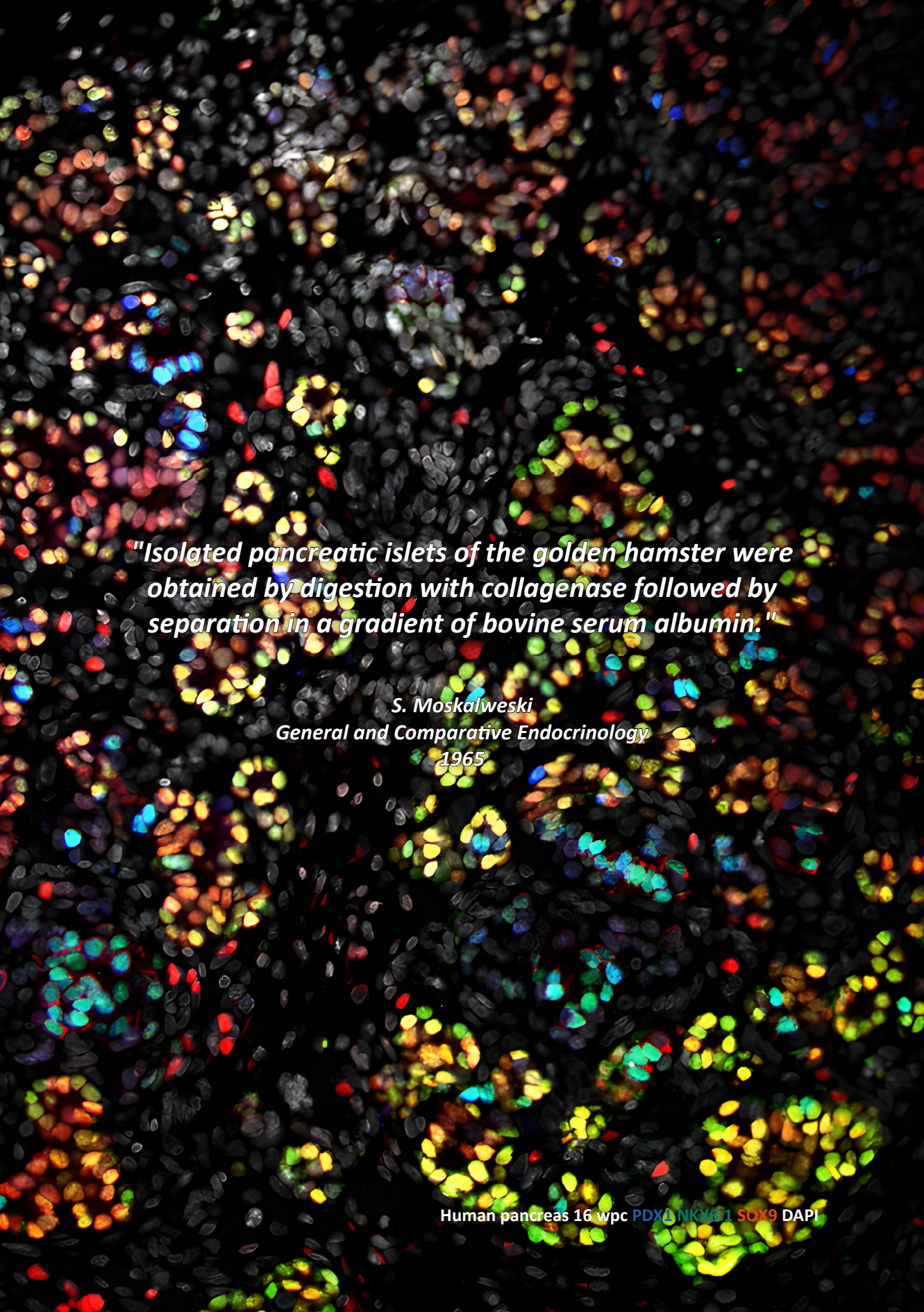
Duct cells in development, regeneration, and transplantation: charting a path to new islets

Balak, J.R.A.


"Isolated pancreatic islets of the golden hamster were obtained by digestion with collagenase followed by separation in a gradient of bovine serum albumin."
S. Moskalweski
General and Comparative Endocrinology
1965
Human pancreas 16 wpc PDX1 NKX6.1 SOX9 DAPI

CHAPTER| 6

Differentiation of Human Pancreatic Ductal Cells Towards a Beta Cell Phenotype Using INGAP, FGF7 and a GLP-1R Agonist

Jeetindra R.A. Balak[1], Hendrica A.M. Töns[1], H. Siebe Spijker[1], Cindy J.M. Loomans[2], Françoise Carlotti[1], Eelco J.P. de Koning[1,2]

[1]Dept of Internal Medicine, Leiden University Medical Center, Leiden, the Netherlands
[2]Hubrecht Institute, Utrecht, the Netherlands

Abstract

Putative progenitors in the human adult duct compartment of the pancreas are an attractive alternative cell source for beta cell replacement therapy. Potential candidates to improve differentiation protocols are compounds associated with duct-derived beta cell neogenesis. Here we aim to explore the activation of GLP-1R, INGAP and FGF7 pathways as triggers for beta cell differentiation of primary human islet-depleted tissue. Ductal cells were expanded from human islet-depleted tissue as monolayer culture and subsequently differentiated in suspension culture. We evaluated a multistage protocol based on the use of INGAP, the GLP-1R agonist liraglutide and FGF7, in comparison with a basic serum-free differentiation medium. Upon *ex vivo* differentiation, gene expression of endocrine hormone markers was induced up to 15- to 75-fold in the multistage protocol. In the multistage protocol 6% of the aggregated cells were PDX1^{high}-positive and 60% of these cells also co-expressed NKX6.1. After transplantation in mice PDX1^{high}NKX6.1^{+}Insulin^{+} cells were observed in the graft. Remarkably, differentiated beta cells were found outside duct structures suggesting a delamination process. In conclusion, this study provides evidence that pancreatic endocrine progenitor cells can be formed *ex vivo* from primary human exocrine cells.

Introduction

Type 1 diabetes mellitus (T1DM) is a chronic disease characterised by an autoimmune destruction of the insulin-producing beta cells located in the islets of Langerhans, resulting in hyperglycemia[1]. T1DM is treated by administration of exogenous insulin. In a small group of T1DM patients beta cell replacement (pancreas or islet transplantation) is performed. A lack of donor organs is one of the factors why this treatment occurs rarely. One of the main challenges in making beta cell replacement therapy accessible to a broader group of patients is identifying a renewable source of beta cells.

Alternative sources for beta cells include pluripotent stem cells such as human embryonic stem cells (hESC) or induced pluripotent stem cells (iPSC). Recent developments in this field are encouraging with development of multistage differentiation protocols, yielding pure populations of beta cells[2,3]. However, clinical use of these pluripotent cells is currently limited by ethical concerns and by the risk of malignant formation by residual undifferentiated stem cells when transplanted *in vivo*[4].

Another option would be the use of adult pancreas-specific progenitor cells. It is hypothesized that the adult pancreas harbours cells that are able to develop towards a beta cell phenotype[5-9]. During embryonic development the pancreatic ductal epithelium is able to generate all pancreatic cell types, including the beta cells[10,11]. However, whether the duct cells retain this capacity in the postnatal pancreas is still controversial[12-15]. Human autopsy studies have indicated that in altered metabolic states such as pregnancy or obesity there is a significant increase in the number of beta cells[16-18]. These newly formed beta cells are in close proximity or located in the ducts of the pancreas, indicating that the origin of these cells is in the ductal compartment. Moreover, we recently reported that a novel computational method to exploit single-cell transcriptome data (StemID)

identified pancreatic progenitor cells to be located in the human pancreatic duct cell population[19,20].

