amplification and β-Cell Differentiation of Human Pancreatic Duct-Derived Cells

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Abstract:	Candidate cells that are actively sought for diabetes cell replacement therapy are requested to be available in large quantities and to demonstrate insulin secretion capacities. We developed a new system for robust expansion and β-cell differentiation of human pancreatic duct cells (hDCs). Purified hDCs expressing CA19-9 are expanded after plating into endothelial growth-promoting media. The proliferating human duct-derived cells (HDDCs) show evidence of a partial epithelial-mesenchymal transition and are able to produce 100 x 109 cells within 1 month. After 3 passages, uniform cultures of HDDCs subjected to a 4-step protocol acquire characteristic features of β-cells with secretion of human insulin into culture media. Our protocol represents a fast and reliable method for production of insulin-producing cells with potential for diabetes cell therapy.

Author Comments:	In the revised version of the manuscript, we addressed the comments that were under the scope of our protocol manuscript. Comments on previously published material were not considered. Furthermore, we believe some comments (e.g. from reviewers #3 and #4) were not accurate in the current context of beta-cell biology. Anyway, we are willing to revise again some parts (or figures) of the manuscript if it is considered necessary for improving the understanding of our procedures.
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Cover Letter

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IREC, Pôle Pédiatrie, 1200 Brussels, Belgium

Brussels, February 27th, 2015

Dear Editor,

We are submitting an original article entitled “In Vitro Amplification and β-Cell Differentiation of Human Pancreatic Duct-Derived Cells” by Corritore E, Dugnani E, Valentina P, Liberati D, Dodge RR, Yatoh S, Piemonti L, Bonner-Weir S, Sokal EM, Lysy PA, to be considered for publication in *JoVE*.

Cell therapy is emerging as a possible therapeutic solution for diabetes but current candidates have limitations in terms of safety. Pancreatic epithelial cells have β-cell differentiation capacities but have not been significantly expanded.

Here we report a system that allows expansion of adult human pancreatic duct cells to clinically useful levels. The proliferating cells showed evidence of partial epithelial-mesenchymal transition and could be driven *in vitro* towards β-like cells with a 2-week differentiation protocol (with no genetic modifications). Importantly, the insulin-positive cells – estimated at 2-3% of the starting population – secrete insulin in the culture media. We estimate that our adult human cells have therapeutic potential for diabetes because they are easily isolated, highly expandable, and reprogrammed in a timely manner.

We think our study will be of general interest to the scientific community, particularly those interested in cell therapy for diabetes. Our protocol is unique and requires cell isolation and cell culture skills. Therefore, a video protocol is an important complement to our previously published work to ease access to our findings and allow reproduction.

EC and LPA wrote the manuscript and analyzed data; DE, PV, PL provided human tissues; OYL and YS participated in the elaboration of the duct cell isolation protocol; BWS and SEM participated to scientific discussions and reviewed the manuscript.

Mary Struziak helped us in the elaboration of the paper. Please find below a list of proposed reviewers.

We thank you for considering our work for publication in *JoVE*.

Sincerely,

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SHORT ABSTRACT:
Diabetes cell therapy is in search of new cell sources with unlimited supply and potential to reverse chronic hyperglycemia. This procedure describes how to reliably expand human pancreatic duct cells via an epithelial-mesenchymal transition, and to produce insulin-secreting cells from these expanded cultures using a four-step molecule-based protocol.

LONG ABSTRACT:

Candidate cells that are actively sought for diabetes cell replacement therapy are requested to be available in large quantities and to demonstrate insulin secretion capacities. We developed a new system for robust expansion and β-cell differentiation of human pancreatic duct cells (hDCs). Purified hDCs expressing CA19-9 are expanded after plating into endothelial growth-promoting media. The proliferating human duct-derived cells (HDDCs) show evidence of a partial epithelial-mesenchymal transition and are able to produce 1 x 10^{11} cells within 1 month. After 3 passages, uniform cultures of HDDCs subjected to a 4-step protocol acquire characteristic features of β-cells with secretion of human insulin into culture media. Our protocol represents a fast and reliable method for production of insulin-producing cells with potential for diabetes cell therapy.

INTRODUCTION

Diabetes, either type 1 or type 2, is characterized by chronic hyperglycemia which results from a lack of functional insulin secretion by pancreatic β cells. Current treatments fail to avoid glycemic variability and long-term micro- and macrovascular complications, therefore the replacement of pancreatic β cells is an attractive potential therapy[1]. Transplantation of human islets has provided proof-of-concept of diabetes cell therapy but is facing important limitations in terms of donor scarcity. Recent developments in the use of pluripotent stem cells (*i.e.* embryonic or induced) to replace deficient β cells has generated much enthusiasm since these cells achieve high levels of β-cell differentiation after both *in vivo*[2,3] and *in vitro* differentiation protocols[4]. However, clinical use of pluripotent stem cells is still associated with the risk of overgrowth or metastasis of undifferentiated cells. Furthermore, their supply relies on ethically sensitive sampling and costly propagation procedures.

In this context, there is a need for new cell sources being both efficient for β-cell engineering and compatible with clinical procedures. The pancreas itself contains epithelial cells with β-cell differentiation potential[5], the most promising being duct, acinar and alpha cells. Although all three lineages revealed a capacity for β-cell regeneration or neogenesis in specific *in vivo* settings[5], successful *in vitro* culture of purified populations from human tissue was only achieved with duct cells (DCs)[6]. Because robust monolayer expansion of DCs has not been evidenced yet, we focused our work on how to efficiently derive β-like cells from human pancreatic duct cells after an *in vitro* expansion step yielding clinically significant amounts of cells. In fact, only somatic cells capable of producing a critical mass of tissue *in vitro* will be considered for cell transplantation in diabetes-related clinical assays.

PROTOCOL:

The protocol follows the guidelines of Université Catholique de Louvain's human research ethics committee.

1. Preparations

1.1. Prepare human pancreatic exocrine tissue.

1.1.1. Prepare complete HAM's:F12, *i.e.* HAM's:F12 medium supplemented with 1% penicillin/streptomycin (P/S), 2 mM L-glutamine and 1% bovine serum albumin (BSA).

1.1.2. Perform whole pancreas digestion and Ficoll gradient purification (as described elsewhere[7]). Proceed with the isolation using the islet-depleted exocrine tissue.

1.1.3. Dispense the exocrine tissue (mainly cell clusters) as fractions of 20 mL per 50 mL tube. Wash 3 times in 25 mL of complete HAM's:F12 by centrifugation at 50 x g for 5 min, at room temperature (RT).

