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In vitro pancreas organogenesis from dispersed mouse embryonic progenitors

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Corresponding Author:	Anne Grapin-Botton University of Copenhagen Copenhagen N, DENMARK
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	anne.grapin-botton@sund.ku.dk
Corresponding Author's Institution:	University of Copenhagen
Corresponding Author's Secondary Institution:	
First Author:	Chiara Greggio
First Author Secondary Information:	
Other Authors:	Chiara Greggio
	Filippo De Franceschi
	Manuel Figueiredo-Larsen
Order of Authors Secondary Information:	
Abstract:	The pancreas is an essential organ that regulates glucose homeostasis and secretes digestive enzymes. Research on pancreas embryogenesis has led to the development of protocols to produce pancreatic cells from stem cells. The whole embryonic organ can be cultured at multiple stages of development. These culture methods have been useful to test drugs and to image developmental processes. However the expansion of the organ is very limited and morphogenesis is not faithfully recapitulated since the organ flattens. We propose three-dimensional (3D) culture conditions that enable the efficient expansion of dissociated mouse embryonic pancreatic progenitors. By manipulating the composition of the culture medium it is possible to generate either hollow spheres, mainly composed of pancreatic progenitors expanding in their initial state, or, complex organoids which progress to more mature expanding progenitors and differentiate into endocrine, acinar and ductal cells and which spontaneously self-organize to resemble the embryonic pancreas. We show here that the in vitro process recapitulates many aspects of natural pancreas development. This culture system is suitable to investigate how cells cooperate to form an organ by reducing its initial complexity to few progenitors. It is a model that reproduces the 3D architecture of the pancreas and that is therefore useful to study morphogenesis, including polarization of epithelial structures and branching. It is also appropriate to assess the response to mechanical cues of the niche such as stiffness and the effects on cell´s tensegrity.

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Author Comments:	We have revised the manuscript according to the reviewers´comments and thank them for their work. With regards to the movie files, we are including 3 different movie files corresponding to the same movie, hoping that one will be usable. Please use only one.
Additional Information:	
Question	**Response**

Cover Letter

FACULTY OF HEALTH SCIENCES
UNIVERSITY OF COPENHAGEN

Anne Grapin-Botton
DanStem
University of Copenhagen
Blegdamsvej 3B
DK-2200 Copenhagen N
Denmark
anne.grapin-botton@sund.ku.dk

26 NOVEMBER 2013

THE DANISH STEM CELL CENTER (DANSTEM)

UNIVERSITY OF COPENHAGEN
BLEGDAMSVEJ 3B
DK-2200 COPENHAGEN

DIR +45 35327300

Dear Dr. Singh,

We are submitting a revised version of our article entitled " **In vitro pancreas organogenesis from dispersed mouse embryonic progenitors**" for your consideration for publication in JoVE.

All reviewers were overall very positive but made useful comments that will improve the manuscript. We are indicating in the rebutal how we addressed their comments as well as the editorial comments.

We look forward to hearing your thoughts about the suitability of our manuscript for publication in JoVE.

Yours sincerely,

Anne Grapin-Botton.

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TITLE: In vitro pancreas organogenesis from dispersed mouse embryonic progenitors

AUTHORS:

Greggio, Chiara[1*]
[1]Ecole Polytechnique Fédérale de Lausanne, School of Life Sciences, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland, chiara.greggio@unil.ch

De Franceschi, Filippo[1*]
[1]Ecole Polytechnique Fédérale de Lausanne, School of Life Sciences, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland, filippo.defranceschi@rd.nestle.com

Figueiredo-Larsen, Manuel[2*]
[2]DanStem, University of Copenhagen, 3B Blegdamsvej, DK-2200 Copenhagen N, Denmark, manuel.figueiredo@sund.ku.dk

Grapin-Botton, Anne[1,2]
[1]Ecole Polytechnique Fédérale de Lausanne, School of Life Sciences, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland.
and
[2]DanStem, University of Copenhagen, 3B Blegdamsvej, DK-2200 Copenhagen N, Denmark, anne.grapin-botton@sund.ku.dk

[*] Equal contribution

CORRESPONDING AUTHOR:

Grapin-Botton, Anne
DanStem, University of Copenhagen, 3B Blegdamsvej, DK-2200 Copenhagen N, Denmark,
anne.grapin-botton@sund.ku.dk
Tel +45 21 15 28 63

KEYWORDS:

Pancreas, Progenitors, Branching Epithelium, Development, Organ Culture, 3D Culture, Diabetes, Differentiation, Morphogenesis, Cell organization, Beta Cell.

SHORT ABSTRACT:

The three-dimensional culture method described in this protocol recapitulates pancreas development from dispersed embryonic mouse pancreas progenitors, including their substantial expansion, differentiation and morphogenesis into a branched organ. This method is amenable to imaging, functional interference and manipulation of the niche.

LONG ABSTRACT:
The pancreas is an essential organ that regulates glucose homeostasis and secretes digestive enzymes. Research on pancreas embryogenesis has led to the development of protocols to produce pancreatic cells from stem cells [1]. The whole embryonic organ can be cultured at multiple stages of development [2-4]. These culture methods have been useful to test drugs and to image developmental processes. However the expansion of the organ is very limited and morphogenesis is not faithfully recapitulated since the organ flattens.