In an effort to find compounds that can induce beta cell neogenesis several animal models of diabetes and pancreatic injury models have been developed, inducing regeneration of the pancreas[21-23]. These models have resulted in the discovery of compounds associated with duct-derived beta cell neogenesis. One of these models is the pancreatic duct obstruction model in hamsters, in which new beta cells were formed in close proximity of the duct[24]. The bioactive peptide islet neogenesis associated protein (INGAP) was isolated from the regenerating pancreas and has demonstrated to reduce hyperglycemia in diabetic rodents and to improve glucose homeostasis in patients with diabetes mellitus in clinical trials[25-27]. Also, *in vitro* experiments with a pancreatic ductal cell line demonstrated differentiation into endocrine cells when treated with this peptide[28]. Another neuropeptide is glucagon-like peptide-1 (GLP-1), an incretin endogenously produced by L-cells in the intestine. GLP-1 receptor agonists (GLP-1 RA) are widely used for its insulinotropic properties in patients with type 2 diabetes mellitus[29]. In several rodent models of diabetes, administration of GLP-1 or its long-acting receptor agonist exendin-4, significantly increased beta cell mass. Individual beta cells were found in ducts and in small clusters outside islets, next to an increased proliferation of duct and acinar tissue, suggesting GLP-1 has an effect on both the proliferation and differentiation of pancreatic cells[30,31]. Moreover, *in vitro* studies using the pancreatic ductal cell lines ARIP and PANC-1 showed increased beta cell differentiation with GLP1-R agonist treatment[32]. Fibroblast growth factor 7 (FGF7) is known for its regulation of proliferation and differentiation of cells. During development it is expressed in the pancreatic mesenchyme where it promotes growth, morphogenesis and differentiation of pancreatic exocrine cells by activation of the NOTCH pathway[33,34]. *In vitro* studies with pluripotent cells demonstrate that FGF7 promotes differentiation of PDX1-expressing pancreatic progenitor cells[35].

The vast majority of our understanding of beta cell neogenesis is derived from research performed with animals or cell lines due to limited access to primary human tissue. For development of a potentially translatable therapeutic approach of duct-derived beta cell neogenesis, it is essential to determine the effects of these agents on human tissue. A differentiation protocol that included INGAP, FGF7 and a GLP-1R agonist was successfully used to differentiate human embryonic stem cells towards insulin-producing beta cell[36]. When applying this protocol, the differentiating cells underwent a phase in which the majority of cells expressed KRT19 (ductal marker), prior to endocrine differentiation. We hypothesized that the differentiation of putative progenitors located in the ductal compartment of the human pancreas can also be triggered by using these agents that stimulate islet neogenesis. Here, we evaluated the effects on primary human islet-depleted tissue of three compounds associated with duct-derived beta cell neogenesis *in vivo*, *i.e.,* INGAP, GLP-1, and FGF7.

Materials and methods

Expansion of islet-depleted tissue

Human donor pancreata were processed at in the Good Manufacturing Practice facility of our institute according to the Ricordi method[37]. Tissue was only used if consent for research was indicated on the Eurotransplant organ donor forms. Organ donors were between 14 and 61 years of age, had a BMI between 20 and 34 kg/m^2 and did not have a medical history of diabetes mellitus (**Table 1**). The donor pancreata were enzymatically and mechanically digested followed by islet purification using a Ficoll gradient. Islet purity was directly assessed after digestion using dithizone (DTZ) staining and islet-depleted tissue fractions were cultured overnight in culture bags containing 4 mL of tissue and 200 mL Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin. Next, islet-depleted tissue was washed with PBS and single cells were obtained by trypsinisation and filtered using 70 µm filters. Samples of single cells were collected for RNA isolation and formalin fixation. Single cells were plated in tissue-culture-treated T75 tissue culture flasks (Corning) at a density of 15 x 10^6 cells per flask in Endothelial Basal Medium-2 (EBM-2) (Clonetics) supplemented with 10% FCS, 100U/mL penicillin and 100 µg/mL streptomycin. The cells were cultured at 37 °C under a humidified condition of 95% air and 5% CO_2 until confluency for 5-7 days. The medium was changed the day after plating and subsequently every 2 days.

Table 1. Donor characteristics

Donor number	Sex	Age (years)	BMI (kg/m^2)	Cause of death
1	M	36	27	Cardiac arrest
2	F	14	20	Trauma
3	F	20	25	Trauma
4	M	51	34	Stroke
5	F	61	23	Trauma
6	M	17	20	Stroke
7	F	47	26	Stroke
8	F	42	22	Stroke
9	M	46	25	Suicide
10	F	45	24	Brain tumour
11	M	27	30	Suicide

Ex vivo differentiation of expanded islet-depleted tissue

The cells in monolayer were washed with PBS before incubation in 8 mL of non-enzymatic cell dissociation solution (NCDS) (Sigma) to detach the cells from the plastic surface. Cells were collected in DMEM containing 10% FCS, 100U/mL penicillin and 100 µg/mL streptomycin. After

washing, cells were counted and samples for RNA isolation and formalin fixation were obtained. The rest of the cells were used for differentiation by 3D culture in suspension in 6-well low attachment plates (Corning).

For differentiation, two protocols were compared (**Supplemental Figure 1**). The control protocol was based on differentiation culture medium described and used for primary human ductal cells and consisted of three weeks of culture in serum-free DMEM (Life technologies) supplemented with 24 mM glucose, 1 µg/mL insulin, transferrin, selenium (ITS, Sigma), 10 mM nicotinamide, 2 g/L human albumin (Cealb, Sanquin) and 200 U/mL penicillin with 200 µg/mL streptomycin[38]. This protocol was compared to a multistage protocol based on a human embryonic stem cell differentiation protocol, starting with 2 weeks of culture with DMEM/F12 (Life technologies) supplemented with 17 mM glucose, 1 µg/mL insulin, transferrin, selenium (ITS, Sigma), 10 mM nicotinamide, 2 g/L human albumin (Cealb, Sanquin), 200 nM INGAP (Sequence: IGLHDPSHGTLPNGS, synthesized at the peptide facility of our institute), 10 nM Liraglutide (GLP-1R agonist, Novo Nordisk) and 10 ng/µL fibroblast growth factor 7 (FGF7, R&D), followed by 1 week of culture with RPMI1640 (Life technologies) with B27 1× (Life technologies) and 10 mM nicotinamide[36]. Medium for both protocols was refreshed every two days.

Transplantation of *ex vivo* differentiated aggregates

All animal experiments were approved by the committee for animal ethics of our institute. After 3 weeks of *ex vivo* differentiation aggregates were washed with PBS and cell pellets of approximately 200 µL were transplanted under the kidney capsule of normoglycaemic 8-12 weeks old NOD/SCID mice. After one month of *in vivo* maturation the kidneys with the grafts were removed and processed for immunohistochemistry.

Quantitative real-time PCR

RNA was extracted using the RNeasy mini kit (Qiagen) following the manufacturer's protocol. The amount and quality of the RNA extracted was assessed using a Nanodrop ND-1000 spectrometer (NanoDrop technologies). 1 µg RNA was used for reverse transcription into cDNA using M-MLV RTase (Life technologies). PCR reactions were run in duplicate using a mix of 1/20 of the cDNA, 1 mM forward and reverse primer, 6.25 µL SYBR Green Master Mix (Qiagen) and sterile water up to a total volume of 12.5 µL. The primer sequences can be found in **Table 2**. The PCR reactions were performed on a LightCycler 480II (Roche, Basel, Switzerland). Acquired Ct values were normalized against the housekeeper gene GAPDH. The normalized Ct values were quantified using the delta-delta Ct method. Gene expression is presented relative to gene expression in cells after expansion.

