**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 106 CA19-9+ cells at P0, we could obtain 2.5 x 109 HDDCs at P5 and harvest 53 x 106 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 10¹⁰-10¹¹ when starting from 10⁸ cells, represents 10²-10³-fold expansion. Fig. 1A shows that cells are capable of 15-20 population doublings, which represent 3X10⁴-10⁶-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 10¹¹ 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 106 CA19-9+ duct cells, and obtain up to 1011 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 109 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