We propose three-dimensional (3D) culture conditions that enable the efficient expansion of dissociated mouse embryonic pancreatic progenitors. By manipulating the composition of the culture medium it is possible to generate either hollow spheres, mainly composed of pancreatic progenitors expanding in their initial state, or, complex organoids which progress to more mature expanding progenitors and differentiate into endocrine, acinar and ductal cells and which spontaneously self-organize to resemble the embryonic pancreas.

We show here that the *in vitro* process recapitulates many aspects of natural pancreas development. This culture system is suitable to investigate how cells cooperate to form an organ by reducing its initial complexity to few progenitors. It is a model that reproduces the 3D architecture of the pancreas and that is therefore useful to study morphogenesis, including polarization of epithelial structures and branching. It is also appropriate to assess the response to mechanical cues of the niche such as stiffness and the effects on cell´s tensegrity.

INTRODUCTION:
Organ culture provides a useful model that bridges the gaps between the complex but highly relevant *in vivo* investigations and the convenient but approximate simulation of cell line models. In the case of the pancreas, there is no cell line perfectly equivalent to pancreas progenitors although there are transformed cell lines simulating endocrine and exocrine cells. The adult whole pancreas cannot be cultured; isolated endocrine islets can be maintained for few weeks without cell proliferation and tissue slices can be kept *in vitro* for few hours [5]. Embryonic pancreas culture has been widely used not only to study its development, but also to investigate epithelial-mesenchymal interactions [4,6,7], to image processes [8] or to chemically interfere with them [9]. Two organ culture methods are mainly used: the first consists in culturing pancreatic buds on fibronectin coated plates [2], which is convenient for imaging purposes; the second option is to culture the organs on filters at the air-liquid interface [3,4] which best preserves morphogenesis. Although very useful, these methods lead to a certain degree of flattening; the expansion of progenitors is very limited as compared to the normal development and the starting population is complex comprising all types of pancreatic cells and mesenchymal cells.

The ability to culture and expand dispersed primary cells is valuable to study lineage relationships and uncover the intrinsic properties of isolated cell types [10]. Sugiyama et al. [11] could maintain pancreas progenitors and endocrine progenitors that retained some functional characters for 3-5 days in culture on feeder layers. Pancreatospheres, akin to neurospheres [12] and mammospheres [13], have been expanded from adult islets and ductal cells although the nature of the progenitors/stem cells that generate these spheres is not clear. In addition, in contrast with physiological development, the pancreatospheres contained some neurons [14,15]. Spheres were also recently produced from embryonic pancreas progenitors [16,17] and regenerating pancreata[18]

with good progenitor expansion and subsequent differentiation but failed to recapitulate morphogenesis.

3D models from dispersed and often defined cells that self-organize into miniaturized organs have recently flourished and simulate the development or adult turnover of multiple organs such as the intestine [19,20], the stomach [21], the liver [22], the prostate [23] and the trachea [24]. In some instances, developmental morphogenesis and differentiation have been recapitulated in 3D from ES cells, as is the case of optic cups [25], intestine [26] or brain [27].

Here, we describe a method to expand dissociated multipotent pancreatic progenitors in a 3D Matrigel scaffold where they can differentiate and self-organize.

PROTOCOL

This protocol aims to grow pancreatic organoids derived from murine E10.5 dissociated epithelial pancreatic cells.

The protocol requires ethical approval for animal experimentation.

1. Dissection of Dorsal Pancreatic Bud from E10.5 mouse embryos

1.1) Sacrifice timed-pregnant mice at embryonic day (E) 10.5, open the abdomen with a pair of scissors, remove the two uterine horns and place them in a 10 cm Petri dish filled with cold phosphate buffer saline (PBS) or Dulbecco modified essential medium (DMEM) kept on ice. The total experiment from the sacrifice to cell seeding is done in 60-90 min to prevent cell damage.

1.2) Separate the uterus into individual embryo segments using small scissors. Transfer one embryo to a 35 mm Petri dish with cold PBS and visualize it under a dissecting microscope with illumination from above. With dissection forceps remove the surrounding muscle, decidua and yolk sac and expose the embryo. Place the embryo back into cold DMEM medium. Embryos can easily be transferred using a 3 ml plastic transfer pipette/dropper. Isolate every individual embryo in a similar manner before continuing.

1.3) Place an embryo into a clean 35 mm Petri dish in PBS.

1.3.1) Use thin forceps (0.05 mm width) to remove the forelimb. Gently insert the forceps in the opening to detach the digestive tract from the spinal cord region (Figure 1). OPTIONAL: To more conveniently see the stomach, remove the upper body of the embryo, down to the heart and the tail region below the yolk stalk.

1.3.2) Locate the stomach, liver and intestine. Using the forceps, isolate the gastrointestinal tract from the stomach to the intestine and place it in cold DMEM on ice (Figure 1). The dorsal pancreatic bud is attached dorsally, posterior to the stomach (Figure 1).

1.3.3) Isolate every individual gastrointestinal tract in a similar manner before continuing. If the embryos need to be genotyped, collect the tail at the time of dissection and keep every individual gastrointestinal tract in a different well in a 24-well plate with cold DMEM on ice.