Table 2. Human primer sequences

Gene	Forward primer	Reverse primer
CK19	CTACAGCCACTACTACACGAC	CAGAGCCTGTTCCGTCTCAAA
FOXA2	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA
GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG
GCG	CAAGGCAGCTGGCAACGT	CTGGTGAATGTGCCCTGTGA
HNF6	CGCTCCGCTTAGCAGCAT	GTGTTGCCTCTATCCTTCCCAT
INS	GCAGCCTTTGTGAACCAACA	TTCCCCGCACACTAGGTAGAGA
MAFA	CTTCAGCAAGGAGGAGGTCATC	GCGTAGCCGCGGTTCTT
NKX6.1	CTGGCCTGTACCCCTCATCA	CTTCCCGTCTTTGTCCAACAA
PDX1	CCATGGATGAAGTCTACCAAAGCT	CGTGAGATGTACTTGTTGAATAGGAACT
PTF1A	AGGCCCAGAAGGTCATC	AGGGAGGCCATAATCAGG
SOX9	AGTACCCGCACTTGCACAAC	ACTTGTAATCCGGGTGGTCCTT

Tissue fixation and immunohistochemistry

Single cells or aggregates were fixed with 4% formalin (Klinipath) for 30 minutes. The mouse kidney containing the transplanted graft was fixed with 4% formalin overnight. After fixation the samples were washed with PBS and suspended in a 2% agar solution, before being processed and embedded in paraffin for sectioning of 4 μm slides. Immunofluorescence staining was performed using the following primary antibodies: 1:1000 mouse anti Synaptophysin (Millipore), 1:250 rabbit anti KRT19 (Abcam), 1:5 goat anti PDX1 (R&D systems), 1:500 mouse anti NKX6.1 (DSHB Hybridoma Bank), 1:200 guinea pig anti Insulin (Santa Cruz), 1:200 rabbit anti Glucagon (Vector Laboratories). The secondary antibodies that were used: 1:1000 goat anti mouse Alexa Fluor 568 (Molecular Probes), 1:1000 goat anti guinea pig Alexa Fluor 647 (Molecular Probes), 1:200 streptavidin Alexa Fluor 488 (Invitrogen), 1:400 TRITC anti guinea pig (Jackson ImmunoResearch), 1:500 goat anti rabbit Pacific Blue (Molecular Probes), 1:1000 goat anti rabbit Alexa Fluor 488 (Molecular Probes). Antibodies were diluted in PBS with 1% lamb serum (Invitrogen). Antigen retrieval was performed by boiling the slides in 0.01 M citrate buffer. Nuclei were stained with a 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). Microscopic images were taken with a Leica DM5500 microscope (Leica Inc., Germany) and confocal microscopy was performed on a Zeiss LSM 7 MP (Zeiss, Germany). Fluorescent images were processed and analysed using ImageJ (http://rsbweb.nih.gov/ij) and Zen Lite software (Zeiss, Germany).

Flow cytometry

Dissociated tissue was fixed with 4% formalin for 15 minutes at room temperature and permeabilised with 0.1% saponin for 30 minutes. Cells were washed with 1% BSA/0.1% saponin and filtered with 40 μm filters (BD Bioscience). Single cells were incubated with 1:100 mouse anti KRT19 (DakoCytomation) and subsequently with 1:1000 goat anti mouse Alexa Fluor 488 (Invitrogen).

Flow cytometry was performed on a BD FACSCalibur (BD Bioscience). Flow cytometry data was analysed with FlowJo X (Tree Star).

Statistical analysis

Data are represented as means ± SEM. Statistical significance was determined using a one-tailed Student's t-test or two-way ANOVA followed by a Bonferroni multiple comparison test where appropriate. Differences were considered to be statistically significant when $P \leq 0.05$. All statistical analyses was performed with GraphPad Prism 6.0 (GraphPad Software Inc).

Results

Monolayer expansion of human islet-depleted tissue yields KRT19-positive ductal cells

Crude islet-depleted pancreatic tissue was cultured overnight. Prior to cell dissociation the crude islet-depleted tissue contained 20-30% of KRT19-positive cells (keratin 19, a marker for ductal cells) (**Supplemental Figure 2A, Supplemental Figure 2B**)[39]. The tissue was then dispersed to single cells and plated on tissue culture-treated plastic. The day after plating, cells attached to the plastic surface to a confluency of approximately 10%, whereas non-adherent cells and other dead cells were discarded with medium change. Cells cultured as monolayer expanded rapidly reached confluency after 5-6 days and showed a cobblestone morphology characteristic of ductal cells (**Figure 1A, Supplemental Figure 3**). Immunostainings showed that post-expansion 90% of the cells were KRT19-positive, a marker for ductal cells, and no cells were positive for the beta cell marker C-peptide despite the low amount of C-peptide-positive and synaptophysin-positive cells (2.9%) immediately after cell dispersion before expansion (**Figure 1B, Supplemental Figure 2A, Supplemental Figure 2B**). No acinar cells were found by amylase immunostainings (data not shown). Also, gene expression of endocrine and acinar markers decreased markedly after expansion in contrast to the enrichment for KRT19 (**Figure 1C-F**).

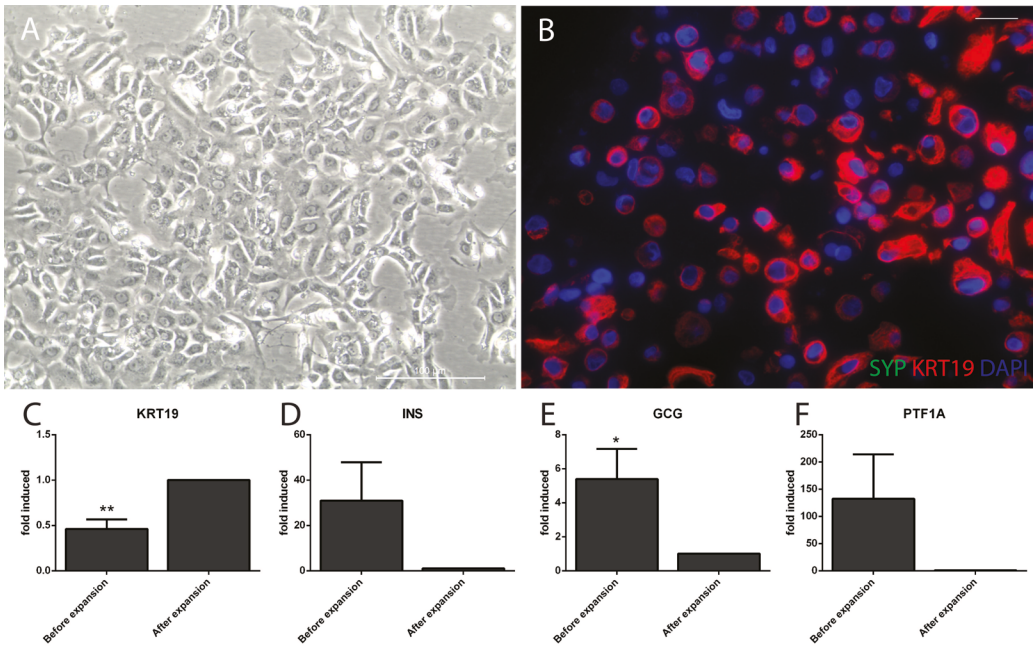


Figure 1. Adherent culture of human islet-depleted tissue leads to enrichment of KRT19-positive cells

(**a**) Islet-depleted tissue cultured for 5 days in monolayer showing cobblestone morphology typical for ductal cells. Scale bar = 100 µm. (**b**) Immunofluorescent staining of the cell population after expansion for keratin 19 (KRT19; ductal marker, red) and synaptophysin (SYP; endocrine marker, green) demonstrating most cells were KRT19-positive and no SYP-positive cells were observed. Scale bar = 20 µm. (**c-f**) Gene expression of islet-depleted tissue before and after expansion. After expansion there is enrichment for KRT19 (duct marker) and a decrease in INS and GCG (insulin and glucagon; endocrine hormones) and a decrease in PTF1A (acinar marker). Values are fold induction (after expansion=1). (n=3 donors) (*$P \leq 0.05$ vs after expansion, **$P \leq 0.01$ vs after expansion).

Differentiation with the multistage protocol yields cells with a more endocrine phenotype

Expanded cells could be efficiently detached from the plastic surface. The next step was to trigger differentiation of these cells towards an endocrine phenotype using a selection of compounds associated with islet neogenesis in the multistage protocol. In a first set of preliminary experiments, the single compounds GLP-1R agonist and INGAP were added to the medium and compared to medium in which both compounds were added for one week. These experiments indicated a higher induction of endocrine marker gene expression when compounds were combined (**Supplemental Figure 4**) (n=3 donors); therefore a complete protocol for multiple weeks was designed. With this protocol cells autonomously formed aggregates within 2-3 days (**Figure 2A**) (n=9 donors). Larger and more aggregates were found in the multistage protocol compared to the control protocol.