1.1.4. Carefully remove the supernatant with an aspiration pipette (cell pellet may dissolve during procedure) in order to leave a 10 mL cell suspension in the tubes. Resuspend 10 mL of cell pellets in 20 mL of complete HAM's:F12. Gently mix by pipetting up and down with a 10 mL pipette.

1.1.5. Plate 1 mL of this "packed tissue volume" in a 175 cm^2 flask for suspension culture with 24 mL of complete HAM's:F12 and culture overnight at 37 °C in a humidified atmosphere with 5% CO_2.

1.1.6. The following day, collect and centrifuge the exocrine cell suspension (packed tissue volume does not attach) at 50 x g for 5 min (RT). Carefully discard the supernatant and proceed with step 2 for the isolation procedure.

1.2. Prepare solutions for human pancreatic duct cell isolation.

1.2.1. Prepare phosphate buffered saline (PBS) buffer solution by adding 375 mg of ethylenediaminetetraacetic acid (EDTA) and 2.5 g of BSA to 500 mL of 1X Dulbecco's Phosphate-Buffered Saline. Stir the solution at room temperature (RT) for 1 h, then filter (with a 0.2 µm syringe filter) and keep sterile at 4 °C.

1.2.2. Prepare culture medium, *i.e.* Connaught Medical Research Laboratories (CMRL) medium containing 10% fetal bovine serum and 1% P/S, and store at 4 °C.

2. Isolation of human pancreatic duct cells

2.1. Cell dispersion

2.1.1. Wash the exocrine cell suspension 2 times with culture medium by centrifugation at 180 x g for 5 min. Aspirate the supernatant with an aspiration pipette and resuspend the pellet obtained from each 175 cm^2 flask in 5 mL of culture medium.

2.1.2. Divide the exocrine tissue as fractions of 1 mL cell suspension per 50 mL tube containing 40 mL of culture medium. Centrifuge at 50 x g for 5 min.

2.1.3. Carefully aspirate the supernatant and low-density components with an aspiration pipette and resuspend in 10 mL of 0.05% Trypsin/EDTA.

2.1.4. Vortex the 50 mL tubes for 15 s.

2.1.5. Place the tubes in a 37 °C shaking incubator at 200 rpm for 20 min.

2.1.6. Add 10 mL of cold culture medium to each 50 mL tube (to halt the enzymatic reaction). Gently but carefully mix the tissue by pipetting up and down with a 10 mL pipette.

2.1.7. Filter the cell suspensions by passing it through 40 µm cell strainers placed into new 50 mL tubes. Collect every set of 2 tubes into a new tube. Centrifuge for 3 min at 130 x g.

2.1.8. Aspirate the supernatant with an aspiration pipette and wash cell pellets by adding 5 mL of PBS buffer solution (pre-warmed at RT) and by centrifuging cells at 130 x g for 3 min.

2.2. Cell isolation

2.2.1. Aspirate the supernatant and resuspend cell pellets with 2 mL of PBS buffer solution containing mouse anti-human CA19-9 antibody at a 1:200 concentration. Incubate the antibody-reacting cell suspensions at 4 °C for 45 min while rotating at 100 rpm in an appropriate 50 mL tube shaker.

2.2.2. Add 10 mL of PBS buffer solution to the 50 mL tubes and gently mix the solutions by pipetting up and down with a 10 mL pipette. Centrifuge at 130 x g for 3 min.

2.2.3. Discard the supernatant with an aspiration pipette and add 600 µL of PBS buffer solution containing rat anti-mouse IgG1 microbeads at 1:5 concentration. Carefully mix the solution and incubate at 4 °C for 45 min while rotating at 100 rpm.

2.2.4. Wash the antibody-reacting cell suspensions carefully by adding 5 mL of PBS buffer solution and tapping. Centrifuge at 130 x g for 3 min.

2.2.5. Aspirate the supernatant with an aspiration pipette and add 20 mL of PBS buffer solution (at RT).

2.2.6. Pass the cell suspensions through 40 µm cell strainers into new 50 mL tubes.

2.2.7. Place 2 or 3 LS separation columns into appropriate magnetic separators attached to the magnetic-assisted cell sorting system. Use 1 column per three 50 mL tubes. Equilibrate each column by loading 5 mL of PBS buffer solution (wait until all the solution has gone through the column).

2.2.8. Load the cell suspension into each column (max 5 mL). Collect flow-through into 50 mL tubes and mark the tubes as containing the CA19-9-negative cell population. Keep the columns wet during the entire procedure.

2.2.9. Once the cell suspension has been completely collected, wash the separation columns 3 times with 3 mL of PBS buffer solution.

2.2.10. Remove each column from the magnetic separators and place it into new 50 mL tubes. Apply 5 mL of PBS buffer solution into each column and firmly push out the cell suspension using the plungers supplied with the columns. Mark the 50 mL tubes as containing the CA19-9-positive cell population.

2.2.11. Centrifuge at 130 x g for 5 min, aspirate the supernatant using an aspiration pipette and add 5 to 10 mL of culture medium into each 50 mL tube depending on the volume of cell pellet and on the desired cell concentration.

2.2.12. While counting cells with hemocytometer after cell suspension, perform viability control with trypan blue exclusion test using 1:2 trypan blue dye diluted in PBS.

2.2.12.1. To do so, gently mix the cell suspension by pipetting up and down with a 10 mL pipette. Take 50 µL of cell suspension and add it to a 1.5 mL tube containing 50 µL of trypan blue dye (final concentration of 1:2).

2.2.12.2. Mix with a 100 µL pipette and immediately evaluate the percentage of white cells (live cells) in the cell suspension, considering that blue cells (marked with trypan blue) are dead cells.

3. Expansion of human pancreatic duct-derived cells (HDDCs)

3.1) Plate fresh CA19-9$^+$ duct cells (DCs) on tissue culture-treated dishes at 3 x 10^5 viable cells/cm^2 in endothelial growth medium (EGM-2-MV) without hydrocortisone (20 mL in 75 cm^2 flask, 7 mL in 25 cm^2 flask, and 3 mL in one well of a 6-well plate).

3.2) After 72-96 h and then twice weekly, change endothelial growth medium.

3.3) When proliferating HDDCs reach 80% confluence (within maximum 14 days), detach cells using 0.05% trypsin/EDTA (5 mL in 75 cm^2 flask, 2 mL in 25 cm^2 flask, and 1 mL in one well of a 6-well plate) at 37 °C.

3.3.1. For this purpose, first aspirate endothelial growth medium using an aspiration pipette and immediately add PBS (5 mL in 75 cm^2 flask, 2 mL in 25 cm^2 flask, and 1 mL in one well of a 6-well plate) at RT.