1.4) Place one gastrointestinal tract in a clean 35 mm Petri dish in cold PBS. Now use illumination from below in bright field to visualize and dissect the dorsal pancreatic bud under the dissection microscope. These conditions will optimize the visualization of volumes. Using electrolytically-sharpened tungsten needles or 20G syringe needles, isolate the pancreatic bud with as little mesenchyme as possible around the epithelium (Figure 1).

1.4.1) Transfer the isolated bud into a petri dish containing a cold dispase solution (1.25 mg/ml) for 2-3 min. From this point, transfer can most conveniently be done with flame-pulled 50 µl glass capillaries attached to a mouth-controlled flexible tube. Alternatively, but with more risk of losing the bud, use a 10 µl automatic pipette (Pipetman) with appropriate plastic tip.

1.4.2) Perform pancreatic bud aspiration and ejection under microscopic control. Put the pancreatic bud back in PBS. Further clean the isolated pancreatic bud from the mesenchyme with the needles and gentle aspiration using the glass capillary (Figure 1).

1.4.3) When the entire mesenchyme is removed, rinse the pancreatic bud in cold PBS; transfer each bud to cold DMEM in individual wells (60-well mini-trays filled with 10 µl of cold DMEM). It is important not to remove the mesenchyme in dispase which makes the tissue very sticky.

2. Plating and culture of dispersed cells

2.1) Transfer the dissected epithelia from all embryos with a pipette into conical wells of 60-well mini-trays filled with 10 µl PBS for rinsing.

2.1.1) Transfer the bud into 10 µl of Trypsin 0.05 % and let it incubate at 37 °C for 4 min. Inactivate the trypsin by transferring the bud into a well with 10 µl DMEM + 10% fetal calf serum (FCS).

2.1.2) Dissociate the cell suspension by pipetting through a thin capillary pulled with a pipette puller. It is important to avoid bubbles at this stage while pipetting up and down to dissociate the cells. Pancreas organoids optimally start from small groups of 5-15 cells and therefore partial dissociation is recommended (Figure 1).

2.2) Pool the cells from several embryos into an Eppendorf tube in order to minimize differences due to individual processing. Dilute the cell suspension in chilled Matrigel at a 1:3 ratio. Aliquot this mixture to a 96 well plate, 8 µl/well or in a plate optimized for imaging (see below).

2.3) Incubate the plate at 37 °C for 5 min, allowing the Matrigel to thicken. Fill the wells with 70 µl of medium of choice (organoid or sphere, see table of specific material/equipment) and leave in a humidified environment containing 5% CO_2 and 95% air at 37 °C.

2.4) Replace the medium every 4th day. Monitor the growing pancreatic colonies daily and document the process by imaging.

2.5) Small molecules or proteins of interest can be added to the medium at this stage for interference experiments, as reported previously [28].

3. Imaging of the progression of organoid development

3.1) Image organoids either daily or by time lapse microscopy using a fluorescent time-lapse microscope. For time lapse imaging, use an XY(Z) automated inverted fluorescent microscope.

3.2) Deposit small droplets of 3 µl in 4-well plates or glass-bottom plates filled with 2-5 ml medium. Image with a 10x long distance objective. Note: Transgenic mice expressing fluorescent tracers can be used. Movie 1 shows for example the initial expansion of organoids

from Pdx1-Ngn3-ER^{TM}-ires-nGFP^{+} mice[4]. The nuclear GFP enables the user to track cells as individual objects but similar principles can be applied to track cells with membrane fluorescence such as mT/mG mice[29].

3.3) Start time-lapse imaging 3 hours after seeding the cells to avoid focus drifts and set the software controlling automation to take 1 picture/hour for 3 or more days at manually defined positions. For every position, acquire a differential interference contrast (DIC) image as well as the GFP signal, reporting fluorescent marker expression.

4. Recovery of organoids for histology

4.1) Place the 96 well plate on ice and remove the medium, replacing it with ice cold PBS. This partially depolymerizes Matrigel.

4.2) Gently aspirate each individual organoid, removing the surrounding Matrigel using a 1000 µl tip in order to not disrupt the overall architecture. Transferred each organoid to a well on ice cold PBS. Direct fixation in Matrigel is also possible.

4.3) Fix the organoid for 15 minutes in 4% paraformaldehyde (PFA), cryopreserve it in sucrose and embed it in gelatin. Process each organoid for cryosectioning and histology as previously described (Johansson et al., 2007).

5. Recovery of organoids for PCR and biochemistry

5.1) Place the 96 well plate on ice and remove the medium. Add 60 µl of RNAlater per well in order to stabilize and protect cellular RNA.

5.2) Disrupt the gel in each well mechanically by partially depolymerizing it on ice. Either recover individual organoids using a 1000 µl tip in order to not disrupt the overall architecture or recover the entire well (with Matrigel) by disrupting the gel mechanically with a 200 µl tip. Use a 1000 µl tip to transfer the well content into an RNAse free-non-sticky Eppendorf tube kept on ice.

5.3) Wash the wells with 60 µl RNAlater and add the remaining content to the same Eppendorf tube.

5.4) Spin the tubes for 5 min at 500-1000 g at 4 °C.