Immunostaining for Ki67 showed the presence of proliferating cells in the first two weeks when cells were treated with the multistage protocol (**Figure 2B-2D**), whereas proliferating cells were only incidentally found in the control protocol (**Supplemental Figure 5**). Upon *ex vivo* differentiation, gene expression of the duct marker KRT19 decreased (**Figure 3I**). PTF1A gene expression (a marker of early progenitor cells and restricted to acinar cells in the adult pancreas) and NGN3 (endocrine progenitor marker) initially increased during the first week, and decreased the second and third week of differentiation in the multistage protocol (**Figure 3D-E**). Pancreatic progenitor markers SOX9, FOXA2, HNF6 were upregulated throughout the three weeks of differentiation (**Figure 3A-C**). Gene expression of the beta cell markers PDX1, NKX6.1, MAFA were all upregulated in the multistage protocol (**Figure 3F-H**), as well as genes for the endocrine hormones insulin and glucagon that were induced up to 15- to 75-fold in the multistage protocol after three weeks (**Figure 3J-K**). No major differences were observed at the transcriptional level between the multistage protocol and the control protocol (**Figure 3A-K**). Differentiation was also examined at the protein level. In both protocols, approximately 6% of the cells were highly positive for the beta cell marker PDX1 (PDX1^{high}) (**Figure 4A-E**). Interestingly, 60% of these PDX1^{high} cells also expressed the beta cell marker NKX6.1 in the multistage protocol; in contrast to only 20% in control aggregates (**Figure 4G**). Only sporadic insulin-positive cells were found with the multistage protocol at this stage. Cells that were differentiated towards an endocrine cell fate lost their KRT19 expression. Overall, these findings suggest that the multistage protocol is able to trigger the differentiation of human islet-depleted tissue towards an endocrine phenotype.

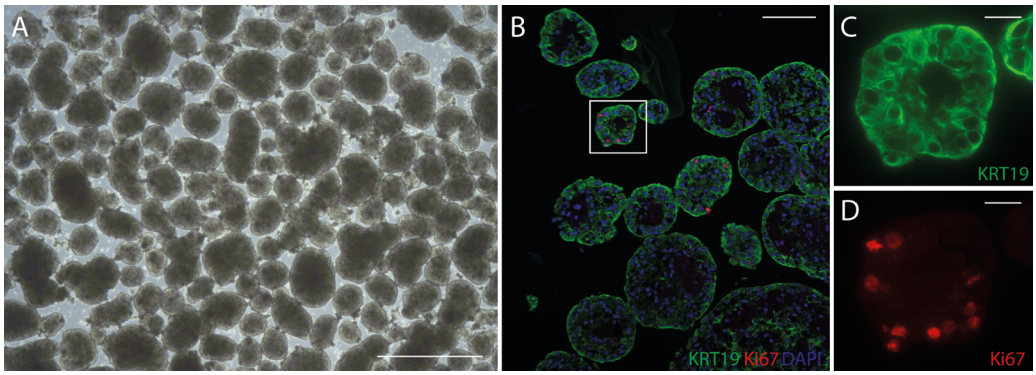


Figure 2. Cells cultured in suspension with the multistage protocol yield aggregates with proliferating cells

(**a**) Brightfield image of aggregates at 1 week of differentiation with the multistage protocol demonstrating heterogeneous aggregates. Scale bar = 100 µm. (**b**) Immunofluorescent staining of aggregates differentiated for 2 weeks with the multistage protocol for: KRT19 (ductal marker, green), Ki67 (proliferation marker, red) and DAPI (nuclear marker, blue), demonstrating proliferating ductal cells. Scale bar = 100 µm. (**c-d**) Magnification of B, showing single channel KRT19-positive cells and Ki67-positive cells. Scale bar = 20 µm. (n=3 donors).

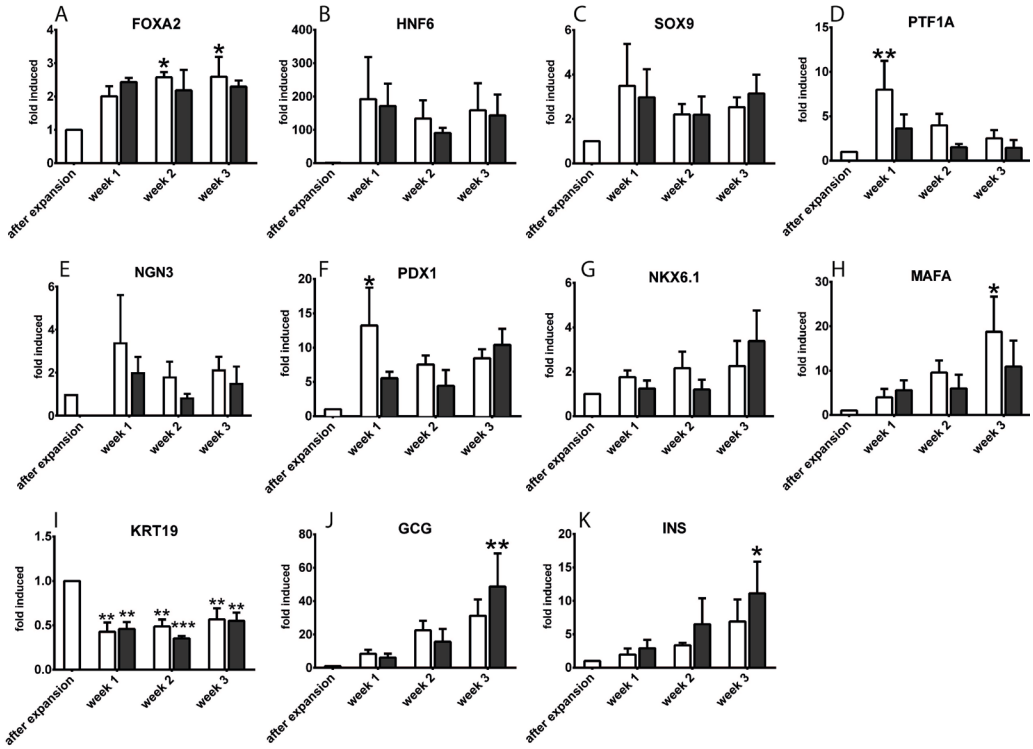


Figure 3. Gene expression during differentiation using the multistage protocol
(**a-c**) Increase in the SOX9, FOXA2 and HNF6 (pancreatic progenitor markers) during differentiation. (**d-e**) Initial increase and then decrease of PTF1A (progenitor marker in progenitor cells, restricted to acinar cells in adult cells) and NGN3 (endocrine progenitor marker). (**f-h**) Increase in the beta cell transcription factors PDX1, NKX6.1, MAFA. (**i-k**) Decrease in KRT19 (ductal marker) and increase in GCG and INS (endocrine hormones). Control aggregates depicted as white bars, multistage protocol aggregates as black bars. Values are fold induction (after expansion=1). (N=3 donors). (**P* ≤ 0.05 vs after expansion, ***P* ≤ 0.01 vs after expansion, ****P* ≤ 0.001 vs after expansion).

In vivo differentiation of *ex vivo* pre-differentiated cells

Prior studies have shown that maturation of progenitor cells can occur after transplantation *in vivo*[2,38,40]. In order to further differentiate the cells from the multistage differentiation protocol towards a beta cell phenotype, pre-differentiated aggregates were transplanted under the kidney capsule of NOD/SCID mice for a month. Immunostainings of the grafts showed that the transplanted aggregates had formed large ductal structures (**Figure 5A**). Up to 5% of the graft consisted of PDX1^{high}NKX6.1^{+}Insulin^{+} cells, most differentiated cells were found in the duct lining (**Figure 5A**). Interestingly, PDX1^{high}NKX6.1^{+}Insulin^{+} cells were also found in close proximity but separate from the duct structure, suggesting that delamination had occurred (**Figure 5B**). Serum was harvested at 1 and 4 weeks post-transplantation for a C-peptide ELISA (Mercodia). The amount of C-peptide in the serum was undetectable for the detection level of the assay (data not shown)