3.3.2. After rinsing, discard PBS and add 0.05% trypsin/EDTA into the flask (aliquot trypsin/EDTA and use immediately after thawing). Do not exceed 3 min for the trypsin/EDTA incubation; discard adherent cells remaining after trypsin incubation.

3.4) Collect the detached cell suspension and add it into a 50 mL tube containing 40 mL PBS. Centrifuge at 190 x g for 4 min.

3.5) Discard the supernatant using an aspiration pipette and count the cells using hemocytometer. Perform subculture by plating HDDCs into a new flask or a culture plate at 5000 cells/cm^2 in endothelial growth medium.

4. β-cell differentiation of HDDCs

4.1) Plate HDDCs at 1.5 x 10^4 cells/cm^2 on uncoated tissue culture-treated 6-well or 12-well dishes in basal medium (BM), *i.e.* Dulbecco's modified Eagle medium (DMEM)/F12 containing 0.1% bovine serum albumin (BSA), 10 mM nicotinamide, 1x Insulin-Transferrin-Selenium A Supplement (ITS), and 1% P/S. Prepare a volume of 12 mL per 6-well or 12-well plate. Let the cells adhere overnight.

4.2) The next day (day 1), prepare the solution for the first step of differentiation (medium #1): BM + 100 ng/dL activin A + 1 µM wortmannin + 10 µM SB-216763. Prepare a volume of 12 mL per 6-well or 12-well plate.

4.2.1. For all differentiation steps, use freshly prepared medium only. Perform medium change with medium #1 within the test wells and leave BM in control wells. Before medium change, check confluence of HDDCs in the test wells and make sure the cells are not be less than 30% confluent.

4.3) On day 4, change the cells in test wells with medium #2: BM + 5 µM PNU-74654. Change control wells with BM.

4.4) On day 7, change the cells in test wells with medium #3: BM + 5 ng/mL bFGF + 5 µM forskolin.

4.5) On day 10, change the cells in test wells with medium #4: BM + 2 µM retinoic acid.

4.6) On day 13, process the cells for analyses (analysis of β-cell markers by qPCR, immunostaining, and ELISA as described elsewhere[8]).

4.7) Alternatively, on day 1 after plating, incubate HDDCs with pre-differentiation medium, *i.e.* BM containing 1 µM A83-01 and 0.5 mM valproic acid.

4.7.1. On day 4, start differentiation protocol with successive incubations with media #1 to #4 (as in steps 4.1 to 4.6), for 13 days.

REPRESENTATIVE RESULTS

Efficient proliferation of HDDCs

Production of HDDCs is highly efficient and occurs with all exocrine donors yielding a DC viability >60% after isolation. After plating, epithelial cells show a cobblestone-like morphology and perform 2-3 rounds of proliferation. While a majority of DCs enters in senescence, about 30% of these cells will modify their morphology to become spindle-shaped and to start proliferate. If DC isolation is performed carefully (flow cytometry analysis of purified DCs should show >95% CA19-9^+ cells), spindle-shaped cells arise throughout culture dishes and not as scattered clusters. At the end of initial plating (before 1^{st} passage), cultures consist of a mix of epithelial-like cells and fusiform cells. By 1^{st} passage, cultures are uniformly spindle-shaped.

Quality control of HDDC cultures includes the examination of proliferation rate, of DC and mesenchymal gene expression, and flow cytometry analysis. Proliferation rate is estimated by calculation of cumulative population doublings (CPDs) using the following equation: $([\log_{10}(N_H) - \log_{10}(N_I)]/\log_{10}(2))$, where N_I is inoculum number

and N_H is cell harvest number[9]. Expansion of HDDCs is robust from passage 2 to 7 (with a yield of 15 CPDs) and then decline (**Figure 1A**). After P9, HDDCs reach a plateau at 18 CPDs. Young donors (< 5 years of age) show higher CPDs at early passages but have similar growth pattern. Importantly, HDDCs do not have clonal expansion capacities. HDDCs show progressive loss of epithelial markers (*e.g. CDH1*, *CAII*, *CD133*, *CFTR*) with expansion but maintain low *CK19* and *SOX9* expression (**Figure 1B**), which are absent in mesenchymal lineages. A gain of *vimentin* but not of *fibroblast-specific protein 1* gene expression is also a key feature of proliferating HDDCs. Uniform cultures of HDDCs (from passage 1) have a phenotype of partial epithelial-mesenchymal transition (EMT), which is confirmed by flow cytometry analysis showing incomplete mesenchymal signature ($CD90^+/CD105^+/CD73^{low}$ phenotype) and co-expression of epithelial and mesenchymal proteins ($CK19^+/CD90^+$ phenotype).

β-cell differentiation of HDDCs

The differentiation protocol (called 'R') was designed with the aim of recapitulating embryonic pancreas development on the basis of specific growth factors and molecules with identified metabolic activities. After this four-step 13-day procedure, HDDCs acquire β-cell-like features identified by gene and protein expression assays. It is recommended to apply differentiation protocol on HDDC cultures from P2 to P5 to avoid residual β-cell contamination and to use HDDCs at their maximal plasticity potential.

After β-cell differentiation protocol, HDDCs show *de novo* expression of β-cell-specific genes (*insulin*, *synaptophysin [SYP]*, *PDX1*, *MAFA*, *NGN3*, *NKX6.1*) with negligible levels of glucagon (*GCG*) expression (**Figure 2A**). R-differentiated HDDC populations contain up to 3% $insulin^+$ cells by immunostaining (**Figures 2B, 2C**). The insulin-producing cells are either dispersed as single cells or clustered and have epithelial-like (arrowhead, **Figure 2B**) or spindle-shaped (arrows, **Figure 2B**) morphology. Co-staining analysis shows co-expression of $insulin^+$ cells with PDX1 and MAFA but not with glucagon, somatostatin, or pancreatic polypeptide. Some $MAFA^+/insulin^-$ cells are also typically observed after R protocol. At day 13 of differentiation procedure, HDDCs have insulin secretion capacities under basal glucose (2.8 mM) conditions.

An alternative protocol has been designed, with pre-R incubation with A83-01 and VPA. This A83-01/VPA-R protocol improves β-cell-specific gene expression but not frequency of $insulin^+$ cells or secretion capacities. Furthermore, in adult donors aged 60 or more, pre-incubation with A83-01 may be deleterious for cell viability.