5.5) Remove the supernatant, only leaving 20-30 µl of RNAlater in the tube together with the pellet. For biochemistry, store the samples as dry as possible.

5.6) Do not freeze samples in RNAlater immediately; store at 4 °C overnight (to allow RNAlater to thoroughly penetrate the tissue). The tissue can be stored at -20 °C for long term storage and can later be processed for tissue disruption and extraction of small quantities of RNA.

REPRESENTATIVE RESULTS:

E10.5 dorsal pancreatic progenitors dissociated and seeded in 3D Matrigel recapitulate pancreas development. Progenitors can be most easily followed with fluorescent reporters. In our case we used a transgenic mouse that expresses a nuclear GFP protein controlled by *Pdx1* promoter (*Pdx1-Ngn3-ER^{TM}-nGFP*) (movie 1) in the absence of tamoxifen and thus without activating Neurog3[4] (Figure 2).

With the organoid medium, an initial compaction of small clusters of cells occurs in the first hours. Expansion can then be detected by the enlargement of the clusters in the first 4 days (Figure 2A). From day 5, branches form in the 20% largest organoids. Single cells do not expand and lose *Pdx1* expression while the large clusters retain *Pdx1* expression[28].

In these conditions, progenitors undergo a spectacular morphogenesis with the emergence of branched epithelial structures. This process takes place only when the progenitors are seeded in ≥4-cells clusters, indicating a strong requirement for community signals. Histological analysis reveals that after day 7 of culture, the resulting mini-organs are composed of pancreatic progenitors (SOX9^{+}/HNF1B^{+}/*Pdx1*$^{+}$ cells: Figure 3B) and differentiated cells expressing either exocrine (Amylase^{+}) or endocrine (Insulin^{+} or Glucagon^{+}) markers (Figure 3A,C). The differentiation into endocrine cells is lower than in the endogenous pancreas (around 0.1%) but is increased to 1% when FGF1 is not added to the culture medium [28]. Remarkably, not only do the seeded progenitors differentiate into the expected pancreatic lineages, but they also spontaneously adopt the normal pancreatic architecture. Although E10.5 multipotent pancreas progenitors are not polarized, the cells in culture polarize as demonstrated by the segregation of Mucin1 and aPKC in the membrane facing the central lumen and they organize into a branched tubular network. Regionalized "tip and trunk" domains emerge: HNF1B^{+} progenitors and endocrine cells are localized in the central region, while acinar cells are located at the periphery as a partial or complete crown of cells. The organoids can be maintained in culture for 10 days; after this period, they generally lose their architectural organization and become cystic (not shown). Passaging can be done after partial dissociation but quickly leads to cyst formation, a phenomenon that is reduced by the addition of the BMP inhibitor Noggin [28].

With the sphere medium, expansion is more frequent and is seen from 2% of single cells; nevertheless the efficiency of sphere formation correlates with the size of the seeded clusters[28]. At day 2/3, a lumen is detected in the small clusters and expands thereafter, leading to largely mono-layered hollow spheres with occasional local multilayered areas (Figure 2B). These spheres collapse when retrieved from Matrigel (Figure 3D-H). Under these conditions, the resulting structures are mainly composed of pancreatic progenitors, with a small percentage of differentiated exocrine and endocrine cells at day 7 (Figure 3D-H). Progenitors in the spheres also become apically polarized, as demonstrated by the segregation of aPKC at the membrane facing the central lumen of all cells (Figure 3F). Pancreatospheres can be passaged at least twice (not shown).

Figure Legends:

Figure 1: Schematic representation of the procedure.
The gastro-intestinal tract is initially dissected from the embryo and subsequently the dorsal pancreatic bud is isolated. The mesenchyme is removed and the pancreatic progenitors are dissociated using trypsin. The resulting partially-dispersed cells are then seeded at low density in growth factor-depleted Matrigel. Scale bars: 1.00 mm except for the resulting organoid picture where the scale bar is 200 µm.

Figure 2: Progress of culture over time.
A small cluster of pancreatic progenitors grown with the organoid medium and followed with time-lapse microscopy from 3 hr after plating, for 60 hrs. In the bottom panels, an organoid after 7 days of culture. Scale bar: 200 µm applies to all panels in **A**. **B.** Example of a sphere followed in a 60 hrs time-lapse and captured at day 7 of culture. Scale bar: 200µm applies to all panels in B.

Figure 3: Histology.
A-C Serial sections of a 7-day organoid stained for progenitor (B – HNF1B) and differentiation (A – amylase, C- insulin) markers. The organoid is composed of epithelial (A – E-cadherin) and apically polarized (B – mucin1) cells. The dashed line corresponds to the non-acinar central region (A), where HNF1B (B) and endocrine (C) cells are detected. **D-H** Sections of 7-day spheres stained for progenitor (G – HNF1B; H – SOX9) and endocrine (D – insulin and glucagon) markers. The spheres are composed of apically polarized (D, F – aPKC) epithelial (E – E-cadherin) cells. Scale bar: 50µm.

