**Title:**

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

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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 1011 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 therapy1. 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 protocols4. 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 potential5, 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* settings5, 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. Prepare human pancreatic exocrine tissue.
      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).
      2. Perform whole pancreas digestion and Ficoll gradient purification (as described elsewhere7). Proceed with the isolation using the islet-depleted exocrine tissue.
      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).
      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.
      5. Plate 1 mL of this "packed tissue volume" in a 175 cm2 flask for suspension culture with 24 mL of complete HAM's:F12 and culture overnight at 37 °C in a humidified atmosphere with 5% CO2.
      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.
   2. Prepare solutions for human pancreatic duct cell isolation.
      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.
      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**
   1. Cell dispersion
      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 cm2 flask in 5 mL of culture medium.
      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.
      3. Carefully aspirate the supernatant and low-density components with an aspiration pipette and resuspend in 10 mL of 0.05% Trypsin/EDTA.
      4. Vortex the 50 mL tubes for 15 s.
      5. Place the tubes in a 37 °C shaking incubator at 200 rpm for 20 min.
      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.
      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.
      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. Cell isolation
      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. 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.
      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.
      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.
      5. Aspirate the supernatant with an aspiration pipette and add 20 mL of PBS buffer solution (at RT).
      6. Pass the cell suspensions through 40 µm cell strainers into new 50 mL tubes.
      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).
      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.
      9. Once the cell suspension has been completely collected, wash the separation columns 3 times with 3 mL of PBS buffer solution.
      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.
      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.
      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.
          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. 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 105 viable cells/cm2 in endothelial growth medium (EGM-2-MV) without hydrocortisone (20 mL in 75 cm2 flask, 7 mL in 25 cm2 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 cm2 flask, 2 mL in 25 cm2 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 cm2 flask, 2 mL in 25 cm2 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/cm2 in endothelial growth medium.

1. **β-cell differentiation of HDDCs**

4.1) Plate HDDCs at 1.5 x 104 cells/cm2 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 elsewhere8).

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 1st passage), cultures consist of a mix of epithelial-like cells and fusiform cells. By 1st 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: ([log10(*N*H) − log10 (*N*I)]/log10 (2)), where *N*I is inoculum number and *N*H is cell harvest number9. 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+/CD73low 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 106 purified CA19-9+ cells, we obtain within 1 month from 10 to 100 x 109 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 embryogenesis10, 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 Arxinhibitors11. 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+ cells12, 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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