Figure 1. Expansion and phenotype switch of HDDCs *in vitro* (**A**) CPDs of HDDCs showing a quasi-linear growth from P2 to P7 and a progressive decline from P7 (n=5). Data were presented as mean ± SD. (**B**) Real-time qPCR data showing rapid loss of *E-cadherin* (*CDH1*), *CAII*, *CD133* and *CFTR* gene expression in HDDC populations during subculture. In contrast, *CK19* expression was upregulated during P0 and P1, and from P2 HDDCs maintained low levels of *CK19* and *SOX9* expression (n=4). Data were expressed as mean expression levels compared to freshly isolated DCs.

Figure 2. **β-cell differentiation levels of HDDCs after in vitro 4-step protocol** (**A**) Human pancreatic duct cells were cultured and expanded *in vitro.* Spindle-shaped cells (HDDCs) appeared at P1 after culture in EGM-2MV medium. HDDCs from P2 to P5 were plated in 6-well or 12-well dishes. On day 1, the cells were incubated with Activin A, Wortmannin and SB-216763 (STEP 1). On day 4, the medium was changed and the cells were incubated with PNU-74654 (STEP 2). HDDCs were incubated on day 7 with bFGF and forskolin (STEP 3) and on day 10 with retinoic acid (STEP 4) before being collected for analysis on day 13. (**B**) β-cell gene expression analysis using qPCR in HDDCs after incubation with R and A83-01/VPA – R protocols, as compared with controls incubated in BM (*n*=4-6). Data were expressed in log scale with major tick intervals in log. (*) $p<0.05$ compared with A83-01/VPA – R protocol. Significant differences were assessed using an unpaired Student's *t*-test. (**C**) Representative staining of R-differentiated HDDCs showing organization of insulin$^+$ cells. (**D**) Distinctive immunostaining of R-differentiated HDDCs co-expressing insulin and MAFA proteins. Magnification bars: 100 µm.

Table 1. Summary of the key factors used in 'R' protocol for β-cell differentiation of HDDCs

DISCUSSION

This video article provides a procedure for fast and cost-effective β-cell differentiation of pancreatic DCs that undergo proliferation after partial EMT. The present expansion protocol allows massive production of HDDCs: from 100 x 10^6 purified CA19-9$^+$ cells, we obtain within 1 month from 10 to 100 x 10^9 HDDCs at passage 5, when HDDCs maintain differentiation potential. EMT is a *sine qua non* condition for expansion of human DCs, which have only limited proliferation capacities in 2D culture configuration. Besides being a recognized process during embryogenesis[10], EMT has recently been described as a mandatory step for recruitment of new α cells from DCs in the setting of α-to-β differentiation driven by Arx inhibitors[11]. DCs thus have a natural propensity for EMT that can be exploited using the present procedure. Although resulting from EMT, HDDCs demonstrate cell cycle regulation *in vitro* with contact inhibition and senescence after P9. Since HDDCs enter cell cycle arrest after >80% confluence, a daily check of HDDC culture is recommended.

Isolation of HDDCs from human pancreatic exocrine tissue is efficient except in preparations with low viability. In this case, packed tissue volume will not form pellets after centrifugation and most aggregates will be trapped in cell strainers. If homogeneous cell suspension cannot be obtained, purity of CA19-9$^+$ population will be low with negative impact on HDDC emergence and expansion. So far, no parameters from donors (*e.g.* age, gender, BMI) or from pancreas digestion step could be correlated to exocrine tissue viability at initiation of isolation protocol.

Current differentiation protocol of HDDCs yields 2-3% of insulin-secreting cells after a 13-day incubation. As compared to classical protocols with ESCs producing 7% insulin$^+$ cells[12], HDDCs' differentiation procedure is competitive since it bears no ethical or safety issues for clinical translation, and because large quantities of insulin$^+$ cells can be obtained from an initially small fraction of pancreas. The protocol is reliable, especially when applied in passage 2 to 5 HDDCs with uniform spindle-shaped morphology, moderate nucleus-to-cytoplasm ratio, typical growth

pattern, and in confluent cultures at day 1 of differentiation protocol. Differentiation on low-density (<30% confluence) cultures should be avoided, as should be the use of tissues from donors aged >70 years. Although insulin secretion capacities of HDDCs do not reach levels of *bona fide* human islets, this limitation might be overcome in the clinical setting by selection of insulin-producing cells or by modulation of the volume of HDDC-derived tissue considered for transplantation. In both settings, expansion of HDDCs should overcome the relative shortage of insulin$^+$ cells and allow transplantation of a critical mass of insulin-producing tissue. Furthermore, HDDCs' model bears the advantage over pluripotent cells of not being theoretically limited by co-transplantation of partially differentiated populations. These characteristics make HDDCs a promising candidate for translational research procedures and for further research aiming at improving the insulin secretion levels after *in vitro* differentiation protocols.

DISCLOSURES

The authors declare that they have no competing financial interests.

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Figure 1
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Figure 1