DISCUSSION:

Large-scale production of functional beta cells *in vitro* is still ineffective [1]. In this challenging context, developmental biology studies may help deciphering the exact signals that are required for the differentiation of functional beta cells. This protocol allows for the maintenance, expansion and differentiation of embryonic pancreatic progenitors *in vitro*. This includes the formation of insulin-producing beta cells that do not co-express other endocrine hormones, have high levels of Pdx1, express the pro-convertases that mature insulin and have the processed insulin [28]. Important key factors within the system are the activity of FGF (exogenously stimulated by added FGF potentiated by heparin) and Notch (endogenous) signaling pathways, as well as ROCK-inhibition by Y-27632: in the absence of those factors, no or very limited numbers of organoids and spheres were generated[28]. The requirement for FGF and Notch activity is easily understood based on their importance for pancreas development *in vivo*[28]. ROCK inhibitor can be substituted by blebbistatin, thus revealing that hyperactivation of microfilament dynamics upon dissociation leads to both increased cell death, a strong inhibition of the progenitor transcription factor Pdx1 and a lack of expansion. Interestingly, many additional components of the organoid medium were proven to be individually unnecessary, but their combined absence resulted in the loss of epithelial branching[28]. In addition to certain essential components of the medium, it is important to control the level of dissociation of progenitors. Indeed, progenitor proliferation and *Pdx1* maintenance are significantly promoted in groups of more than 4 cells. A compaction can be observed within the first 12 hours and failure

to compact results in failure to maintain *Pdx1* and expand. The ROCK inhibitor is essential for this process.

At the moment organoids do not form after FACS sorting but the efficiency of the system could potentially be improved by reaggregating a controlled number of progenitors. Another critical component of this culture system is the 3D matrix. Cells put on Matrigel or in the Matrigel too close to the bottom of the plate spread and lose Pdx1 expression. Matrigel most likely provides biochemical components, notably laminin as well as mechanical cues[28]. Indeed, the stiffness of the matrix plays a pivotal role. Stiff hydrogels are not permissive for pancreatic progenitors maintenance and expansion [28] and diluted Matrigel is not either. When Matrigel is diluted 1:10 pancreas progenitor cannot be cultured.

The organoid system can be used to test the effects of small molecules and recombinant proteins on pancreatic progenitors in terms of survival, proliferation, differentiation, polarization and branching[28]. It can also be used to test the cooperation of different cell types during pancreas development[28]. We are confident that the accessibility of pancreatic progenitor cells will also allow genetic manipulations, such as viral targeting, as seen in other organoid systems [17,27]. This could be used for screening [17] with a system that enables morphogenesis in contrast to the spheres described previously as well as here[16,1728]. The culture conditions we developed also present the advantage of being serum-free, feeder-free and devoid of mesenchyme and blood vessels thereby reducing the cellular and biochemical complexity. However, there is a limitation in the ability to passage the organoids and thus to obtain large quantities of progenitors. This could be circumvented in the future by adaptations of the protocol to later stages of development where progenitors are more abundant, to sources of pancreatic progenitors produced from embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. This paves the way for a 3D model of human pancreas development.

This system can also potentially be used for the production of pancreatic cells in the future perspective of therapy. In this context, the production of functional beta cells for transplantation would potentially help in diabetes therapy. The adaptations of the system to human ES or iPS cells would be important for this purpose. It is still unclear whether the organoid or sphere conditions should be used. The organoid conditions allow for the production of cells that have several characteristics of mature beta cells but their function remains to be tested. However, these cells are currently not numerous and are mixed among other cells. It is also likely that the early appearance of heterogeneity in the organoid system leads to uncontrolled signaling between cells and is therefore detrimental to production.

The sphere system that maintains progenitors is in principle preferable for controlled expansion and passaging of progenitors but their subsequent differentiation remains to be controlled. Others have recently produced pancreatospheres that can be efficiently differentiated. It will be important to compare the nature of the spheres obtained in the current protocol devoid of feeders and serum to spheres obtained with the other protocols [16,17]. From a therapeutic point of view, the complexity and biological origin of Matrigel may constitute an issue of reproducibility, health and scalability. Preliminary results have shown that soft hydrogels functionalized with laminin are permissive for pancreatic progenitor expansion *in vitro*. Further optimization is required as these gels are not yet as efficient as Matrigel[28].

The production of beta cells in their natural context could also potentially be useful to test drugs that boost beta cell activity or increase their survival or proliferation but for this purpose it will be important to first test the degree of maturity of the beta cells, to increase the efficiency of their differentiation and to first test whether the culture conditions presented here better maintain islets than the current suspension cultures. Producing the exocrine pancreas could also be useful to develop drugs to target pancreatic cancers and pancreatitis. Here again, the degree of maturity of the exocrine cells produced needs to be thoroughly investigated.

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DISCLOSURES:
The authors have nothing to disclose.