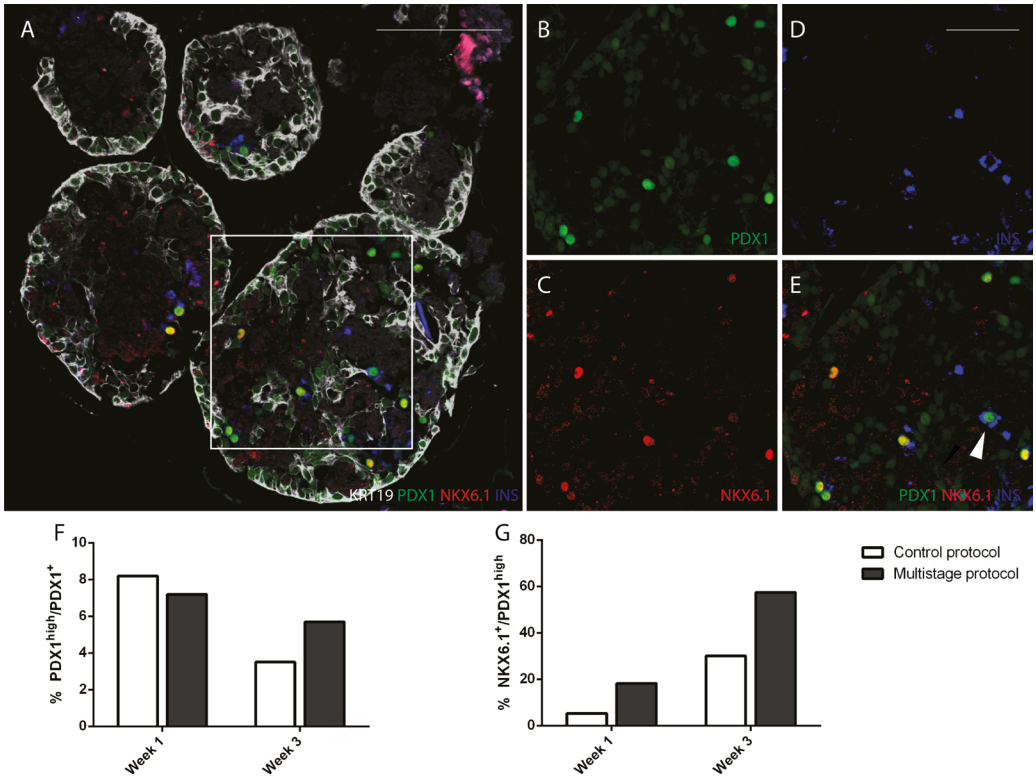


Figure 4. Aggregates differentiated for three weeks with the multistage protocol yield more pancreatic progenitor cells

(**a**) Immunofluorescent staining of aggregates after three weeks of differentiation for KRT19 (ductal marker, white), NKX6.1 (beta cell marker, red), PDX1 (beta cell marker, green) and insulin (beta cell hormone, blue). Scale bar = 100 µm. (**b-e**) Magnification of figure A showing single channel fluorescent images of PDX1^{high}, NKX6.1^{+} and Insulin^{+} cells. Arrowhead in E indicates a PDX1^{high}NKX6.1^{+}Insulin^{+}. Scale bar = 50 µm. (**f-g**) Quantification of PDX1^{high}-positive cells and PDX1^{high}/NKX6.1^{+} cells, indicating more PDX1^{high}NKX6.1^{+} cells in the multistage protocol (800 cells quantified in control aggregates, 1000 cells in multiprotocol aggregates). (n=1 donor)

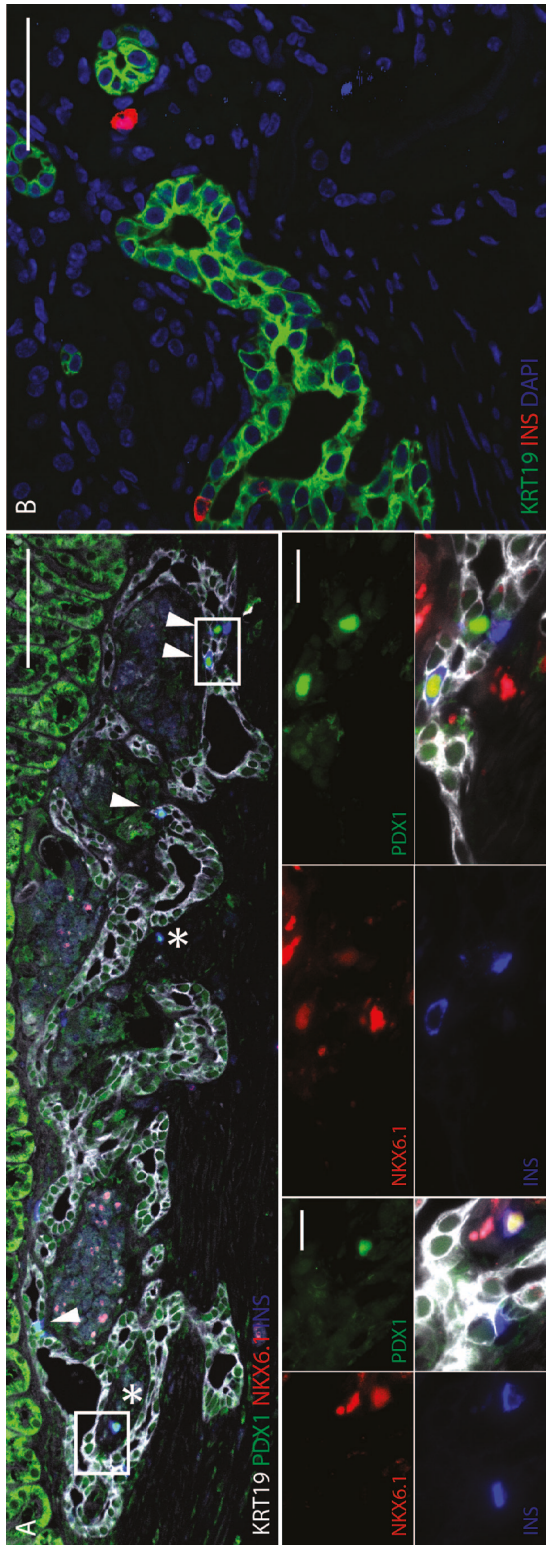

A
KRT19 PDX1 NKX6.1 INS
NKX6.1
PDX1
NKX6.1
PDX1
INS
INS
B
KRT19 INS DAPI

Figure 5. After one month of *in vivo* maturation cells pre-differentiated towards pancreatic progenitor cells become PDX^{high}NKX6.1^{+}insulin^{+} cells
(**a**) Immunofluorescent staining of graft retrieved after one-week maturation under the kidney capsule for KRT19 (ductal marker, white), NKX6.1 (beta cell marker, red), PDX1 (beta cell marker, green) and insulin (beta cell hormone, blue). Triple positive cells are inside and outside the duct lining. Arrowheads in A indicate PDX1^{high}NKX6.1^{+}Insulin^{+} cells in the duct structure and the asterisk indicate delaminating PDX1^{high}NKX6.1^{+}Insulin^{+} cells. Scale bar = 100 µm. Magnification shows single channel fluorescent images. Scale bar = 20 µm. (**b**) graft retrieved after one month ***in vivo*** maturation showing a delaminating insulin-positive cell. Scale bar = 50 µm (n=1 donor transplanted in 5 mice).

Discussion

Islet transplantation is a promising treatment for patients with T1DM, but a lack of donor tissue is one of the major hurdles for widespread use. Therefore, there is an urgent need for an alternative source of beta cells. There have been very few studies published on beta cell neogenesis and primary human exocrine tissue. The differentiation medium we used as control in this study is derived from a protocol published in 2007[38]. A more recent study showed improved differentiation of human duct cells to beta-like cells, but upon genetic manipulation (overexpression of transcription factors)[41]. The goal of our study was to improve the current differentiation protocols using a non-genetic approach by using beta cell neogenesis agents that have been demonstrated to stimulate endocrine differentiation from ductal cells in rodent models of diabetes, ductal cell lines, and human embryonic stem cells. Here we developed a multistage protocol that allows the generation of pancreatic endocrine progenitor cells (PDX1^{high} NKX6.1^{+}) from human islet-depleted tissue using a non-genetic approach using beta cell neogenesis agents that have been demonstrated to stimulate endocrine differentiation in rodent models of diabetes, ductal cell lines, and human embryonic stem cells. Upon maturation *in vivo*, differentiated beta cells were found outside duct structures suggesting a delamination process.