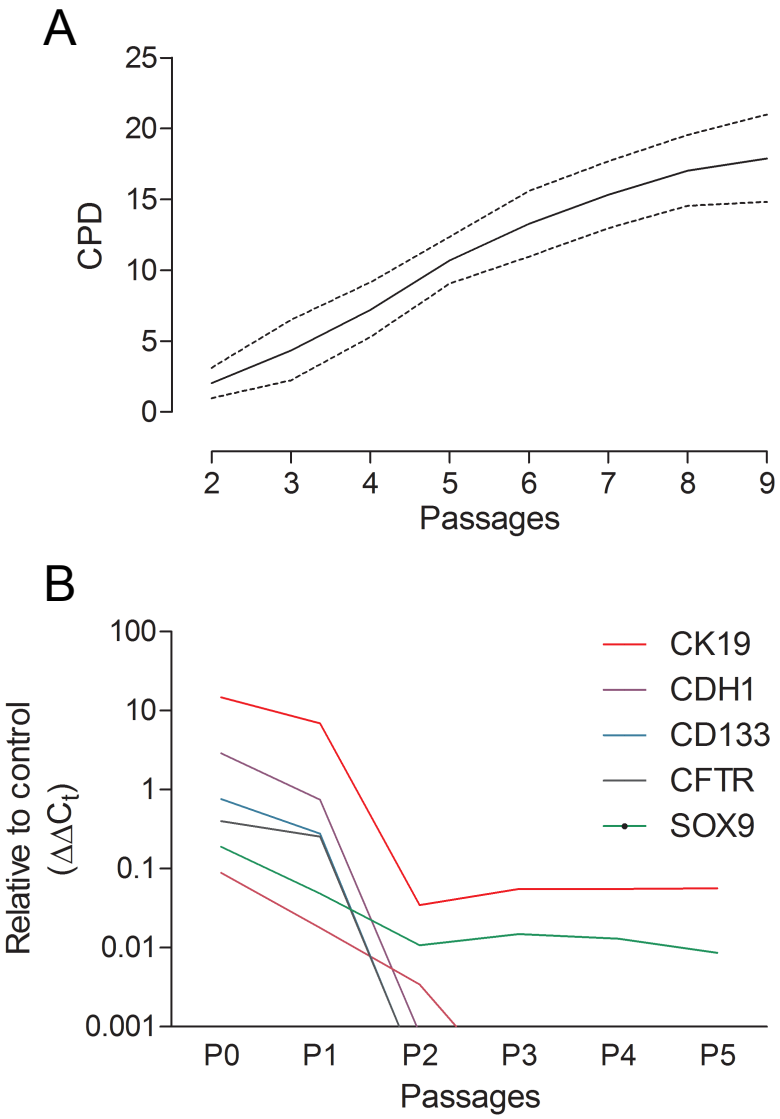

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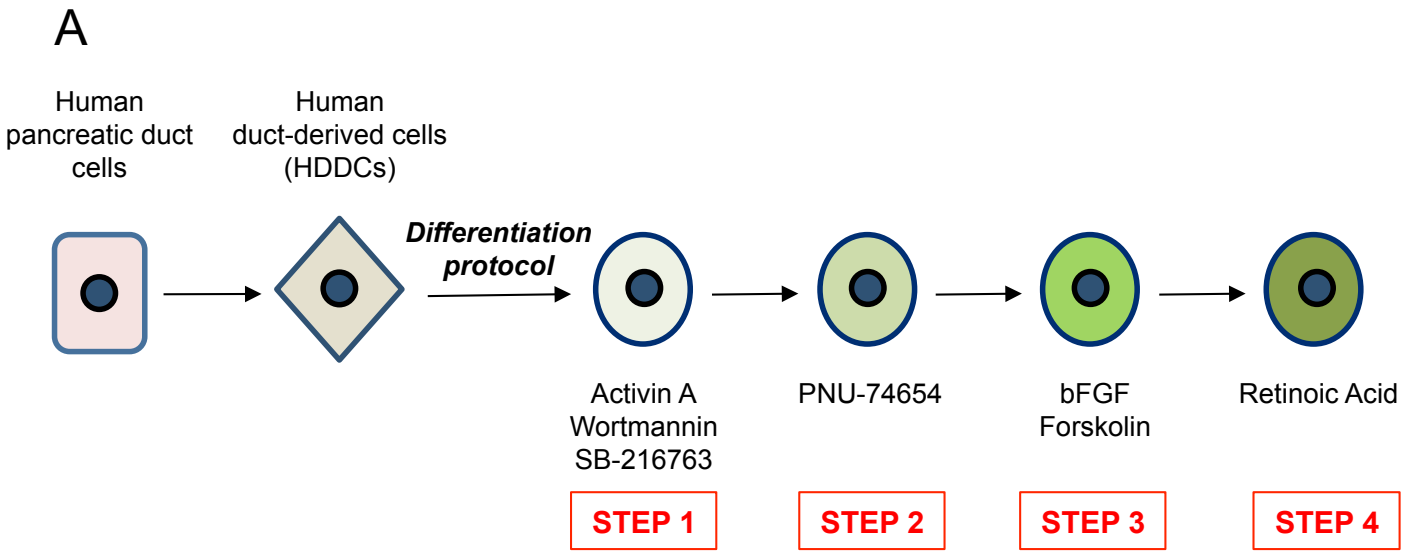
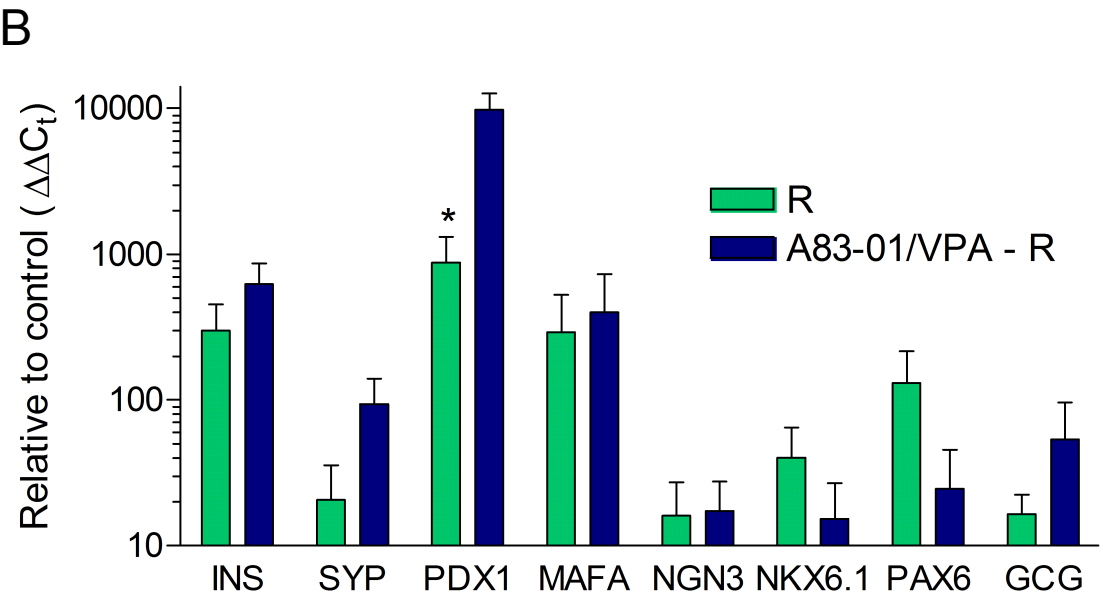
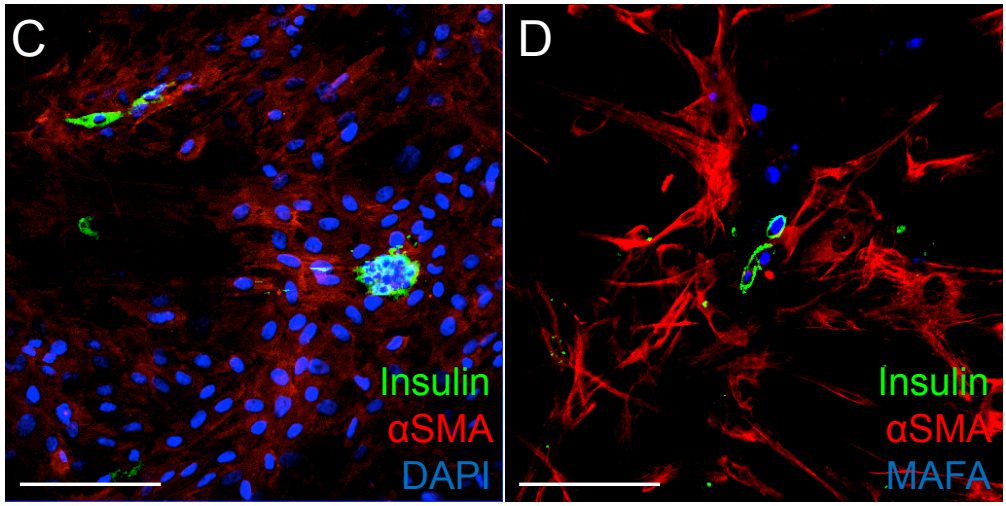
Figure 2