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*Figure 1

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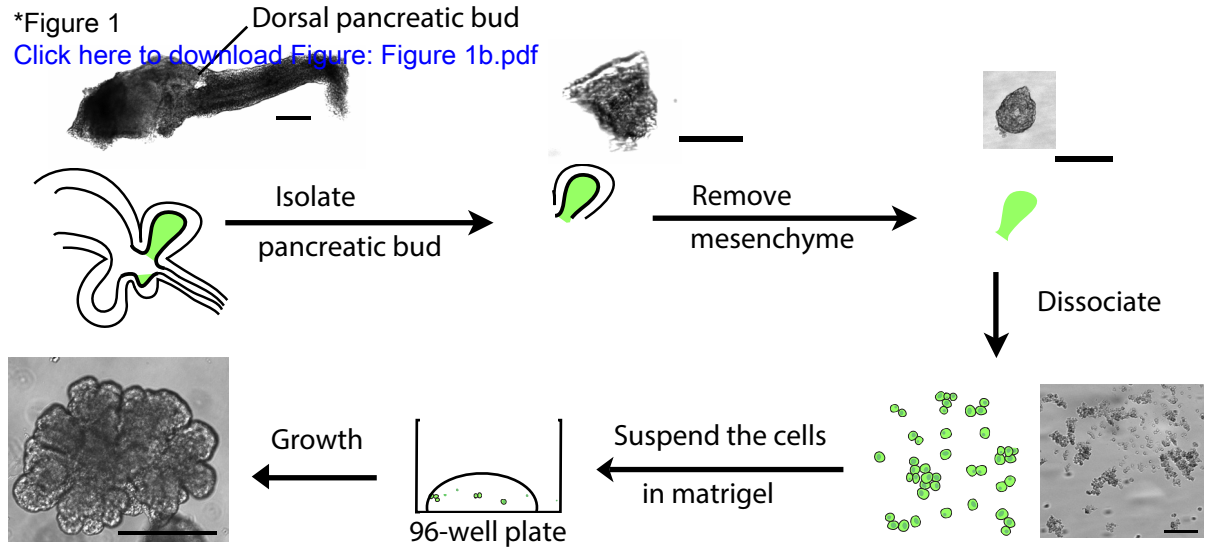

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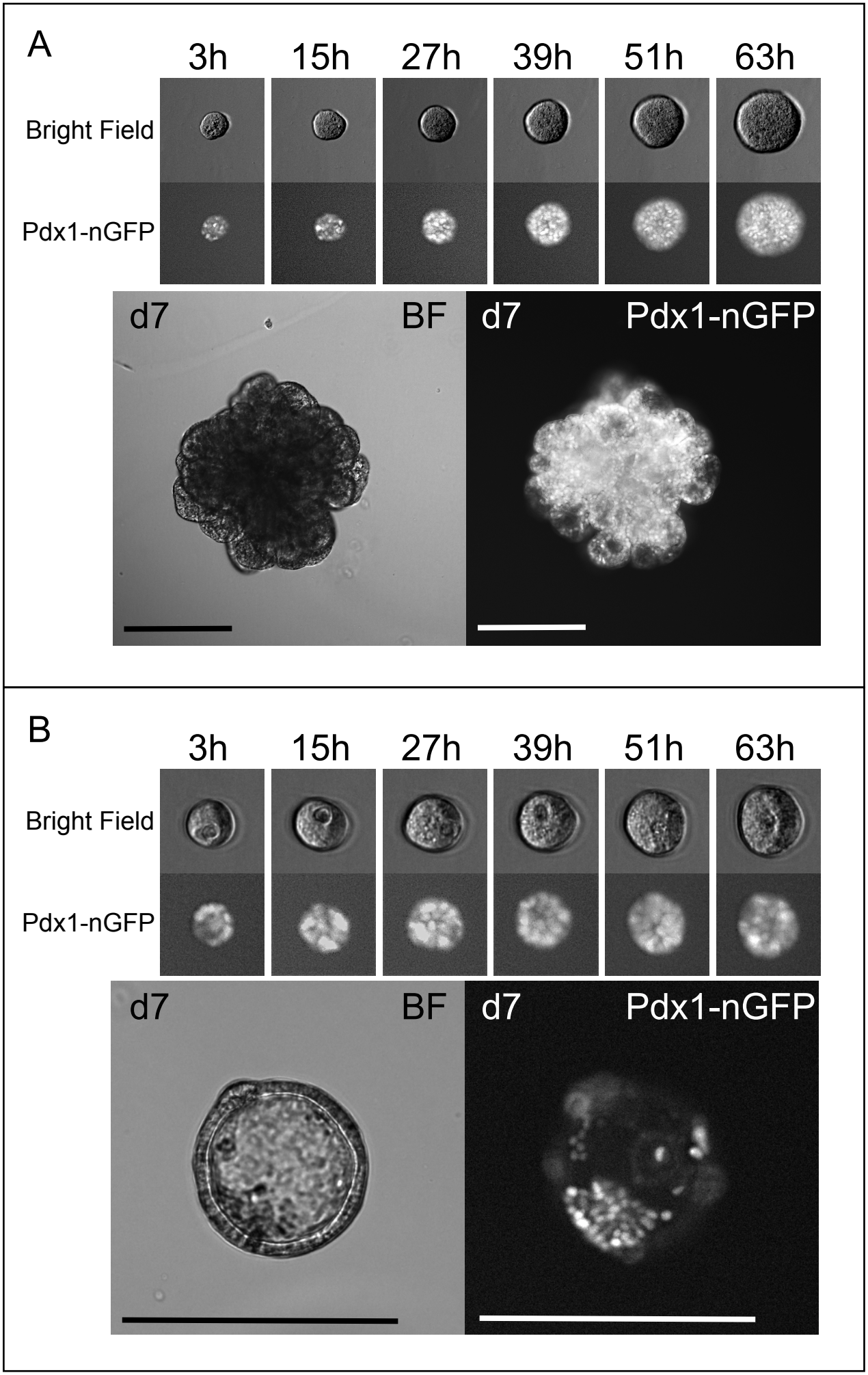