The co-expression of PDX1 and NKX6.1 is a crucial stage in the formation of beta cells. During embryonic development pancreatic endocrine progenitor cells co-express PDX1 and NKX6.1, a combination of transcription factors that becomes restricted to beta cells in the adult pancreas. In the hESC field it has been demonstrated that only cells co-expressing PDX1 and NKX6.1 (pancreatic progenitor cells) are capable of differentiating into mature beta cells[2,40]. Human adult duct cells normally do not co-express these transcription factors. However, after three weeks of *ex vivo* pre-differentiation we find an increase in cells co-expressing both transcription factors, indicating that our protocol with beta cell neogenesis agents can trigger the differentiation of human adult primary exocrine tissue towards pancreatic progenitor cells *ex vivo.* Moreover, pancreatic progenitor markers such as SOX9, HNF1B, HNF6 were upregulated. PTF1A, a progenitor marker restricted to acinar cells in the adult pancreas and NGN3, an endocrine progenitor marker, were transiently upregulated before endocrine differentiation, suggesting a dedifferentiation process prior to differentiation. By transplanting these progenitor cells *in vivo* we confirm that these progenitor cells can differentiate further into insulin-positive cells.

Additionally, we observed insulin-positive cells outside the duct lining after one month of *in vivo* maturation, indicating that these cells have delaminated. During development endocrine cells are formed within the progenitor ductal epithelium, and subsequently delaminate from the epithelium and migrate to the surrounding mesenchyme where they cluster together and form the islets. In prior studies with pre-differentiated crude duct aggregates and *in vivo* maturation under the kidney capsule newly formed beta cells were only found in the ductal lining[38]. Even though clusters of insulin-positive cells indicating early islet formation were not observed yet, the fact that single insulin-positive cells were delaminated from the ductal epithelium indicates that the novel multistage protocol triggers a higher degree of endocrine maturation of the cells.

The number of endocrine cells that are formed is currently too limited for sufficient clinical impact, although it cannot be excluded that *in vivo* differentiation for a longer time period than a month would increase this frequency. Up to 5% of the cells were found insulin positive after *in vivo* maturation. These insulin-positive cells maintained PDX1^{high}NKX6.1^{+}, indicative of maturity. The origin of the differentiated cells with an endocrine phenotype in our samples is unclear, as our starting population is crude exocrine tissue. The pancreatic duct compartment is a heterogeneous compartment, consisting of smaller and larger sized ducts[42]. Evidence points towards different intrinsic properties of the cells lining these bigger and smaller ducts, and it is hypothesized that the progenitor capacity is only restricted to a small subpopulation of ductal cells[43,44]. Efficient selection and expansion using cell surface markers of a possible progenitor population might result in a higher yield of pancreatic progenitor cells and eventually more beta cells. Due to lack of current knowledge on which cell subpopulation would be the most prone to differentiate to beta cells and the required cell surface markers to isolate for these populations, we chose to start expanding cells from an unsorted cell population (*i.e.*, islet-depleted tissue) in contrast to other studies[41]. The expansion method we use has been used by other groups to selectively expand ductal cells[45,46]. After expansion, 90% of the cells we harvest are KRT19-positive in our study. However, by using this crude and unbiased approach we cannot exclude the possibility of a contaminating cell population (*e.g.*, acinar cells) to contribute to the endocrine cell formation.

Moreover, our current basic understanding of the precise spatiotemporal cues that play a role during embryonic development is insufficient to develop a complete protocol for efficient, proper beta cell specification from adult human ductal cells. The exact pathways activated by GLP-1R agonist and INGAP are not completely elucidated. Prior studies indicate that activation of the GLP-1R in hESC leads to differentiation by activation of the hedgehog, cyclic adenosine monophosphate (cAMP) and phosphatidylinositol-3-kinase (PI3K) signaling pathways[47,48]. Also , INGAP has shown to generate endocrine cells from KRT19^{+} cells by activation of the PI3K pathway[49,50]. A downstream activator of PI3K signaling pathway is Akt1, which is a regulator of neurogenin3 (NGN3, key transcription factor for endocrine differentiation) expression, indicating that this pathway is involved in endocrine formation[51]. This is supported by other studies demonstrating that the Akt1 pathway is necessary for beta cell growth and differentiation *in vitro* and *in vivo*[52-54]. Further studies on islet biology and discovery of additional signaling pathways involved in beta cell specification are

needed to optimise the selection of compounds to further increase the efficiency of differentiation of adult human ductal cells to beta cells.

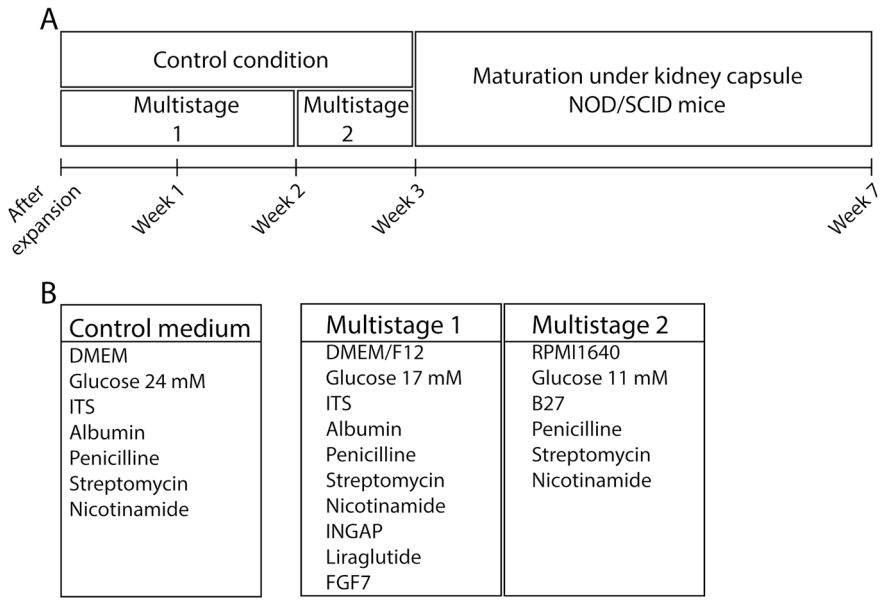
Finally, promising breakthroughs are made in the hESC field, where new protocols are developed for formation of islets from pluripotent cells. These newly formed islets are getting close to fully functional beta cells, and are capable of physiological insulin secretion in response to glucose[55]. However, there is still a risk of undifferentiated pluripotent cells forming a teratoma when transplanted *in vivo*. Interestingly the pluripotent cells in this culture undergo an intermediate stage in which they acquire a ductal phenotype, expressing KRT19, followed by a stage in which they express other pancreatic progenitor markers such as PDX1 and NKX6.1[2,36], as in our study. Building upon these protocols should allow the further development of differentiation protocols for primary human adult ductal cells, a cell type already committed to a pancreatic cell fate, thus likely making the differentiation process shorter and with a lower risk of tumour formation when transplanted *in vivo* due to limited pluripotency.