Table
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Differentiation factors	Protocol Steps	Function
Activin A	Step 1	TGF-β family member involved in growth and differentiation processes
Wortmannin	Step 1	Phosphorylation PI3K, activation of growth and differentiation pathways
SB-216763	Step 1	Selective inhibition of GSK3
PNU-74654	Step 2	Inhibition of Wnt/ beta-catenin pathway
bFGF	Step 3	Growth factor involved in proliferation and differentiation pathways
Forskolin	Step 3	Activation of adenylyl cyclase (AC)
Retinoic Acid	Step 4	Metabolite of vitamin A, required for growth and embryonic development

TGF-β, transforming growth factor beta; PI3K, phosphatidylinositol-3-kinases; GSK3, glycogen synthase kinase 3;

bFGF, basic fibroblast g

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
A83-01 (Alk5i)	Tocris	2939	
Bovine serum albumin	Sigma	A2153-100G	
Cell strainers, 40 µm	Sartorius	16531	
CMRL medium 1066	Life Technologies	21530027	
DMEM/F12 medium	Life Technologies	A13708-01	
EDTA	Sigma	ED2SC-100G	
EGM-2-MV Bulletkit medium	Lonza	CC-3202	use without hydrocortisone
Ficoll Histopaque	GE Healthcare	17-0891-01	
Forskolin	Tocris	1099	
Guinea Pig anti-human insulin	Dako	A056401	
HAM's:F12 medium	Life Technologies	31765-035	
Recombinant human activin A	Peprotech	120-14E-10µg	
Recombinant human bFGF (FGF-2)	Peprotech	100-18B-10µg	
Insulin-Transferring-Selenium A Supplement	Sanbio	SCC0803	
LS columns	Miltenyi Biotec	130-042-401	
Mouse anti-aSMA-Cy3	Sigma	C6198-.2ML	
Mouse anti-human CA19-9	Life Technologies	18-7265	
Mouse anti-human CD73 PE-Cy7	Becton Dickinson	561258	
Mouse anti-human CD90 APC-conjugated	Becton Dickinson	559869	
CD105 PerCP-Cy5.5	Becton Dickinson	560819	
Nicotinamide	Sigma	N5535-100G	

Penicillin/streptomycin	Life Technologies	15070063
PNU-74654	Santa Cruz	sc258020
Rabbit anti-human MAFA	Abcam	ab26405
Rat anti-mouse IgG1 microbeads	Miltenyi Biotec	130-047-102
Retinoic acid	Sigma	R2625-50MG
SB-216763	Santa Cruz	sc200646
Trypsin/EDTA	Life Technologies	25300-054
Valproic acid	Sigma	P4543-10G
Wortmannin	Sigma	W1628-1MG

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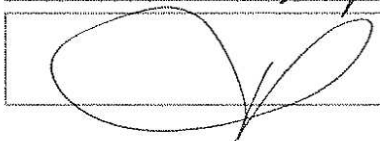
Signature: 

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Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Corritore et al. describe here a method for generating large numbers of human beta cells from exocrine tissue, using techniques to isolate pancreatic duct cells, expand them, and then differentiate into beta cells. This method will be very valuable for researchers who wish to use beta cells, but either don't have access to human islets, or need more cells than typically afforded by islet isolation. I have a few minor comments/suggestions below, but otherwise this manuscript is well explained and suitable for publication in JoVE.

Major Concerns:
No major concerns

Minor Concerns:
1. Some of the centrifugation steps have seemingly arbitrarily precise g values (e.g. 129g spin). Is that because of the centrifuge used, and the calculation from rpm? That might be explained a little, so the reader can understand why it should be, for example, 46g and not 50g.

We thank the reviewer for this useful comment. Changes have been made to the revised version of the manuscript with simplification of the 'g' values.

2. During cell isolation, it sounds like the suggestion is to use 600uL PBS with secondary antibody in a 50mL tube. Is that the most appropriate size, with such little volume? I'd be concerned about losing it.

Seconday antibodies coupled with microbeads need a minimal volume for correct antigen recognition. Volumes were used as per Miltenyi Biotec's instructions.

3. During expansion step, please indicate that EGM-2-MV media is commercially available. You define other media in previous steps, so it should either be defined, or the reader should be referred to a particular company.

As per JoVE's instructions, the manuscript is submitted with a separate file providing details about the material used for our research. This will be accessible to the readers.

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The paper entitled "In vitro amplification and b-cell differentiation of human pancreatic duct-derived cells" by Corritore et al was reviewed. The authors aimed to provide a new method to access sufficient quantity of progenitor-like cells for clinical cell replacement therapy. The protocol was well written and one believed that any laboratory who would like to perform the isolation procedure could undoubtedly

reproduce the fair successful outcome following the protocol described. So, one would suggest the editorial office to consider for acceptance this article after the authors make the following minor revisions.

Major Concerns:
No

Minor Concerns:
A. Line 156 : Volume of the HAM's:F2 medium?

Volume of HAM's:F2 medium is 25 mL. Changes were made accordingly to the revised version of the manuscript.

B. Line 164, "packed tissue volume" is required for further clarification. In addition, from Step 1.1.4 to 1.1.5, did the author mean 10ml reagent from Step 1.1.4 could be plated on 10x T175 flasks in Step 1.1.5?

The protocol of DC isolation and HDDC expansion can efficiently be performed with 1 mL of the cell suspension obtained in 1.1.4. We described the protocol as such to allow pancreas isolation centers to distribute the exocrine tissue. However, DC isolation and HDDC expansion can also be made with the complete exocrine tissue; this results in higher numbers of purified cells.