*Figure 3
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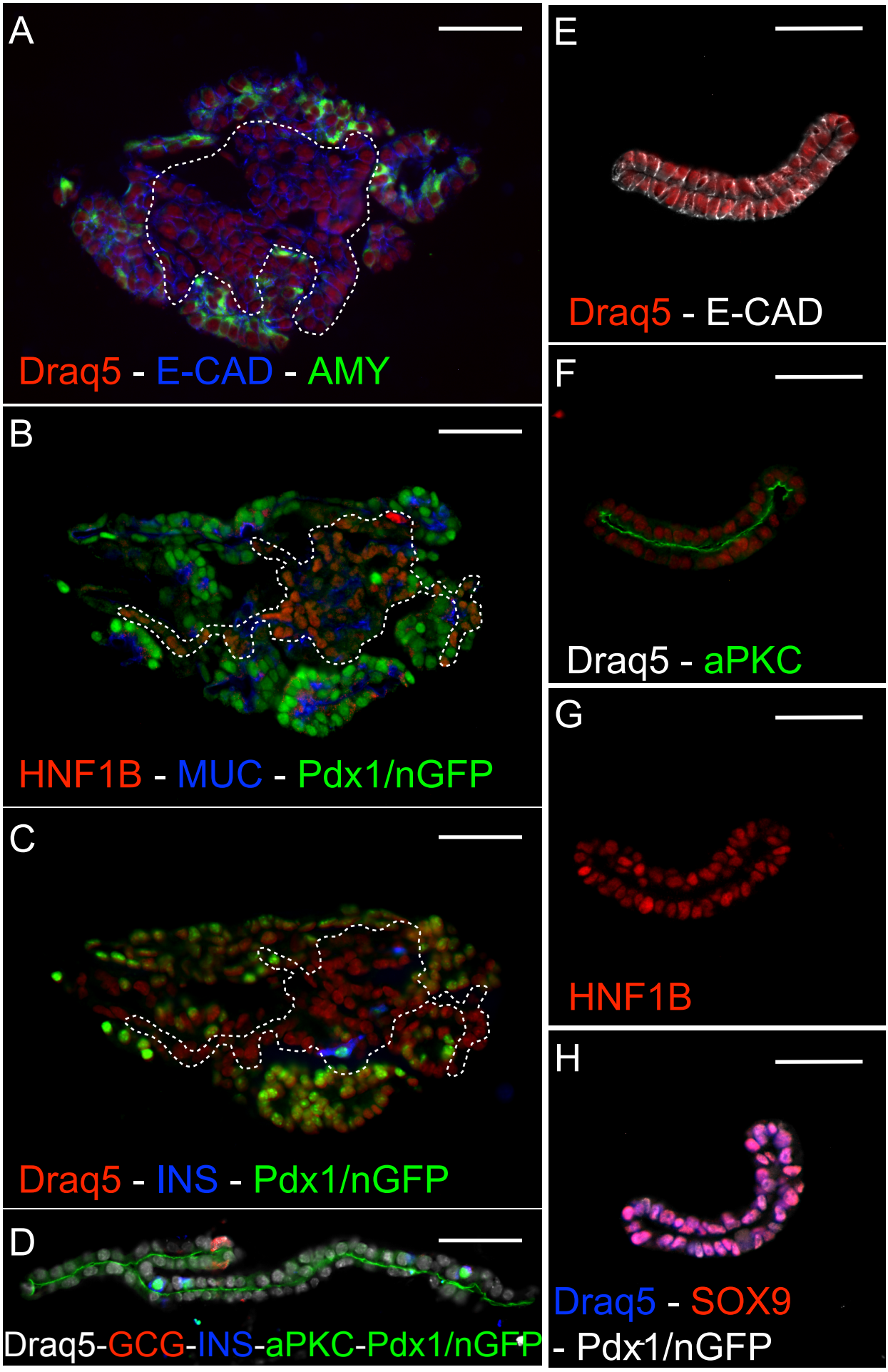

*Excel Spreadsheet- Table of Materials/Equipment
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Organoid medium