References

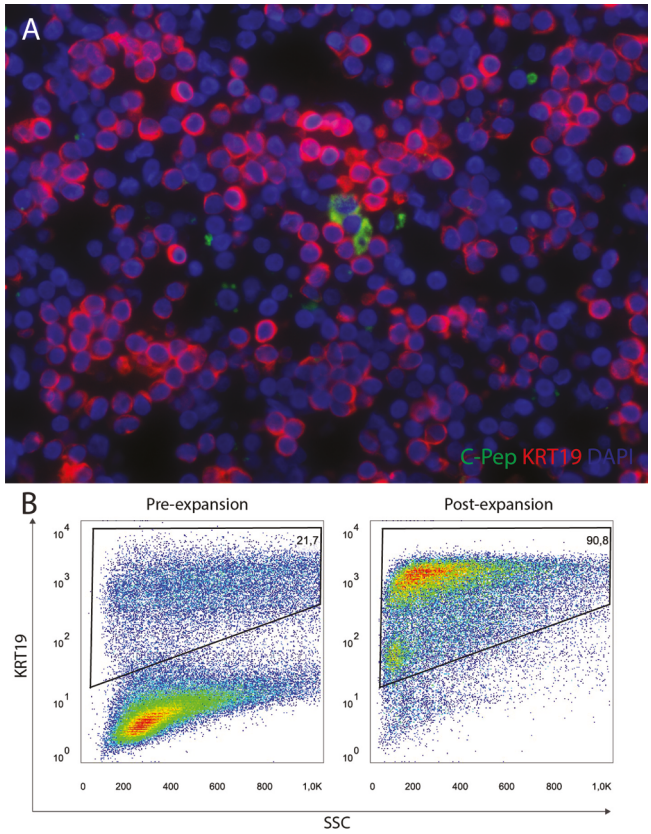
1. Atkinson, M.A., Eisenbarth, G.S. & Michels, A.W. Type 1 diabetes. *Lancet* **383**, 69-82 (2014).
2. Rezania, A.*, et al.* Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. *Nature Biotechnology* (2014).
3. Pagliuca, F.W.*, et al.* Generation of functional human pancreatic beta cells in vitro. *Cell* **159**, 428-439 (2014).
4. Kroon, E.*, et al.* Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nature biotechnology* **26**, 443-452 (2008).
5. Bonner-Weir, S.*, et al.* The pancreatic ductal epithelium serves as a potential pool of progenitor cells. *Pediatric diabetes* **5 Suppl 2**, 16-22 (2004).
6. Bonner-Weir, S. In vitro cultivation of human islets from expanded ductal tissue. *Proceedings of the National Academy of Sciences* **97**, 7999-8004 (2000).
7. Bouwens, L. & Pipeleers, D.G. Extra-insular beta cells associated with ductules are frequent in adult human pancreas. *Diabetologia* **41**, 629-633 (1998).
8. Heremans, Y.*, et al.* Recapitulation of embryonic neuroendocrine differentiation in adult human pancreatic duct cells expressing neurogenin 3. *J Cell Biol* **159**, 303-312 (2002).
9. Bogdani, M.*, et al.* Formation of insulin-positive cells in implants of human pancreatic duct cell preparations from young donors. *Diabetologia* **46**, 830-838 (2003).
10. Herrera, P.L. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. *Development* **127**, 2317-2322 (2000).
11. Gu, G., Dubauskaite, J. & Melton, D.A. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development* **129**, 2447-2457 (2002).
12. Kopp, J.L.*, et al.* Sox9+ ductal cells are multipotent progenitors throughout development but do not produce new endocrine cells in the normal or injured adult pancreas. *Development (Cambridge, England)* **138**, 653-665 (2011).
13. Solar, M.*, et al.* Pancreatic exocrine duct cells give rise to insulin-producing beta cells during embryogenesis but not after birth. *Developmental cell* **17**, 849-860 (2009).
14. Furuyama, K.*, et al.* Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. *Nature genetics* **43**, 34-41 (2011).
15. Dor, Y., Brown, J., Martinez, O.I. & Melton, D.a. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. *Nature* **429**, 41-46 (2004).
16. Butler, A.E.*, et al.* Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. *Diabetologia* **53**, 2167-2176 (2010).
17. Saisho, Y.*, et al.* beta-cell mass and turnover in humans: effects of obesity and aging. *Diabetes Care* **36**, 111-117 (2013).
18. Butler, A.E.*, et al.* B-Cell Deficit and Increased -Cell Apoptosis in Humans With Type 2 Diabetes. *Diabetes* **52**, 102-110 (2003).
19. Grun, D.*, et al.* De Novo Prediction of Stem Cell Identity using Single-Cell Transcriptome Data. *Cell Stem Cell* **19**, 266-277 (2016).
20. Muraro, M.J.*, et al.* A Single-Cell Transcriptome Atlas of the Human Pancreas. *Cell Syst* **3**, 385-394 e383 (2016).
21. Xu, X.*, et al.* Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. *Cell* **132**, 197-207 (2008).
22. Inada, A.*, et al.* Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 19915-19919 (2008).
23. Rovira, M.*, et al.* Isolation and characterization of centroacinar/terminal ductal progenitor cells in adult mouse pancreas. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 75-80 (2010).
24. Rosenberg, L., Brown, R.A. & Duguid, W.P. A new approach to the induction of duct epithelial hyperplasia and nesidioblastosis by cellophane wrapping of the hamster pancreas. *J Surg Res* **35**, 63-72 (1983).
25. Rafaeloff, R.*, et al.* Cloning and sequencing of the pancreatic islet neogenesis associated protein (INGAP) gene and its expression in islet neogenesis in hamsters. *The Journal of clinical investigation* **99**, 2100-2109 (1997).
26. Rosenberg, L.*, et al.* A pentadecapeptide fragment of islet neogenesis-associated protein increases beta-cell mass and reverses diabetes in C57BL/6J mice. *Ann Surg* **240**, 875-884 (2004).
27. Dungan, K.M., Buse, J.B. & Ratner, R.E. Effects of therapy in type 1 and type 2 diabetes mellitus with a peptide derived from islet neogenesis associated protein (INGAP). *Diabetes Metab Res Rev* **25**, 558-565 (2009).
28. Assouline-Thomas, B.*, et al.* Islet Neogenesis Associated Protein (INGAP) induces the differentiation of an adult human pancreatic ductal cell line into insulin-expressing cells through stepwise activation of key transcription factors for embryonic beta cell development. *Differentiation* **90**, 1-14 (2015).
29. Gutniak, M., Orskov, C., Holst, J.J., Ahren, B. & Efendic, S. Antidiabetogenic effect of glucagon-like peptide-1 (7-36)amide in normal subjects and patients with diabetes mellitus. *N Engl J Med* **326**, 1316-1322 (1992).
30. Xu, G., Stoffers, D.A., Habener, J.F. & Bonner-Weir, S. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. *Diabetes* **48**, 2270-2276 (1999).
31. Wang, Y.*, et al.* Glucagon-like peptide-1 can reverse the age-related decline in glucose tolerance in rats. *J Clin Invest* **99**, 2883-2889 (1997).
32. Hui, H., Wright, C. & Perfetti, R. Glucagon-Like Peptide 1 Induces Differentiation of Islet Duodenal Homeobox-1-Positive Pancreatic Ductal Cells Into Insulin-Secreting Cells. *Diabetes* **50**, 785-796 (2001).
33. Miralles, F., Czernichow, P., Ozaki, K., Itoh, N. & Scharfmann, R. Signaling through fibroblast growth factor receptor 2b plays a key role in the development of the exocrine pancreas. *Proc Natl Acad Sci U S A* **96**, 6267-6272 (1999).
34. Ye, F., Duvillie, B. & Scharfmann, R. Fibroblast growth factors 7 and 10 are expressed in the human embryonic pancreatic mesenchyme and promote the proliferation of embryonic pancreatic epithelial cells. *Diabetologia* **48**, 277-281 (2005).
35. Shirasawa, S.*, et al.* A novel stepwise differentiation of functional pancreatic exocrine cells from embryonic stem cells. *Stem Cells Dev* **20**, 1071-1078 (2011).
36. Xu, X., Browning, V. & Odorico, J.S. Activin, BMP and FGF pathways cooperate to promote endoderm and pancreatic lineage cell differentiation from human embryonic stem cells. *Mechanisms of development*,