C. One would suggest to move 1.2 (Line 172) before 1.1

We described media (1.2) after exocrine preparation (1.1) to avoid confusion regarding the nature of media required for exocrine preparation step. Anyway, if requested, this could be changed.

D. Line 217, please clarify how to "rotating at 100rpm"

We rotate with a 50 mL tube shaker. This was specified in the text of the revised manuscript (line 218).

E. Line 225, please identify "rat anti-mouse IgG1 microbeads", any conjugation such as magnetically label on the beads?

As per JoVE's instructions, the manuscript is submitted with a separate file providing details about the material used for our research. This will be accessible to the readers. As per Miltenyi's technique, microbeads – due to their superparamagnetic nature – are naturally attracted by a magnet that allows the separation technique.

F. Line 238, "prime each column…." should be changed to "equilibrate each column….."

Changes have been made to the revised version of the manuscript (line 238).

G. Line 272, the receipt of EGM-2-MV should be further defined or relevant reference needs to be stated.

As per JoVE's instructions, the manuscript is submitted with a separate file providing details about the material used for our research. This will be accessible to the readers.

H. Line 294, define "endothelial growth medium"

Since our purpose in the present manuscript is to provide a protocol for cell culture and that the endothelial growth medium is referred as being EGM-2-MV (please see line 272 and references accessible in the supplementary data), we believe that it is not appropriate to extend on the content of the endothelial growth medium.

I. Line 298, is "uncoated tissue culture-treated 6-well or 12-well dishes" petri dishes? Is this for suspension culture?

As described in the title of the protocol, this plating procedure is for β-cell differentiation (in adherent cells).

J. In "REPRESENTATIVE RESULTS", one would suggest author to add pictures of cultured cells at various stages. For example: the cell images after plating, after 24-hr culture, before change to #1-~~#~~4 medium, etc.…

The present paper is a protocol paper that provides a thorough description of techniques that were used to achieve a scientific study published elsewhere. Our past research is referenced (ref #8) in the present manuscript and all necessary data were described in our past study (ref #8). We thus believe that it is not the topic of the present manuscript to re-assess these data.

Additional Comments to Authors:
No

Reviewer #3:
Manuscript Summary:
The manuscript provides a detailed culture method to derive human ductal cells into insulin cells, a promise for cell-based therapy for the treatment of diabetes.

Major Concerns:
A very impressive and detailed method, albeit important questions should be addressed before acceptance:
1- It is unclear the proportion of starting, intermediate and end cell populations. It would be insightful to provide pictures, and supporting data (IHCs, gene expression data, FACS) of biomarkers of the different cell differentiation stages (lines 329-337). Also, please provide more details of the quality control process (339-340).
Albeit changes in cell morphology during the culture process is described in the text, please provide pictures, and indicate the implications of the morphological changes.

The present paper is a protocol paper that provides a thorough description of techniques that were used to achieve a scientific study published elsewhere. Our past research is referenced (ref #8) in the present manuscript and all necessary data were described in our past study (ref #8). We thus believe that it is not the topic of the present manuscript to re-assess these data.

Please explain how what did you define that HDDCs do not have clonal capacities (line 347)

We used the in vitro serial dilution system to determine the capacity of HDDCs to develop clonal clusters under single-cell conditions.

2- It would be useful to provide a summary diagram of key steps of the culture process, and to provide a summary table of key factors used at each step. A brief explanation of why each factor is used would be helpful.

These elements were incorporated into the revised version of the manuscript as Figure 2A (summary diagram) and Table 1 (key differentiation factors).

3- It is not clear in the protocol (lines 215-255) how you go from magnetic bead-anti-CA19-9+ - ductal cell complexes, into ductal cell cultures. In other words, how do you separate cell-magnetic beads after the isolation?

Magnetic-assisted cell separation uses monoclonal antibodies that cannot be dissociated from the purified cells. The CA19-9^{+} cells were plated after MACS sorting and dissociation from the antibodies occurred naturally in the early proliferation steps (duct cells undergo 2-3 rounds of proliferation during the first week).

Images of isolated cells should be provided.

Please refer to our comment on previously published data.

Also, quantification of insulin cells at this stage would be key to determine that the starting population is pure ductal cells.

Insulin expression analysis on early passaged duct cells and HDDCs were performed, as described in our Cellular Reprogramming paper (2014). These data showed that no insulin (gene + protein) could be detected from passage 1 onward.

Assuming that your purity is 95%, and you carry over insulin cells from the original preparation, how could you rule out that the 2-3% insulin cells after 13 days culture are not part of the original culture?

This eventuality is not possible mathematically according to the proliferation capacity of HDDCs. Again this is described in our Cellular Reprogramming paper, as such: "In our system, the question arises whether the β-cells found after differentiation were residual contaminating cells carried through the culture. Whereas initial freshly isolated CA19-9^{+} cell populations had occasional acinar and β-cells but no mesenchymal cells, our characterization was performed after extensive proliferation and no insulin mRNA was detected from P1. Simple mathematical calculations ruled out the possibility. Approximately 2% insulin-producing cells are observed in HDDCs differentiated at P5 with either R or A83- 01/VPA-R protocols. HDDCs from P0 to P5 achieve 10.7 population doublings, so if we started with 1 x 10^{6} CA19-9^{+} cells at P0, we could obtain 2.5 x 10^{9} HDDCs at P5 and harvest 53 x 10^{6} insulin^{+} cells. For residual islet or DCs to be the origin of those cells, they would need to perform at

least 5.7 population doublings in 32 days, while maintaining their epithelial phenotype and 100% efficiency of β-cell differentiation, all of which is quite unlikely."

4- Line 376: The message could be misinterpreted. Glucose-induced insulin secretion at 2.8 mM glucose is an indication of undifferentiated insulin cells. Have you attempted GSIS?

We have performed GSIS and observed insulin secretion independently of glucose concentration (2.8 or 20.2 mM), suggesting that insulin secretion by 'R'-differentiated HDDCs was not regulated by external glucose, as it is the case with some protocols for embryonic stem cell differentiation. It is confusing to us what the reviewer means by 'undifferentiated insulin cells'.

5- Figure 1: Please indicate error bars, and if any statistical method was applied.

As mentioned in Figure 1 legend, Fig. 1A is presented as mean ± SD. Figure 1B reflects expression changes of duct cell markers after passaging and is a correlate to previously published data (Figure 2, Cellular Reprogramming, 2014, ref #8). No error bars or statistics were inserted since the objective of the present manuscript is to provide the reader with an example of what is expected after HDDC subculture, rather than to show precise data described elsewhere. However, if requested by the reviewer, this Figure 1B could be changed with actual data.