Name of Material	Company	Catalog Number	Stock Concentration	Concentration in final medium	Volume of stock	Comments/Description
Penicillin-Streptomycin	Gibco	15070-063	100%	1%	50 µl	Stock keept at -20°C
KnockOut Serum replacement (supplement)	Gibco	10828-028	100%	10%	500 µl	Stock keept at -20°C
2-mercaptoethanol	Sigma Aldrich	3148-25ML	14.3 M	0.1 mM	1 µl	Stock keept at 4°C
Phorbol Myristate Acetate (PMA)	Calbiotech	524400-1MG	16 µM	16 nM	5 µl	Stock keept at -20°C
Y-27632 (ROCK inhibitor)	Sigma Aldrich	ab120129	50 mM	10 µM	1 µl	Stock keept at -20°C- Attention! Stability/source is a frequent source of problems
EGF	Sigma Aldrich	E9644-2MG	50 µg/ml	25 ng/ml	2.5 µl	Stock keept at -80°C
Recombinant Human R-spondin 1	R&D	4645-RS-025/CF	250 µg/ml	500 ng/ml	10 µl	Stock keept at -80°C
- or -						
Recombinant Mouse R-spondin 1	R&D	3474-RS-050	250 µg/ml	500 ng/ml	10 µl	Stock keept at -80°C
Recombinant Human FGF1 (aFGF)	R&D	232-FA-025	100 µg/ml	25 µg/ml	1.25 µl	Stock keept at -80°C- do not include to increase beta cell production
Heparin (Liquemin)	Drossapharm		2500 U/ml	2.5 U/ml	2 µl	Stock keept at 4°C
Recombinant Human FGF10	R&D	345-FG-025	100 µg/ml	100 ng/ml	5 µl	Stock keept at -80°C
DMEM/F-12	Gibco	21331-020			4412.25 µl	
Total					5000 µl	

Sphere medium

Name of Material	Company	Catalog Number	Stock Concentration	Concentration in final medium	Volume of stock	Comments/Description
Penicillin-Streptomycin	Gibco	15070-063	100%	1%	50 µl	Stock keept at -20°C
B27 x50 (supplement)	Gibco	17504-044	100%	10%	100 µl	Stock keept at -20°C
Recombinant Human FGF2 (bFGF)	R&D	233-FB-025	100 µg/ml	64 ng/ml	3.2 µl	Stock keept at -80°C
Y-27632 (ROCK inhibitor)	Sigma Aldrich	ab120129	50 mM	10 µM	1 µl	Stock keept at -20°C- Attention! Stability/source is a frequent source of problems
DMEM/F-12	Gibco	21331-020			4845.8 µl	
Total					5000 µl	

Other components

Name of Material	Company	Catalog Number	Working Concentration	Comments/Description
Matrigel	Corning	356231		Stock keept at -20°C
Trypsin 0.05%	Gibco	25300-054	0.05%	Stock keept at 4°C
RNAlater - RNA stabilizing reagent	Qiagen	76104		Store at room temperature
Dispase	Sigma Aldrich	D4818-2MG	1.25 mg/ml	Stock keept at -20°C
BSA for reconstitution	Milipore	81-068		For reconstituition of cytokines - Stock keept at -20°C
Fetal calf serum (FCS)	Gibco	16141079		Stock keept at -20°C

Equipment

Name of Material	Company	Catalog Number
60 well MicroWell trays	Sigma Aldrich	M0815-100EA
4-well plates	Thermo Scientific	176740
95-well plates F bottom	Greiner Bio	6555180
Glas bottom plates	Ibidi	81158
Disposal micropittes	Blaubrand	708745

Microscopes used

Cell^R imaging station (motorized inverted Olympus IX81 stand) equipped with a Hamamatsu ORCA ER B7W camera and the Ludin Cube and Box.
Leica DMI6000 B stand surrounded with a Ludin Cube and Box, equipped with a Leica DFC365 FX camera and the AF6000 Expert/Matrix software command interface.

Objectives used

Olympus UPLAN FL NA 0.30 air 9.50 mm 10x long distance
Leica HC PL FLUOTAR NA 0.30 air 11.0 mm 10x long distance

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Author(s): Chiara Greggio, Filippo De Franceschi, Manuel Figueiredo_Larsen and Anne Grapin-Botton

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Name: Anne Grapin-Botton

Department: DanStem

Institution: University of Copenhagen

Article Title: In vitro pancreas organogenesis from dispersed mouse embryonic progenitors

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Reviewers' comments:

Reviewer #1:
This is a well described technical manuscript that follows a recent publication by the same group now in press in Development (Ref 28 of the present MS).
This new technical manuscript perfectly illustrates and complements the one just published and is important for the scientific community.

I do not have major concerns but only very minor ones:
-1- introduction, line 4: please replace acinar by exocrine as it is mainly duct-like cell lines that have been generated

We replaced acinar by exocrine.

-2- introduction, line 5: islets can in fact be maintained in some cases few weeks in culture without cell proliferation. The authors could change their sentence

We changed the sentence to indicate a culture of weeks instead of days.

-3- Table on organoid medium and other component: the table is a bit difficult to read as the lines are not perfectly aligned. Moreover, the term "EFG" should be replaced by "EGF"

We tried to realign the lines and changed the formatting.

Reviewer #2:

Manuscript Summary:

This is an interesting, accurate and important manuscript that describes for the first time a method to expand mouse embryonic pancreas early progenitors and follow organogenesis in vitro.
The methods are extremely well detailed and documented.

Major Concerns:

N/A

Minor Concerns:

N/A

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

This is a clear and detailed description of 3D culture conditions to grow and manipulate embryonic pancreas in vitro. The study provides good evidence of the advantages and disadvantages of the described system and will be useful to those in the pancreas differentiation field as well as those with different areas of expertise.

Major Concerns:

None

Minor Concerns:

In 1.3.1 of the protocol, it would be useful to precisely indicate which inner organs.

We did indicate which inner organs.

Additional Comments to Authors:
N/A