1-16 (2011).
37. Shapiro, A.M.*, et al.* Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *The New England journal of medicine* **343**, 230-238 (2000).
38. Yatoh, S.*, et al.* Differentiation of affinity-purified human pancreatic duct cells to beta-cells. *Diabetes* **56**, 1802-1809 (2007).
39. Ichii, H.*, et al.* Characterization of pancreatic ductal cells in human islet preparations. *Laboratory investigation; a journal of technical methods and pathology* **88**, 1167-1177 (2008).
40. Rezania, A.*, et al.* Enrichment of human embryonic stem cell-derived NKX6.1-expressing pancreatic progenitor cells accelerates the maturation of insulin-secreting cells in vivo. *Stem cells (Dayton, Ohio)*, n/a-n/a (2013).
41. Lee, J.*, et al.* Expansion and conversion of human pancreatic ductal cells into insulin-secreting endocrine cells. *eLife* **2**, 1-22 (2013).
42. Reichert, M. & Rustgi, A.K. Pancreatic ductal cells in development, regeneration, and neoplasia. *The Journal of clinical investigation* **121**, 4572-4578 (2011).
43. Dorrell, C.*, et al.* Isolation of major pancreatic cell types and long-term culture-initiating cells using novel human surface markers. *Stem cell research* **1**, 183-194 (2008).
44. Dorrell, C.*, et al.* Transcriptomes of the major human pancreatic cell types. *Diabetologia* **54**, 2832-2844 (2011).
45. Klein, T.*, et al.* Investigation and characterization of the duct cell-enriching process during serum-free suspension and monolayer culture using the human exocrine pancreas fraction. *Pancreas* **38**, 36-48 (2009).
46. Street, C.N.*, et al.* Enriched human pancreatic ductal cultures obtained from selective death of acinar cells express pancreatic and duodenal homeobox gene-1 age-dependently. *Rev Diabet Stud* **1**, 66-79 (2004).
47. Hui, H.*, et al.* Glucagon like peptide-1-directed human embryonic stem cells differentiation into insulin-producing cells via hedgehog, cAMP, and PI3K pathways. *Pancreas* **39**, 315-322 (2010).
48. Egan, J.M., Bulotta, A., Hui, H. & Perfetti, R. GLP-1 receptor agonists are growth and differentiation factors for pancreatic islet beta cells. *Diabetes/metabolism research and reviews* **19**, 115-123 (2003).
49. Barbosa, H.C.*, et al.* Islet neogenesis-associated protein signaling in neonatal pancreatic rat islets: involvement of the cholinergic pathway. *J Endocrinol* **199**, 299-306 (2008).
50. Jamal, a.-M.*, et al.* Morphogenetic plasticity of adult human pancreatic islets of Langerhans. *Cell death and differentiation* **12**, 702-712 (2005).
51. Vojtek, A.B.*, et al.* Akt regulates basic helix-loop-helix transcription factor-coactivator complex formation and activity during neuronal differentiation. *Mol Cell Biol* **23**, 4417-4427 (2003).
52. Bernal-Mizrachi, E., Wen, W., Stahlhut, S., Welling, C.M. & Permutt, M.A. Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *J Clin Invest* **108**, 1631-1638 (2001).
53. Tuttle, R.L.*, et al.* Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat Med* **7**, 1133-1137 (2001).
54. Pende, M.*, et al.* Hypoinsulinaemia, glucose intolerance and diminished beta-cell size in S6K1-deficient mice. *Nature* **408**, 994-997 (2000).
55. Pagliuca, F.W.*, et al.* Generation of functional human pancreatic beta cells in vitro. *Cell* **159**, 428-439 (2014).

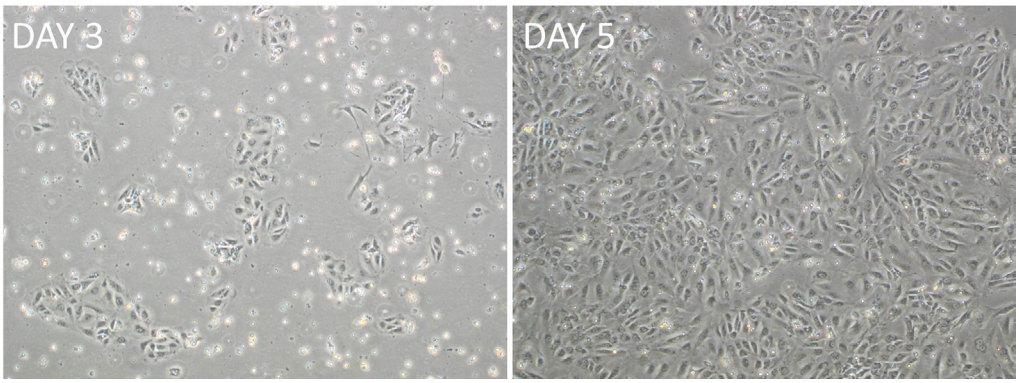
Supplemental information



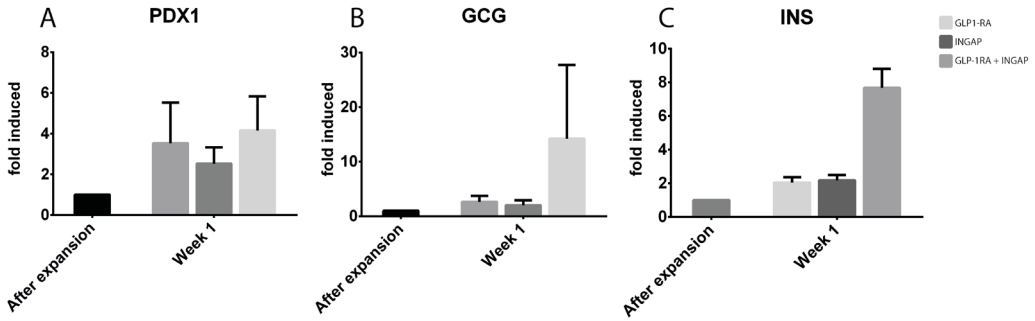
Supplemental Figure 1. Experimental setup
(**a**) Timeline of *ex vivo* differentiation and *in vivo* maturation. (**b**) Medium composition during different stages.



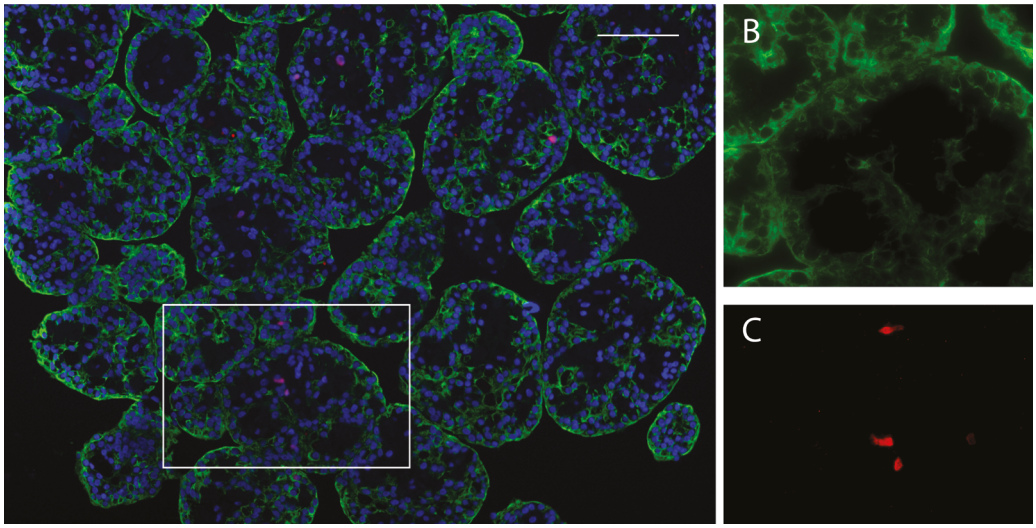
Supplemental Figure 2. Quantification of KRT19+ cells pre-expansion and post-expansion
(**a**) Immunofluorescent staining of the cell population before expansion for keratin-19 (KRT19; ductal marker, red) and synaptophysin (SYP; endocrine marker, green) demonstrating most cells were KRT19 positive and incidentally SYP positive cells were observed. Scale bar = 20 µm. (**b**) Flow cytometry showing 20% KRT19+ cells pre-expansion and 90% KRT19+ cells post expansion. SSC: Side Scatter.



Supplemental Figure 3. In monolayer culture cells selectively attach to the plastic surface and rapidly expand
Brightfield image of a representative monolayer culture at 3 and 5 days after plating.



Supplemental Figure 4. Upregulation of endocrine transcription factors compared between single compounds and combined compounds after one week.
(**a-c**) Combining the compounds in the multistage medium induced a higher upregulation of endocrine gene expression. Values are fold induction (after expansion=1). (N=3 donors).



Supplemental Figure 5. Cells cultured in suspension with the control protocol yield proliferating cells
(**a**) Immunofluorescent staining of aggregates differentiated for 2 weeks with the control protocol for: KRT19 (ductal marker, green), Ki67 (proliferation marker, red) and DAPI (nuclear marker, blue), demonstrating a proliferating ductal cell. Scale bar = 100 µm. (**b-d**) Magnification of B, showing single channel KRT19 positive cells and Ki67 positive cells. Scale bar = 20 µm.