6- Figure 2: Please indicate what statistical method was applied.

Significant differences were assessed using an unpaired Student's t-test. Changes were made accordingly to the revised version of the manuscript (lines 408-409).

A figure should show staining of other islet cell types, glucagon, somatostatin, PP, Epsylon cells.

As stated in line 372 of the submitted manuscript (R1), HDDC-derived insulin$^+$ cells did not co-express glucagon, somatostatin or PP. For this reason, we did not include a picture showing absence of co-staining. It is recognized that epsilon cells are very scarce in human healthy pancreas and therefore very difficult to detect after stem/progenitor cell differentiation. Furthermore, the influence of ghrelin on glucose homeostasis is still largely unknown.

Please provide pictures at higher magnification.
7- Discussion:
Lines 415 to 419 could be omitted.

In the discussion, it is a requirement from JoVE to include comments about the pro's and con's of the described technology. One may wonder if HDDCs may have unregulated cell growth after EMT (a recognized process for tumorigenesis). Our comments from lines 415 to 419 was meant to confirm the maintenance of contact inhibition (the absence of which would suggest transformation) but also to stress out the need for daily check of cultures since confluence may impede further plasticity.

Line 423: please change the wording "insulin-secreting to insulin-expressing". Also

found in other parts of the text.

In line 429 of the submitted manuscript (R1), we mentioned "2-3% of insulin-secreting cells", since our 'R'-differentiated HDDCs secrete insulin in culture media. The word "secreting" is used a second time in the short abstract.

Line 433: HDDCs are still not fully differentiated, despite your encouraging cell yield, you should state the need to improve further the differentiation/specification protocol.

Changes were made accordingly to the revised version of the manuscript (lines 455-457).

Line 438: How could you rule out that HDDCs don't keep some sort of pluripotency? How long can your cells keep their markers? For how long have you kept these cells in culture?

Pluripotency markers were analyzed in our previous work (Cellular Reprogramming, 2014). These data showed that HDDCs do not express pluripotency markers. We believe the other questions fall out of the scope of the present protocol paper.

8- Very little is disclosed about donor information. Age, BMI, gender,... and cell yield should be stated in the form of a table.

Again, these data are available elsewhere (Cellular Reprogramming, 2014). We believe the other questions fall out of the scope of the present protocol paper.

Minor Concerns:
Line 156: please add model of centrifuge, it's not easy to get rpm if you don't know centrifuge radius or model. Also, do you have different acceleration and deceleration speeds? Please indicate, it can make a huge difference for cell preparations.

We changed the 'g' values for better understanding by the readers.

Additional Comments to Authors:
N/A

Reviewer #4:
Manuscript Summary:
This manuscript describes a method for isolation and expansion of human pancreatic duct cells, as well as a method for their differentiation into insulin-producing cells.

Major Concerns:
The methods are clearly described. However, the result interpretation is somewhat unclear. The cell doubling time is not specified. Cell yield from the expansion stage, reaching 10^{10}-10^{11} when starting from 10^{8} cells, represents 10^{2}-10^{3}-fold expansion. Fig. 1A shows that cells are capable of 15-20 population doublings, which represent $3X10^{4}$-10^{6}-fold expansion. The discrepancy is likely caused by cell death. This should be clearly explained and discussed. Also, stating that cells "…are able to produce 10^{11} cells within 1 month" is meaningless without specifying the starting

number.

We thank the reviewer for these useful comments. As stated in the text, we start from 100 x 10^6 CA19-9+ duct cells, and obtain up to 10^{11} HDDCs at passage 5, through an EMT process. There is thus a phenotype switch and an initial cell loss at the beginning of duct cell culture. This was clearly stated in the text in lines 335-337 of the submitted manuscript: "While a majority of DCs enters in senescence, about 30% of these cells will modify their morphology to become spindle-shaped and to start proliferate."

The differentiation protocol was performed following 3 passages, representing about 5 population doublings, which are equivalent to a theoretical 30-fold expansion. It would be useful to know if the cells are capable of differentiation following the full expansion capacity. Otherwise the statements on cell expansion per se are not very significant.

In lines 410-411, we mentioned obtaining "within 1 month from 10 to 100 x 10^9 HDDCs at passage 5, when HDDCs maintain differentiation potential." We thus have full-fledged differentiation capacity after expansion at P5.

The logic of applying differentiation conditions designed for pluripotent cells to pancreatic cells should be explained. Duct cells that underwent EMT are not equivalent to ES cells in their gene expression and epigenetic profiles.

In our previous work (Cellular Reprogramming, 2014), which this present protocol paper refers to, we provided the explanation on this matter, as such: "We defined culture conditions that induced β-cell differentiation in HDDCs. The R protocol was designed to recapitulate pancreatic development (Champeris Tsaniras and Jones, 2010) because β-cell neogenesis from DCs occurs in rodents after pancreatic injury by recapitulating development (Bonner-Weir et al., 1993; Li et al., 2010b)."

The differentiation protocol results in 3% of insulin-positive cells. This is compared to 7% achieved with ES cells, however the efficiency of ES cell differentiation protocols reported in recent publications is much higher. Also, given that the starting cell type here is pancreatic, rather than pluripotent, this is a rather low efficiency.

Comparison with embryonic stem cells is provided in the discussion, with reference to validated papers and known differentiation levels (a yield of 7% is widely accepted in the context of embryonic stem cell differentiation). Besides recent publications (e.g. from the Melton's group in 2014) showing high rates of β-cell reprogramming, current strategies fail to achieve massive insulin-producing cell output. In this setting, we believe our handy and efficient reprogramming strategy is competitive and at low-risk in the setting of clinical translation.

Finally, the resulting cells have been characterized only for expression of a small number of genes, and there is no information on insulin content and secretion. Staining for other islet hormones would be informative, to determine if the cells are monohormonal, given that the ES cell differentiation protocol often leads to polyhormonal cells. If the cells are polyhormonal or not glucose-responsive they may be of limited use.

These data are available elsewhere (Cellular Reprogramming, 2014). We believe the other questions fall out of the scope of the present protocol paper. Furthermore, the submitted manuscript stipulated that "co-staining analysis shows co-expression of insulin+ cells with PDX1 and MAFA but not with glucagon, somatostatin, or pancreatic polypeptide (lines 377-378).

The number of different donors used to reproduce these results is not specified.

Numbers of donors are provided in the figure legends. More information on donors (age, BMI, etc) is available elsewhere (Cellular Reprogramming, 2014).

Minor Concerns:
N/A

Additional Comments to Authors:
N/A